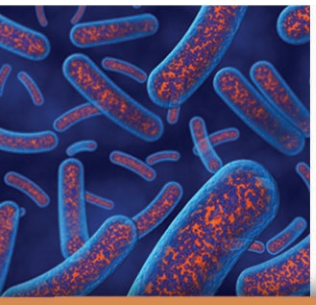
Training Manual

Summer Internship Programme on

Advanced Techniques in Microbiology, Biochemistry, Biotechnology and Bioinformatics



Course Coordinators

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7 May – 5 June, 2018

ICAR-Indian Institute of Spices Research
Kozhikode- 673012, Kerala, India

SUMMER INTERNSHIP PROGRAMME ON

ADVANCED TECHNIQUES

IN

MICROBIOLOGY, BIOCHEMISTRY, BIOTECHNOLOGY AND

BIOINFORMATICS

TRAINING MANUAL

ICAR - INDIAN INSTITUTE OF SPICES RESEARCH
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FACULTY MEMBERS OF THE PROGRAMME

1. Dr. K Johnson George, Principal Scientist, Division of Crop Improvement and Biotechnology
2. Dr. R Suseela Bhai, Principal Scientist, Division of Crop Protection
3. Dr. A Ishwara Bhat, Principal Scientist, Division of Crop Protection
4. Dr. K S Krishnamurthy, Principal Scientist, Division of Crop Production and Post Harvest Technology
5. Dr. N K Leela, Principal Scientist, Division of Crop Production and Post Harvest Technology
6. Dr. V Srinivasan, Principal Scientist, Division of Crop Production and Post Harvest Technology
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25. Mr. I P Vijesh Kumar, Division of Crop Protection
26. Dr. Ann Jasmine, Division of Crop Production and Post Harvest Technology
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30. Ms. Prashinamol, Division of Crop Improvement and Biotechnology
31. Ms. Aparna, Division of Crop Improvement and Biotechnology
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Summer Internship programme: Advanced techniques in Microbiology, Biochemistry, Biotechnology and Bioinformatics 7th May to 5th June, 2018				
Date	**Time**	**Subject**	**Faculty**	**Venue**
07-05-2018 Monday	9.30 - 10.00	Registration	Trainees	MCH, CF
	10.00 - 11.00	Inauguration	All	SJ Hall
	11.00 - 11.15	Tea break	All	SJ Hall
	11.15 - 12.00	Pre evaluation	Dr. P. Rajeev	MCH, CF
	12.00 - 1.00	Briefing the course	Coordinators	MCH, CF
	2.00 - 4.30	Biosafety laboratory procedures	Dr. A. I. Bhat	Central facility
08-05-2018 Tuesday	9.45 - 11.15	Plant Secondary Metabolites – Chemistry of Flavour and Fragrance (T)	Dr. N.K. Leela	MCH, CF
	11.30 - 1.00	Quantitative estimation of Plant Phenolics using Folin-ciocalteu reagent (P)	Dr. Ann Jasmine	Biochemistry
	2.00 - 4.30	Extraction of Essential oil/oleoresin (P)	Dr. N. K. Leela	Biochemistry
9-05-2018 Wednesday	9.45 - 11.15	Introduction to Spices Biochemistry (T)	Dr. T. J. Zachariah	MCH, CF
	11.30 - 1.00	Analytical Techniques in Plant Biochemistry – Spectrophotometer, HPLC, GC-MS, LC-MS (T)	Dr. N.K. Leela	MCH, CF
	2.00 - 4.30	Total antioxidant activity using DPPH radical scavenging assay (P)	Dr. Ann Jasmine	Biochemistry
10-05-2018 Thursday	9.45 - 11.15	Moulding Brains for Science(T)	Dr. Anees, K	MCH, CF
	11.30 - 1.00	Advances in Protein Chemistry(T)	Dr. Anees, K	MCH, CF
	2.00 - 3.30	Biosynthetic Pathway Discovery (T)	Dr. Anees, K	MCH, CF
	3.30 - 4.30	Soxhlet extraction of plant secondary metabolites (P)	Dr. Ann Jasmine	Biochemistry
11-05-2018 Friday	9.45 - 11.15	Metabolic pathways of Plant Secondary metabolite synthesis (T)	Ms. Sivaranjani. R	MCH, CF
	11.30 - 1.00	Analytical Techniques in Plant Biochemistry – Basis of column chromatography (T)	Dr. Ann Jasmine	MCH, CF
	2.00 - 4.30	Post harvest processing of spices (T)	Dr. Jayashree	MCH, CF
12-05-2018 & 13-05-2018 (Holidays)				
14-05-2018 Monday	9.45 - 11.15	Nutraceuticals and Phytopharmaceuticals (T)	Ms. Sivaranjani. R	MCH, CF
	11.30 - 1.00	Phytochemical extraction and analysis (T)	Ms. Sivaranjani. R	MCH, CF
	2.00 - 4.30	Phytochemical extraction and analysis (P)	Ms. Sivaranjani. R	Biochemistry
15-05-2018 Tuesday	10.30 - 1.00	Nanotechnology and its use in agriculture	Dr. A. Jeevalatha	MCH, CF
	2.00 - 4.30	Exposure to National Active Germplasm site	Mrs.Aarthi/ Mr.Nissar	Peruvannamuzhi farm
16-05-2018 Wednesday	9.45 - 11.15	Extraction of enzymes and Proteins from Plant samples and Isozyme analysis (T)	Dr. K. S. Krishnamurthy	MCH, CF
	11.30 - 1.00	Plant hormones (T)	Dr. K. S. Krishnamurthy	MCH, CF
	2.00 - 4.30	Estimation of protein content (P)	Dr. K. S. Krishnamurthy	Biochemistry
17-05-2018	9.45 - 11.15	Fundamentals in plant tissue culture (T)	Dr. Sharon Aravind	MCH, CF

Thursday	11.30 - 1.00	Fundamentals in plant tissue culture (P)	Dr. Sharon Aravind	Tissue culture lab
	2.00 - 4.30	Fundamentals in plant tissue culture (P)	Dr. Sharon Aravind	Tissue culture lab
18-05-2018 Friday	9.45 - 11.15	Carbon sequestration (T)	Dr. V. Srinivasan	MCH, CF
	11.30 - 1.00	Impact of climate changes on physiology of crop plants(T)	Dr. M. Alagupalamuthirsolai	MCH, CF
	2.00 - 4.30	Production of hydrolytic enzymes by biocontrol agents	Dr. R. Suseela Bhai	Plant Pathology
19-05-2018 Saturday	9.45 - 11.15	Introduction of plant pathogens and diseases (T)	Dr. Praveena	MCH, CF
	11.30 - 1.00	Drought tolerance in crop plants (T)	Dr. M. Alagupalamuthirsolai	MCH, CF
	2.00 - 4.30	Biocontrol agents – its production and commercialization (P)	Dr. R. Praveena	Biocontrol lab
20-5-2018 (Holiday)				
21-05-2018 Monday	9.45 - 10.45	Role of functional soil microbes(T)	Dr. C. Sarathambal	MCH, CF
	11.00 - 12.00	Advances in food pathogen detection techniques(T)	Dr. C. Sarathambal	MCH, CF
	12.00 - 1.00	Analytical skills and creative thinking for a career in Science(T)	Dr. Lijo Thomas	MCH, CF
	2.00 - 4.30	Screening of Aspergillus sp. isolates for aflatoxin production	Dr. C. Sarathambal	Plant Pathology
22-5-2018 Tuesday	9.45 - 11.15	Morphological and molecular characterization of plant pathogens (T)	Dr. R. Suseela Bhai	MCH, CF
	11.30 - 1.00	Plant-pathogen interactions (T)	Dr. A. Jeevalatha	MCH, CF
	2.00 - 4.30	Isolation and identification of *Phytophthora (P)* (or) Isolation of Pathogens from soil, root and leaf (P)	Dr. R. Praveena	Biocontrol lab
23-5-2018 Wednesday	9.45 - 11.15	Principles of DNA isolation and PCR analysis(T)	Dr. T. E. Sheeja	MCH, CF
	11.30- 1.00	Identification of *Phytophthora* species through PCR (P)	Dr. A. Jeevalatha	Central facility
	2.00 – 4.30	Isolation of DNA from Plant samples (P)	Dr. T. E. Sheeja	Central facility
24-5-2018 Thursday	9.45 – 11.15	Introduction to actinomycetes and its biocontrol potential(T)	Dr. R. Suseela Bhai	MCH, CF
	11.30 - 1.00	PCR amplification of DNA (P)	Dr. T. E. Sheeja	Central facility
	2.00 - 4.30	Gel Electrophoresis of PCR product (P)	Dr. T.E. Sheeja	Central facility
25-5-2018 Friday	9.45 - 10.45	Introduction to Plant endophytes and its significance (T)	Dr. Mohammed Faisal Peeran	MCH, CF
	11.00 - 12.00	Microbial toxins, enzyme production and growth regulation in relation to pathogen (T)	Dr. C. N. Biju	MCH, CF
	12.00 - 1.00	Mechanism of host plant resistance to insect pests (T)	Dr. Balaji Rajkumar	MCH, CF
	2.00 - 4.30	Entomopathogens as biocontrol agents (T&P)	Dr. Balaji Rajkumar	MCH, CF
26-5-2018 Saturday	9.45 - 11.15	Introduction to plant viruses (T)	Dr. A.I. Bhat	MCH, CF
	11.30 - 1.00	Molecular techniques for plant virus detection	Dr. A.I. Bhat	MCH, CF

		(T)		
	2.00 - 4.30	Molecular techniques for plant virus detection (P)	Dr. A.I. Bhat	MPP lab
27-5-2018 (Holiday)				
28-5-2018 Monday	9.45 - 11.15	Principles of RNA isolation and qPCR analysis (T)	Dr. D. Prasath	MCH, CF
	11.30 - 1.00	Plant RNA isolation (P)	Dr. T.E. Sheeja	Central facility
	2.00 - 4.30	Plant RNA isolation (P)	Dr. T.E. Sheeja	Central facility
29-5-2018 Tuesday	9.45 - 11.15	Principles of genetics (T)	Dr. M. S. Shivakumar	MCH, CF
	11.30 - 1.00	Development of molecular maps and marker assisted selection in crop plants(T)	Dr. M. S. Shivakumar	MCH, CF
	2.00 - 4.30	qPCR analysis (P)	Dr. D. Prasath	Central facility
30-5-2018 Wednesday	9.45 - 11.15	Biofortification via conventional breeding and second generation GM crops (T)	Mrs. Aarthi	MCH, CF
	11.30 - 1.00	Cloning of PCR products (T)and (P)	Dr. A. I. Bhat	MCH, CF
	2.00 - 4.30	Cloning of PCR products (P)	Dr. A. I. Bhat	MPP lab
31-5-2018 Thursday	9.45 - 11.15	Gene discovery through transcriptome analysis (T)	Dr. K. Johnson George	MCH, CF
	11.30 - 1.00	Next Generation Sequencing (T)	Dr. A. I. Bhat	MCH, CF
	2.00 - 4.30	Term paper presentation	Trainees	MCH, CF
1-6-2018 Friday	9.45 - 11.15	Genome editing through CRISPR/Cos system (T)	Dr. A. I. Bhat	MCH, CF
	11.30 - 1.00	RNA interference in insect pest management (T)	Dr. T. K. Jacob	MCH, CF
	2.00 - 4.30	Term paper presentation	Trainees	MCH, CF
2-6-2018 Saturday	9.45 - 11.15	Introduction to bioinformatics (T)	Mr.I.P.Vijesh Kumar	MCH, CF
	11.30 - 1.00	Primer design and validation, Sequence databases and analysis (T&P)	Ms. Blessy	DISC
	2.00 - 4.30	Introduction to chemi-informatics ,Molecular docking and computer aided drug designing (T&P)	Dr. Prathiba	DISC
3-6-2018 (Holiday)				
4-6-2018 Monday	9.45 - 11.15	IPR and patenting (T)	Dr. T. E. Sheeja	MCH, CF
	11.30 - 1.00	Proteomics and metagenomics (T)	Mrs. P. Umadevi	MCH, CF
	2.00 - 4.30	Protein extraction for 2-D gel electrophoresis (P)	Mrs. P. Umadevi	Central facility
5-6-2018 Tuesday	9.30 - 10.30	Post evaluation	Dr. P.Rajeev	MCH, CF
	10.30 - 1.00	Valedictory function and distribution of certificate		SJ Hall

Tea break: Morning - 11.15-11.30 AM, Evening - 3.15-3.30 PM (ATIC)
Lunch break: 1.00 - 2.00 PM (Canteen)
MCH,CF - Mini conference hall, Central Facility
MPP lab - Molecular Plant Pathology lab
T - Theory; P - Practical

LABORATORY BIOSAFETY PROCEDURES

A I Bhat

A new culture of safety consciousness, accountability, organization and education has developed in the laboratories of the government and academic institutions. Now, programmes are being implemented to train laboratory personnel and to monitor the handling of chemicals from the moment they are ordered until their departure for ultimate treatment or disposal. Learning to participate in this culture of habitual risk assessment, experiment planning, and consideration of worst-case possibilities for oneself and one's fellow workers is as much a part of scientific education as learning the theoretical background of experiments or the step-by-step protocols for doing them in a professional and craftsman like manner.

Forming the foundation for a lifelong attitude of safety consciousness, risk assessment, and prudent laboratory practices should be an integral part of every stage of scientific education--in the classroom, in textbooks, and in the laboratory, from the earliest exposures in primary or secondary school through graduate and postdoctoral training. Teaching and academic institutions have this essential and unique responsibility.

Advanced training in safety is an important component of education through research. Safety training must be a continuing process; it should become an integral part of the daily activities of laboratory workers and those who are accountable for them. As a student or laboratory worker learns a new protocol, safe practices relevant to it should also be emphasized in the normal setting of the laboratory, with the careful guidance of a mentor and the shared responsibility of colleagues.

There are laboratory safety guidelines that are the foundation for biological safety in any laboratory using infectious agents, recombinant DNA, or biological toxins. Additions to the basic practices are required to enhance containment of particular agents or toxins that pose greater risks. Individual research plans that are submitted to the Institutional Biosafety Committee are reviewed to determine whether the safety measures that are proposed are appropriate to the risks inherent in the research procedures.

All the biotechnological laboratories must have a biosafety committee approved by the Department of Biotechnology, Government of India. Many of the standard laboratories have their biosafety practices.

General procedures for working with hazardous chemicals

1. Personal behaviour

Professional standards of personal behavior are required in any laboratory:

1. Avoid distracting or startling other workers.

2. Do not allow practical jokes and horseplay at any time.
3. Use laboratory equipment only for its designated purpose.
4. Do not allow visitors, including children and pets, in laboratories where hazardous substances are stored or are in use or hazardous activities are in progress.

2. Minimizing Exposure to Chemicals

Precautions should be taken to avoid exposure by the principal routes, that is, contact with eyes and skin, inhalation, and ingestion.

a) Avoiding Eye Injury

Eye protection should be required for all personnel and visitors in all locations where chemicals are stored or used. Researchers should assess the risks associated with an experiment and use the appropriate level of eye protection:

1. Safety glasses with side shields provide the minimum protection acceptable for regular use.
2. Safety splash goggles or face shields should be worn when carrying out operations in which there is any danger from splashing chemicals or flying particles.

b) Minimizing Skin Contact

Wear gloves whenever handling hazardous chemicals, sharp-edged objects, very hot or very cold materials, toxic chemicals, and substances of unknown toxicity

c) Avoiding Ingestion of Hazardous Chemicals

Eating, drinking, smoking, gum chewing, applying cosmetics, and taking medicine in laboratories where hazardous chemicals are used should be strictly prohibited. Laboratory water sources and deionized laboratory water should not be used for drinking water.

1. Laboratory chemicals should never be tasted.
2. A pipet bulb or aspirator should be used to pipet chemicals or to start a siphon; pipetting should never be done by mouth.
3. Hands should be washed with soap and water immediately after working with any laboratory chemicals, even if gloves have been worn.

d) Avoiding Inhalation of Hazardous Chemicals

1. Toxic chemicals or compounds of unknown toxicity should never be smelled.
2. Procedures involving volatile toxic substances and operations involving solid or liquid toxic substances that may result in the generation of aerosols should be conducted in a laboratory hood.
3. Dusts should be recognized as potentially contaminated and hazardous.

3. Clothing and Protective Apparel

It is advisable to wear a laboratory coat when working with hazardous chemicals. This is particularly important if personal clothing leaves skin exposed. Apparel giving additional protection (e.g., nonpermeable laboratory aprons) is required for work with certain hazardous substances.

4. Housekeeping-Rules

1. Never obstruct access to exits and emergency equipment such as fire extinguishers and safety showers.

2. Clean work areas (including floors) regularly. Properly label and store all chemicals.

5. Disposal of Chemicals

The overriding principle governing the handling of waste in prudent laboratory practice is that *no activity should begin unless a plan for the disposal of nonhazardous and hazardous waste has been formulated.* Each category of waste has certain appropriate disposal methods. In choosing among these methods, several general principles apply, but local considerations can strongly influence the application of these rules:

6. Handling Flammable Substances

Flammable substances present one of the most widespread hazards encountered in the laboratory. Because flammable materials are employed in so many common laboratory operations, basic prudent laboratory practice should always assume the presence of fire hazard unless a review of the materials and operations in the laboratory verifies the absence of significant hazard.

Working with biohazardous and radioactive materials

Biohazardous Materials

For even the most experienced laboratory worker, a careful review of the publication *Biosafety in Microbiological and Biomedical Laboratorie* should be a prerequisite for beginning any laboratory activity involving a microorganism. It defines four levels of control that are appropriate for safe laboratory work with microorganisms that present occupational risks ranging from no risk of disease for normal healthy individuals to high individual risk of life-threatening disease, and it recommends guidelines for handling specific agents. The four levels of control, referred to as biosafety levels 1 through 4, describe microbiological practices, safety equipment, and features of laboratory facilities for the corresponding level of risk associated with handling a particular agent. The selection of a biosafety level is influenced by several characteristics of the infectious agent, the most important of which are the severity of the disease, the documented mode of transmission of the infectious agent, the availability of protective immunization or effective therapy, and the relative risk of exposure created by manipulations used in handling the agent.

Biosafety Level 1 (BSL-1)

Biosafety level 1 is the basic level of protection appropriate only for agents that are not known to cause disease in normal, healthy humans and of minimal potential hazard to laboratory personnel and the environment. The laboratory is not necessarily separated from the general traffic patterns in the building. Work is generally conducted on open bench tops using standard microbiological practices. Special containment equipment or facility design is neither required nor generally used. Laboratory personnel have specific training in the procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology or a related science.

Biosafety Level 2 (BSL-2)

Biosafety Level 2 is similar to Biosafety Level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment. It differs from BSL-1 in that (1) laboratory personnel have specific training in handling pathogenic agents and are directed by

competent scientists (2) access to the laboratory is limited when work is being conducted (3) extreme precautions are taken with contaminated sharp items and (4) certain procedures in which infectious aerosols or splashes may be created are conducted in biological safety cabinets or other physical containment equipment.

Biosafety level 2 is appropriate for handling a broad spectrum of moderate-risk agents that cause human disease by ingestion or through percutaneous or mucous membrane exposure. Hepatitis B virus, human immunodeficiency virus (HIV), and salmonellae and toxoplasma spp. are representative of agents assigned to this biosafety level. Extreme precaution with needles or sharp instruments is emphasized at this level. A higher level of control may be indicated when some of these agents, especially HIV, are grown and concentrated.

Biosafety Level 3 (BSL-3)

Biosafety Level 3 is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Laboratory personnel have specific training in handling pathogenic and potentially lethal agents, and are supervised by competent scientists who are experienced in working with these agents.

Biosafety level 3 is appropriate for agents with a potential for respiratory transmission and for agents that may cause serious and potentially lethal infections. Emphasis is placed on the control of aerosols by containing all manipulations. At this level, the facility is designed to control access to the laboratory and includes a specialized ventilation system, such as a **biological safety cabinet**, that minimizes the release of infectious aerosols from the laboratory. The bacterium *Mycobacterium tuberculosis* is an example of an agent for which this higher level of control is appropriate.

All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets or other physical containment devices, or by personnel wearing appropriate personal protective clothing and equipment. The laboratory has special engineering and design features.

It is recognized, however, that some existing facilities may not have all the facility features recommended for Biosafety Level 3 (i.e., double-door access zone and sealed penetrations). In this circumstance, an acceptable level of safety for the conduct of routine procedures, (e.g., diagnostic procedures involving the propagation of an agent for identification, typing, susceptibility testing, etc.), may be achieved in a Biosafety Level 2 facility, providing 1) the exhaust air from the laboratory room is discharged to the outdoors, 2) the ventilation to the laboratory is balanced to provide directional airflow into the room, 3) access to the laboratory is restricted when work is in progress, and 4) the recommended Standard Microbiological Practices, Special Practices, and Safety Equipment for Biosafety Level 3 are rigorously followed. The decision to implement this modification of Biosafety Level 3 recommendations should be made only by the laboratory director.

Biosafety Level 4 (BSL-4)

Biosafety Level 4 is required for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease. Agents with a close or identical antigenic relationship to Biosafety Level 4 agents are handled at this level until sufficient data are obtained either to confirm continued work at this level, or to work with them at a lower level. Members of the laboratory staff have specific and thorough training in handling extremely hazardous infectious agents and they understand the primary and secondary containment functions of the standard and special practices, the containment equipment, and the laboratory design characteristics. They are supervised by competent scientists who are trained and experienced in working with these agents. Access to the laboratory is strictly controlled by the laboratory director. The facility is either in a separate building or in a controlled area within a building, which is completely isolated from all other areas of the building. A specific facility operations manual is prepared or adopted.

Within work areas of the facility, all activities are confined to Class III biological safety cabinets, or Class II biological safety cabinets used with one-piece positive pressure personnel suits ventilated by a life support system. The Biosafety Level 4 laboratory has special engineering and design features to prevent microorganisms from being disseminated into the environment.

There are two models for Biosafety Level 4 laboratories: (A) the Cabinet Laboratory where all handling of the agent is performed in a Class III Biological Safety Cabinet, and (B) the Suit Laboratory where personnel wear a protective suit. Biosafety Level-4 laboratories may be based on either model or a combination of both models in the same facility. If a combination is used, each type must meet all the requirements identified for that type.

Exotic agents that pose a high individual risk of life-threatening disease by the aerosol route and for which no treatment is available are restricted to high containment laboratories that meet biosafety level 4 standards. Worker protection in these laboratories is provided by the use of physically sealed glove boxes or fully enclosed barrier suits that supply breathing air.

Radioactive materials

Prudent practices for working with radioactive materials are similar to those needed to reduce the risk of exposure to toxic chemicals and to biohazards:

1. Know the characteristics of the radioisotopes that are being used, including half-life, types and energies of emitted radiations, the potential for exposure, how to detect contamination, and the annual limit on intake.
2. Protect against exposure to airborne and ingestible radioactive materials.
3. Never eat, drink, smoke, handle contact lenses, apply cosmetics, or take or apply medicine in the laboratory, and keep food, drinks, cosmetics, and tobacco products out of the laboratory entirely so that they cannot become contaminated.
4. Do not pipet by mouth.
5. Provide for safe disposal of waste radionuclides and their solutions.
6. Use protective equipment to minimize exposures.

7. Use equipment that can be manipulated remotely, as well as shielding, glove boxes, and personal protective equipment, including gloves, clothing, and respirators, as appropriate.
8. Plan experiments so as to minimize exposure by reducing the time of exposure, using shielding against exposure, increasing your distance from the radiation, and paying attention to monitoring and decontamination.
9. Keep an accurate inventory of radioisotopes.
10. Record all receipts, transfers, and disposals of radioisotopes.
11. Record surveys.
12. Check workers and the work area each day that radioisotopes are used.
13. Minimize radioactive waste.
14. Plan procedures to use the smallest amount of radioisotope possible.
15. Check waste materials for contamination before discarding.
16. Place only materials with known or suspected radioactive contamination in appropriate radioactive waste containers.
17. Do not generate multihazardous waste (combinations of radioactive, biological, and chemical waste) without first consulting with the designated radiation and chemical safety officers.

Liquefied gases and cryogenic liquids

Cryogenic liquids are materials with boiling points of less than 73°C (100°F). Liquid nitrogen, helium, and argon, and slush mixtures of dry ice with isopropanol are the materials most commonly used in cold traps to condense volatile vapors from a system. In addition, oxygen, hydrogen, and helium are often used in the liquid state.

The primary hazards of cryogenic liquids are fire or explosion, pressure buildup (either slowly or due to rapid conversion of the liquid to the gaseous state), embrittlement of structural materials, frostbite, and asphyxiation. The extreme cold of cryogenic liquids requires special care in their use. The vapor that boils off from a liquid can cause the same problems as the liquid itself.

A special risk to personnel is skin or eye contact with the cryogenic liquid. Because these liquids are prone to splash in use owing to the large volume expansion ratio when the liquid warms up, eye protection, preferably a face shield, should be worn when handling liquefied gases and other cryogenic fluids.

Extreme caution should be exercised in using liquid nitrogen as a coolant for a cold trap. If such a system is opened while the cooling bath is still in contact with the trap, oxygen may condense from the atmosphere. The oxygen could then combine with any organic material in the trap to create a highly explosive mixture. Thus, a system that is connected to a liquid nitrogen trap should not be opened to the atmosphere until the trap has been removed. Also, if the system is closed after even a brief exposure to the atmosphere, some oxygen (or argon) may have already condensed. Then, when the liquid nitrogen bath is removed or when it evaporates, the condensed gases will vaporize, producing a pressure buildup and the potential for explosion. The same explosion hazard can be created if liquid nitrogen is used to cool a flammable mixture that is exposed

Precautions while using microorganisms

Infection followed by disease will depend on the microorganism's ability to multiply in the host and on the host's ability to resist or control the infection. It has proved useful to categorise all microorganisms into 4 groups which define their pathogenicity to humans; only the first group are non-pathogens.

Hazard Group 1: Organisms that is most unlikely to cause human disease

Hazard Group 2: Organisms capable of causing human disease and which may be a hazard to laboratory workers, but are unlikely to spread to the community. Laboratory exposure rarely produces infection and effective prophylaxis or effective treatment is usually available

Hazard Group 3: Organisms that may cause severe human disease and present a serious hazard to laboratory workers. They may present a risk of spread to the community, but there is usually effective prophylaxis or treatment available.

Hazard Group 4: Organisms that cause severe human disease and are a serious hazard to laboratory workers. They may present a high risk of spread to the community, and there is usually no effective prophylaxis or treatment

The intention of this categorisation, which applies to non-modified organisms as well, is to identify appropriate containment, which would be required to protect those working with the organisms. The higher the hazard group, the greater the containment required to control the organism and ensure that it does not infect those working with it.

Examples of some biohazardous chemicals – precautions

Ethidium bromide

1. **Ethidium bromide** is a potent mutagen that has been used for many years as a nucleic acid stain. This material fluoresces a red-orange color under ultraviolet light and with increased fluorescence when bound to double-stranded DNA
2. The powder form is considered an irritant to the upper respiratory tract, eyes, and skin. Ethidium bromide is strongly mutagenic, causing living cell mutations. Even though there is no evidence at this time of human carcinogenicity or teratogenicity, this material should be considered a possible carcinogen or teratogen.
3. Wear a lab coat, eye protection, and nitrile gloves when working with ethidium bromide. Leave lab coats, gloves, and other PPE in the lab, when your work is complete, to prevent the spread of this or other chemicals outside of the lab.
4. When an ultraviolet light source is used in your work with ethidium bromide, added caution is required. As a general rule, avoid exposing unprotected skin and eyes to intense UV sources. If the UV light is aimed upwards, wear a UV protective face shield when you are standing near the source. For prolonged work close to UV light boxes or other intense sources, it may be useful to wrap the end of the lab coat sleeves loosely with masking tape to prevent gaps where the wrist could be exposed.

5. When working with ethidium bromide, minimize the potential for spills. Perform all processes that generate ethidium bromide dusts or mists inside the fume hood to minimize inhalation exposures.
6. Spills of ethidium bromide solutions should be absorbed and decontaminated with soap and water. Avoid raising dust when cleaning up solid spills by mixing with water and then absorbing the solution. All spill cleanup materials and absorbents should be bagged or placed in a sealed container with a hazardous waste label**.**

Decomposition of Ethidium bromide solutions

Method 1

1. Add sufficient water to reduce the concentration.
2. Add 0.2 vol. hypophosphorous acid and 0.12 vol. of fresh 0.5 M sodium nitrate.
3. Incubate for 24 hrs at R.T, add 1 M Sodium bicarbonate in excess.
4. Discard**.**

Method 2

1. Add sufficient water to reduce concentration.
2. Add 1 vol. of 0.5 M $KMnO_4$. Add 1 vol. of 2.5 N HCl.
3. Mix carefully to stand at R.T for hours.
4. Add 1 vol. 2.5 N NaOH.
5. Mix and discard**.**

Acryl amide

1. Warning! Harmful if swallowed, inhaled or absorbed through skin.
2. Affects central and peripheral nervous systems and reproductive system.
3. Causes irritation to skin, eyes and respiratory tract. suspect cancer hazard.
4. May cause cancer. risk of cancer depends on level and duration of exposure.
5. Possible birth defect hazard. may cause birth defects based on animal data.
6. Thermally unstable. may polymerize explosively if heated to the melting point.
7. Keep in a tightly closed container. Store in a cool, dry, ventilated area away from sources of heat or ignition. Protect against physical damage. Store separately from reactive or combustible materials, and out of direct sunlight. Isolate from oxidizing materials and peroxides. Store away from acids and alkalies
8. Wash hands, face, forearms and neck when exiting restricted areas. Shower, dispose of outer clothing, change to clean garments at the end of the day. Avoid cross-contamination of street clothes.
9. Containers of this material may be hazardous when empty since they retain product residues (dust, solids); observe all warnings and precautions listed for the product.

Isopropanol

1. This compound contains no ingredients at concentrations of 0.1% or greater that are carcinogens or suspect carcinogens.

2. Signs/symptoms of overexp: nausea, narcosis, dermatitis, skin irritation, eye irritation, lung irritation, mucous membrane irritation, GI tract irritation.
3. Emergency/first aid proc: Eyes: flush with water for 15 minutes while holding eyelids open. Get medical attention. Skin: Remove contaminated clothing. Wash with soap and water. Other protective equipment: Chemical resistant lab coat is necessary to prevent skin contact.
4. Special hazard precautions: irritation to skin, eyes, lungs, mucous membranes and gi tract. Prolonged exposure may cause dermatitis.

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MOULDING BRAINS FOR SCIENCE

Anees K

"I am enough of an artist to draw freely upon my imagination. Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world."

Albert Einstein

Fundamental constitutional duty of citizen of India is to develop scientific temper, humanism and spirit of enquiry and reform. It implies the importance of developing a scientific temper in every individual. In other words developing scientific temper is as good as being patriotic. Science is nothing but the intellectual and practical activity encompassing the systematic study of the structure and behaviour of the physical and natural world through observation and experiment. Observation and its systematic study is possible only by a creative mind. The creativity thus, plays a vital role in the growth and development of science. Genrally creativity is attributed as an essential quality of an artist. In science, creativity as an attribute has been underrated till recent past. Now in the modern era of innovation creativity is equally valid as scientific acumen of the researcher.

According to famous creative artist George Lois, '*creativity can solve almost any problem. Creative act, the defeat of habit by originality overcomes everything*.' It is very clear that creativity arises out of a problem faced. Problem is the provocative force to think creative. There are many ways to develop creativity. Here, five steps for being creative are explained.

Define the constaints/parameters of the problem

It is assumed that creativity is always out of the box thinking. In fact, many a times, creative thinking has to be inside the box. For this, one has to understand 'the box'. The box refers to a situation or surrounding in which the creative thinker exists. In other words it implies the available resources to solve a problem. This means the solution to the problem should be possible only with the available resources and therefore any wild idea may not work in many cases. Therefore it is very important to understand the constraint and parameters of the problem to understand its dimension and possible solution.

Provoke ideas

First law of motion states that a body at rest will remain at rest, unless acted upon by an external **unbalanced force**. In creativity a habit will remain a habit unless defeated by **originality**. The analogue of an unbalanced force in this case is to originality. The provocation of ideas can be made mainly through brainstorming. Brainstorming has to be conducted with a condition that there is no limit for thought process wherein solving the problem is the sole objective. The term originality is referred as 'po' by many sociologists. It may be seen ine contexts such as Hy**po**thesis, Sup**po**se, **Po**ssible and **Po**etry. In general 'po' can be explained as a **P**rovocation

Operation which is a radical and sensible idea. It is very important to understand the balance between being radical and sensible. Deadlines act as 'po' for creative thinking in many cases. Once the target has to be achieved by a specified time, creativity has been observed to be working more effectively. Many difficult problems are solved by giving an impossible deadline by the authorities to the subordinates. This is being adopted as a top managerial strategy to solve problems.

Give absurd ideas respect

During brainstorming, different ideas will emerge which are absurd, looking at the face value. But it has to be respected in its being original and should be given a thought as an option to solve the problem. This aspect is very important for finding an innovative solution. If in a brainstorming session to start a restaurant one opines to start a restaurant which does not serve food, it has to be thought and discussed deeply regarding the possibility of such an absurd idea. Only if we keep our mind for a long while on absurd ideas, we get better innovative solutions. To stay on thought process for a long while on such ideas are important, rather than just leaving the idea after giving a short discussion.

Be optimistically objective

One has to be optimistically objective to arrive at conclusion. The idea of 'restaurant without food' can only be developed further by adding objectivity with optimism to develop such a concept. First and foremost thing to put an idea in to reality is, the belief that it is definitely possible. Once anybody makes that firm mind set, there are many objective solutions to implement the solution or developing the idea into a practical and sensible action. There is no creative value in being cynical.

Celebrate great ideas regardless of who had them

Creative thinking is a team work in the real sense. Bits and pieces of different ideas from different corners come together to form the final solution. Therefore it is important to share the ideas and give respect to it regardless of who proposed it. The value of a small intervention in the whole solution also has to be recognised and need to be celebrated as a team. Then only creative art will be more effective to generate innovative solutions. In science, the team work is very important to execute grate discoveries and for effective resource management.

Developing scientific temper

Scientific temper can be developed or acquired by any individual by adopting simple strategies. A general development of scientific thought process include Questioning the physical phenomena; Observing the nature of phenomena; Forming a hypothesise; Generating testable predictions; Analysis and Forming a theory. This is what every science graduate do in his academics. But, scientific temper development is not a science graduate's job alone. It can be

achieved by any individual with keen observation capacity. Three basic strategies for developing scientific temper is enlisted here

Sharpen your curiosity

Curiosity quotient is more important than the intelligent quotient for developing scientific temper. The desire to know the unknown with a positive mind set is the underlying principle for successful scientific thinking. We often tend to overlook many things which we do in our daily life. If we know the answer of such silly questions in day to day life we can assume that our curiosity quotient is very high. Some few such example questions are given below:

- Do you know the meaning of national anthem?
- Why do we do "parikrama/pradakshina" in temple & why always in clock-wise?
- Why does a chappati puffs?
- Why do we say "touch wood"?
- Why do we blow off candles on birthday? Though putting off burning light is considered inauspicious in our culture

Believe only if the conclusion is statistically valid

In a religious society it is quite natural to believe and blindly follow all our spiritual actions irrespective of its statistical validity. Here are few questions which forces us to think that are we really checking the statistical validity of our own actions.

- Are some moments or days more auspicious?
- Do mental illness become more severe on no-moon days?
- Why doesn't any insurance companies use the astrological predictions before selling their policies?

Pandit Jawaharlal Nehru has categorically stated that 'Don't believe in things unless they are tried and tested'. The statistics does not implies the significance tests which normal statistician perform in scientific researcher. Normally, if any incident reoccurs or can be reproduced at least 50% times independent of place of action, person who performs it can be more or less be predicted as statistically valid observation.

Oppose public optinasy

This attribute of a personal character is very important for scientific temper development. There are many social norms which are highly unscientific floating around us in the society. Being a social animal we tend to agree to it by obeying or following, or even keeping siloenc eon the issue. One has to develop the courage of questioning such values and norms based on scientific arguments. Are we ready to declare and ask some uneasy questions such as why do we have more temples/mosques/churches than schools and hospitals? Why do we object to sex education in school?

Many a time developing scientific temper is confused as being anti-religious. In fact it is not a conflict between religion and science, rather it is a conflict between you and your irrationality.

SPICES: RICH SOURCE OF BIOACTIVE COMPOUNDS

T John Zachariah

Spices are treasured for the flavor, pungency, aroma and color they impart to food. The delightful flavor and pungency of spices make them indispensable in the preparation of palatable dishes. Over the years, Indian spices have carved out a niche of their medicinal and pharmacological properties in the worldof spices. There are 109 spices listed by International standards Organization (ISO) and the Spices Board, Government of India listed 52 major spices of commercial importance. India produces around 3.8 million tones of spices annually, of this about 10% of the total produce is exported to over 150 countries. The USA, Europe, Australia, Japan, the Middle East and Oceanic countries are the major importers of Indian spices.

Culinary herb and Spice

The leaves of a plant used in cooking are denominated as culinary herbs while any other part of the plant is known as a spice. Spices can be leaf (eg bay leaf), buds (clove), bark (cinnamon), root (ginger), berries (grain of black pepper), seeds (cumin) or even stigma of flower (saffron). Both spices and herbs can be used fresh, dried, whole, chopped or ground and due to their colour, aroma and / or flavor characteristisc are used in the preparation of foods and drinks. Spices and herbs contain proteins, fiber, sugars, essential oils, minerals, pigments besides bioactive compounds such as phenolic acids, sterols and cumarins. Many of the functional properties attributed to spices associate with presence, type and concentration of phenolic compounds although exact composition depend on several factors such as the plant part used, vegetative state, environmental conditions, harvesting techniques etc

Other compounds present in spices are the essential oils which due to their content in terpenes, monoterpenes and susquiterpenes (as hydrocarbons, alcohols, ketones etc.) are responsible for many functional properties. These are composed of more than 70 components some of which may represent more than 85% of total content.

Phenolic Compounds

Phenolic compounds in any forms present in spices and herbs whether simple phenoles, flavons, flavanones, flavanols, anthocyanins etc are responsible for their functional properties. Properties of spices such as antioxidant, antibacterial, antiviral, anti-inflammatory capacities, cardio protective and anti carcinogenic effects and their ability to act as inhibitors of platelet aggregation are significant. Phenolic compounds contribute to colour and taste. Simplest phenols are liquid or solid with a low fusion point and high boiling point as they form hydrogen bonds. They are easily oxidized which is why they frequently appeared to be coloured.

Terpenes

These groups of chemicals are the fundamental components of essential oils. They all result from the condensation of isoprenic units. Among their different form are monoterpenes, diterpenes

and sesquiterpenes. They are extremely volatile and have been demonstrated to possess multiple functional properties including antioxidant, antimicrobial and antiviral capacities.

Antioxidant properties

Spices have shown their ability to slow down process of lipidic oxidation. This process is one of the principal causes of food spoilage and may occur during the storage of raw materials, processing or at the storage of final product. To avoid such spoilage food industry has resorted to synthetic antioxidant formulations, some of the most commonly used being butylated hydroxy toluene (BHT) butylated hydroxyl anisole (BHA) and propyl gallate. However because of their synthetic origin their safety and efficacy are frequently questioned. The result has been a growing interest in compounds of vegetable origin which show antioxidant potential. Possible mechanism of antioxidant action by spices may be by scavenging free radicals, hydrogen donation, the chelating of metallic ions or the capacity to act as substrate of radicals such as superoxide or hydroxyl.

Free radicals, produced as a result of normal biochemical reactions in the body, are implicated in contributing to cancer, atherosclerosis, aging, immune suppression, inflammation, ischemic heart disease, diabetes, hair loss, and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. The human body possesses innate defense mechanisms to counter free radicals in the form of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Vitamin C, vitamin E, selenium,b-carotene, lycopene, lutein and other carotenoids have been used as supplementary antioxidants.

Apart from these, plant secondary metabolites such as flavonoids and terpenoids play an important role in the defense against free radicals

Antibacterial Properties

Many techniques have been used to preserve foods from micro organic attack including refrigeration, freezing, water activity reduction and the restriction of nutrients, acidification, modified atmosphere packaging, fermentation or the addition of antimicrobial compounds. To these may be added new technologies such as high pressure, electric pulses, nanotechnology and irradiation.

Food industry is exploring ways to replace synthetic preservatives by natural ones and spices tend to be the main source of these. Again spices and its essential oil are promising natural antimicrobials.

Role of spices in health benefits

Much health benefit attributes of these common food adjuncts have been recognized in the past few decades by pioneering experimental research involving both animal studies and human trials. These studies documented digestive stimulant action, hypolipidemic effect, antidiabetic influence, antilithogenic property, antioxidant potential, anti-inflammatory property, antimutagenic, and anticarcinogenic potential of spices. Among these, the hypocholesterolemic

and antioxidant properties of a few specific spices have far-reaching nutraceutical value. These beneficial physiological effects also have the potential of possible therapeutic application in a variety of disease conditions.

These group of esoteric food adjuncts have been in use for thousands of years to enhance the sensory quality of foods, the quantity and variety consumed in tropical countries is particularly extensive. These spice ingredients impart characteristic flavor, aroma, or piquancy and color to foods. Some spices, like fenugreek, can also modify the texture of food. It is a common experience that their distinct aroma stimulates the appetite. Not only are spices used as flavorings and seasonings, but many are also used in perfumery, cosmetics, and toiletries. In addition, several spices have long been recognized to possess medicinal properties such tonic, carminative, stomachic antispasmodic, and antihelminthic, as listed in Table 1. Although these observations are largely empirical, these undoubtedly efficacious attributes have earned them pharmacological applications in the indigenous system of medicine not only in India, but in other countries as well.

Table 1: Medicinal properties of spices recognized for a long time

Spice	**Medicinal properties recognized for a long time**
Coriander (*Coriandrum sativum*)	Antidyspeptic, flavorant
Cumin (*Cuminum cymminum*)	Antispasmodic, carminative, digestive stimulant
Fenugreek (*Trigonella foenumgraecum*)	Diuretic, emmenagogue, emollient, useful in heart diseases
Garlic(*Allium sativum*)	Antidyspeptic, antiflatulent, for ear infection, duodenal ulcers, as rubefacient in skin diseases
Ginger(*Zingiber officinale*)	Sialogogue, useful in diseases of heart and blood
Onion(*Allium cepa*)	Diuretic, emmenagogue, expectorant, for bleeding piles
Peppr(*Piper nigrum*)	Antipirectic; Rebefacient
Red pepper(*Capsicum annuum*)	Anti-inflammatory, for pain relief (rheumatism/neuralgia)Useful in indigestion, rubefacient
Turmeric (*Curcuma longa*)	Anti-inflammatory, diuretic, laxative, good for affections of the liver, jaundice, diseases of blood

World Awareness of the Potential of Spices

Today there is greater scientifically validated knowledge on spices phytochemistry, therapeutic effects of their bioactive principles and mechanism of action. Health benefits include carminative action, hypolipidemic effect, antidiabetic property, antilithogenic property, antioxidant potential, anti-inflammatory property, antimutagenic and anticarcinogenic potential. Of these, the hypocholesterolemic and antioxidant properties have far-reaching nutraceutical and therapeutic

value.Most of the medicinal properties are attributed to the secondary metabolites – the essential oils and oleoresins – present in spices, a large number of which have been identified

Medicinal and Pharmacological Properties of Spices

Spices are reputed to possess several medicinal and pharmacological properties and hence find position in the preparation of a number of medicines. Thus, in the indigenous system of Indian medicine (Ayurveda and Unani), spices have a vital role to play. Information regarding the medicinal aspects of spices, which are acceptable in the light of modern knowledge, is described with pharmaceutical codex. In Ayurveda, about 25 spices are used for various herbal preparations and the annual consumption by the Ayurvedic firms in Kerala alone is approximately 3.2 tones.Spices which possess some of the important medicinal properties are listed in Table2.

Table 2: Major spices and their medicinal properties

Medicinal property	**Spices**
Cancer Preventive	Ginger, Black pepper, Nutmeg, Cinnamon, Clove, Turmeric, Cardamom, Vanilla, Allspice, Mace
Antimicrobial	Ginger, Nutmeg, Black pepper, Cinnamon, Vanilla, Turmeric, Clove, Allspice, Cardamom, Mace
Anti-Inflammatory	Black pepper, Cinnamon, Clove, Turmeric, Allspice, Cardamom
Spasmolytic	Cinnamon, Black pepper, Clove, Ginger, Nutmeg, Turmeric
Antioxidant	Vanilla, Ginger, Black pepper, Clove, Turmeric
Antiulcer	Ginger, Black pepper, Turmeric, Cinnamon, Clove, Nutmeg, Vanilla, Allspice, Mace
Hypoglycemic	Cardamom
Antihepatotoxic	Vanilla
Antiallergic	Allspice
Antimigraine	Turmeric, Allspice, Cardamom, Mace
Antiosteoporotic	Black pepper, Allspice, Clove, Cardamom, Mace
Estrogenic/ Androgenic	Cardamom
Immunostimulant	Turmeric, Mace
Antilithic	Allspice
Anti-insomniac	Allspice, Clove, Mace
Antiedemic	Vanilla

Ayurvedic herbal drugs and their sources

Spices have been introduced into the Ayurvedic pharmacopoeia at a very early period in its evolutionary history. Table 3 illustrates the herbal formulations with the herbs from the family Piperaceae.

Table 3: Ingredients in major herbal formulations.	
Formulations	**Ingredients**
Trikatu (Three pungents)	Pepper, long pepper and dry ginger
Pancakola(The five pungents)	Long pepper fruit, long pepper root, wild pepper root, *Plumbago rosea* and dry ginger
Sadusanam (The six hot drugs)	The above mentioned herbs along with pepper

Black pepper (*Piper nigrum)*

The aroma of black pepper is mainly contributed by the volatile oil which varies between 2-5% in the berries. Major pepper oil constituents identified by various researchers are listed below.

Monoterpene hydrocarbons and oxygenated compounds

There are 15 monoterpene hydrocarbons identified so far and they are camphene, δ^3-carene, p-cymene, limonene, myrcene, cis-ocimene, α-phellandrene, β-phellandrene and β-pinenes, sabinene, α and γ-terpinenes, terpinolene and α-thujene.

Dilution and concentration experiments on samples of dried black pepper berries from India and Malaysia as well as enantioselective analysis of optically active monoterpenes indicated (±)-linalool, (+)-α-phellandrene, (-)-limonene, myrcene, (-)-α-pinene, 3-methylbutanal and methylpropanal as the most potent odorants of black pepper. (Zachariah and Parthasarathy, 2008)

Sesquiterpene hydrocarbons and oxygenated compounds

β-caryophyllene is the major sesquiterpene hydrocarbon present in pepper oil. Other sesquiterpene hydrocarbons are also reported from black pepper oil. They are α-cis-bergamotene, α-trans-bergamotene, β-bisabolene, δ and γ-cadinenes, calamenene, α-copaene, α- and β-cubebenes, ar-curcumene, β- and δ-elemenes, β-farnesene, α-guaiene, α- and γ-humulenes, isocaryophyllene, γ-muurolene, α-santalene, α- and β-selinenes, ledene, sesquisabinene and zingiberene.

Miscellaneous compounds

Eugenol, methyl eugenol, myristicin, safrole, benzaldehyde, trans-anethole, piperonal, m-methyl acetophenone, p-methyl acetophenone, n-butyrophenone, benzoic acid, phenyl acetic acid, cinnamic acid and piperonic acid are some of the aromatic compounds characterized in pepper oil. Methyl heptenone, pinol, butyric acid, 3-methyl butyric aicd, hexanoic acid, 2-methyl pentanoic acid, methyl heptanoate, methyl octanoate, 2-undecanone, n-nonane, n-tridecane, n-nonadecane and piperidine are the other compounds identified (Narayanan, 2000).

Wide variation in the chemical composition of pepper oil was observed by different research groups. This can be attributed to the effect of cultivar, agro climatic variation, variation in maturity of raw material, differences in the method of obtaining the oil, non resolution of constituents in early gas chromatographic analyses using packed columns etc. Steam distilled pepper oils usually contain about 70-80 per cent monoterpene hydrocarbons, 20-30 percent sesquiterpene hydrocarbons and less than 4 per cent oxygenated constituents. Oils prepared by vacuum distillation of oleoresin extracts differ in having less monoterpene hydrocarbons and more sesquiterpene hydrocarbons and oxygenated constituents.

The major monoterpene hydrocarbons present in pepper oil are α and β-pinenes, sabinene and limonene.

Sensory evaluation of black pepper essential oil

The odour of pepper oil is described as fresh, dry-woody, warm-spicy and similar to that of the black peppercorn. The flavour is rather dry-wood and lacks the pungency of the spice since the alkaloids are not extracted by steam distillation. Caryophellene rich oils possess sweet floral odours where as oils with high pinene content give turpentine like off-odours. The major odours are refreshing pinene like, fresh green, camphoraceous, citrus/lemon like, warm & spicy, peppery, sharp pungent, woody resinous, turmeric like and musty/mouldy.

Fresh pepper was found to taste and smell differently when compared with dry pepper and pepper oil. The fresh pepper aroma compounds were isolated by Amberlite column chromatography and analyzed by GC and GC/MS. The major compounds were found to be trans-linalool oxide and α-terpineol, whereas the dry black pepper oil contained α- and β-pinenes, d-limonene and β-caryophyllene as major components. Flavour and off-flavour compounds of black and white pepper (*Piper nigrum* L.) were evaluated by Jagella and Grosch. Enantioselective analysis of optically active monoterpenes indicated (±)-linalool, (+)-α-phellandrene, (-)-limonene, myrcene, (-)-α-pinene, 3-methylbutanal and methylpropanal as the most potent odorants of black pepper. The musty/mouldy off-flavour of a sample of black pepper was caused by a mixture consisting of 2, 3-diethyl-5-methylpyrazine (2.9 µg/kg) and 2-isopropyl-3-methoxypyrazine (0.2 µg/kg).

Pungency of Black Pepper

Piperine is the major constituent of pepper oleoresin. The alkaloid piperine is generally accepted as the active "bite" component in black pepper. The homologues and analogues of piperine are minor or trace compounds and their contribution to pungency is small. Despite the controversy over the nature of pungent compounds in pepper, piperine content has been taken as a measure of the total pungency. Piperine is a yellow crystalline substance having a melting point of 128-130^{0}C. Piperine, $C_{17}H_{19}O_3N$, was shown to be a weak base which on hydrolysis with aqueous alkali or nitric acid yielded a volatile base $C_5H_{11}N$, later identified as piperidine.

Cardamom (*Elettaria cardamomum* Maton)

Cardamom is used as an aromatic, carminative and stimulant. The seeds have a warm slightly pungent aromatic flavor. It is mainly used as a flavoring agent in tea and food preparations. Cardamom oil is a precious ingredient in food preparations, perfumery, health foods, medicine and beverages.

Among the cultivated types, 'Malabar' and 'Mysore' are the major international trade groups A third intermediate termed 'Vazhukka' is also treated as international., which is cultivated mainly in Kerala, India. The Malabar and Mysore types differ in the composition of their volatile oils.

The oil from Var. Malabar is more camphory in aroma, due to the higher content of 1,8-cineole Var Mysore or the commercial grade known as "Alleppy green" contains more of α-terpinyl acetate which contributes to the mild spicy flavour.
The volatile oil has little mono-or sesquiterpenic hydrocarbons and is dominated by oxygenated compounds, all of which are potential aroma compounds. The dominance of the ether, 1,8-cineole and the esters, α-terpinyl and linalyl acetates in the composition, make the cardamom volatiles a unique combination.

Ginger (*Zingiber officinale* Rosc.)
The ginger rhizome contains a little steam-volatile oil, fixed (fatty) oil, pungent compounds, resin, proteins, cellulose, pentosans, starch and mineral elements. The fibre and volatile-oil contents and the pungency level are the most important criteria in assessing the suitability of ginger rhizomes for particular processing purposes. The crude fibre content of unpeeled ginger is reported to be in the range of 1.5-6 per cent. The volatile oil content of commercial dried gingers varies from 1-3 per cent.

Ginger oleoresin is prepared from dried ginger by extraction using number of organic solvents. The oleoresin contains the organoleptically important volatile oil and pungent principles together with fatty oil, palmitic and some other free fatty acids, resin and carbohydrates. The yield and the relative abundance of the components of the oleoresin are dependant, however, upon the raw material and the solvent used and on the extraction conditions. Commercial dried gingers have been reported to provide oleoresins in yields of 3.5-10 per cent and to contain 15-30 per cent of volatile oil (Zachariah 2008).

The aroma and flavour of ginger are determined by the composition of its steam-volatile oil which is comprised mainly of sesquiterpene hydrocarbons, monoterpene hydrocarbons and oxygenated monoterpenes. The monoterpene constituents are believed to be the most important contributors to the aroma of ginger, and they tend to be relatively more abundant in the natural oil of the fresh ('green') rhizome than in the essential oil distilled from dried ginger. Oxygenated sesquiterpenes are relatively minor constituents of the volatile oil but appear to be significant contributors to its flavour properties. Ginger oil displays considerable compositional diversity, but is typically characterized by a high content of sesquiterpene hydrocarbons, including zingiberene, ar-curcumene, β -bisabolene, and β -sesquiphellandrene. Reports show the main components as α-zingiberene (21.8%), geranial (9.9%), geraniol (9.4%), β-bisabolene (7.9%), nerol (7.1%), 1,8-cineol (6.2%), α-terpineol (5.6%), borneol (5.4%), β-phellandrene (3.1%), linalool (1.7%), methyl nonyl ketone (1.6%) and camphene (1.4%); the other components accounted for ~1% each of the volatile oil. The lemony note is attributed to citrals together with α-terpineol, while β-sesquiphellandrene and ar-curcumene are regarded as partly responsible for the characteristic ginger flavour. Nerolidol was considered to contribute to the woody note; and cis-and trans-β-sesquiphellandrol were suspected to be significant contributors

to the ginger flavour. In combination, these compounds accounted for 85% of the taste panel's response.

Ginger oleoresin

Ginger oleoresin was extracted from rhizomes with ethanol, isopropanol or liquid carbon dioxide. All oleoresin samples had monoterpenes and sesquiterpenes. Oleoresin is the total soluble extractives in a specified solvent. From the functional point, the best oleoresin is one which contains all the flavour components of the material contributing to aroma, taste, pungency and related sensory factors which when diluted to the original concentration in the original material truly recreates the sensory quality of the original spice.

Pungent principles

The non volatile pungent oil obtained by solvent extraction of the spice was named gingerol. The pungent group includes gingerols, shogaols, paradols and zingerone that produce a 'hot' sensation in the mouth. The gingerols, a series of chemical homologs differentiated by the length of their un- branched alkyl chains, were identified as the major active components in the fresh rhizome. In addition the shogaols, another homologous series and the dehydrated form of gingerols are the predominant pungent constituents in dried ginger. Paradol is similar to gingerol and is formed on hydrogenation of shogaol. Evidence obtained from the investigations of Australian and Japanese gingers suggests that [6] - gingerol is the most abundant principle of ginger and it is accompanied by several other gingerol homologues and analogues, the [8] - and [10] - gingerols being prominent

Turmeric (*Curcuma longa* L.)

India is the major producer and exporter of turmeric at present, even though the crop is grown in several countries *viz;* Pakistan, Malaysia, Myanmar, Vietnam, Thailand, Philippines, Japan, China, Korea, Sri Lanka, Caribbean Islands and Central America. It is estimated officially that about 80% of the world production of turmeric is from India alone.

Epidemiological observations suggest that turmeric consumption may reduce certain forms of cancer and render other protective biological effects in humans, which is attributed to its constituent - curcumin. Thus it is effectively used as anti inflammatory, anti angiogermic, anti oxidant, anti cancerous etc. The curcuminoids, which are administered orally, enter the blood circulation and are present as glucuronides and glucuronide sulfate conjugate forms and hence the physiological effects expressed are due to these conjugates.

Turmeric oil

Dried rhizomes and leaves are used industrially to extract the volatile oil. Dried rhizomes contain 5-6 per cent and leaves contain about 1-1.5 per cent oil. It is generally extracted by steam distillation. GC-MS analysis of rhizome oils from five different *Curcuma* species show variations in the major components *viz*; ar-turmerone (2.6-70.3%), α –turmerone (trace-46.2%)

and zingiberene(trace-36.8%). Various reports indicate turmerone (49%), ar-curcumene (15%) and ar-turmerone (6.4%) as the major compounds.

Turmeric Oleoresin

Turmeric oleoresin is the organic extract of turmeric and is added to food items as a spice and coloring agent. Turmeric oleoresin is essentially used in institutional cooking in meat and fish products and certain products such as mustard, pickles and relish formulas, butter and cheese. This is obtained by the solvent extraction of the ground spice with organic solvents like acetone, ethylene dichloride and ethanol for 4-5 hours.

Curcumin

Curcumin (diferuloylmethane) is the main curcuminoid present in turmeric and responsible for its yellow color. Curcumin has been shown to possess significant anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-mutagenic, anticoagulant and anti-infective effects. Curcumin has also been shown to have significant wound healing properties. It acts on various stages of the natural wound healing process to hasten healing.(Dania et al 2014)

Curcumin or curcuminoids concentrate, for use as a food color, is not a regular article of commerce, because for most current uses the cheaper turmeric oleoresin has been found suitable. Curcumin is included in the list of colors with a restricted use because it has been allotted a low ADI (Acceptable Daily Intake) of 0-1.0 mg/Kg body weight/day. Curcumin gives a bright yellow color even at doses of 5-200 ppm. Curcumin, $C_{21}H_{20}O_6$, m p 184 - 185°C was isolated as early as 1815. The main coloured substance in the rhizomes is curcumin, [1, 7-bis (4- hydroxy-3-methoxy prenyl)-1, 6- heptadiene-3, 5-Dione] and two related demethoxy compounds, demethoxy curcumin and bis-demethoxy curcumin, which belong to the group of diarylheptanoids**.** Besides these three forms of curcuminoids, three minor constituents have also been isolated which are supposed to be geometrical isomers of curcumin.

Cinnamon

The true cinnamon, *Cinnamomum verum* syn. *C. zeylanicum*, is a native of Sri Lanka and south India. The dried inner bark of cinnamon and cassia contains volatile oil, fixed oil, tannin, resin, proteins, cellulose, pentosans, mucilage, starch, calcium oxalate and mineral elements. Cinnamon possesses a delicate, spicy aroma, which is attributed to the volatile oil present in it. They can be broadly classified into monoterpenes, sesquiterpenes, and phenyl propenes. Volatile oil content in cinnamon bark varies from 0.4% to 2.8%. Reports indicate that the oil from stem bark of a commercial sample contained 75.0% cinnamaldehdyde, 5% cinnamyl acetate, 3.3% caryophyllene, 2.4% linalool and 2.2% eugenol. *C. verum* leaves contained 0.24% to 3.0% volatile oil depending on the location and method of distillation. The principal component of leaf oil namely, eugenol, varied from 65% to 92%.New cinnamon varieties *Navashree* and *Nithyasree*. recorded 75-78% eugenol in the leaves.

Clove

Clove (*Syzygium aromaticum* (L.) Merril. & Perry, syn. *Eugenia aromaticum* or *Eugenia caryophyllata*) is one of the most ancient and valuable spices of the Orient. Cloves are best used as whole. The flavour quickly deteriorates once it is powdered. Whole and ground cloves are used to enhance the flavour of meat dishes and rice. Oil of clove is extensively used for flavourig of all kinds of food products such as meats, sausages, baked goods, confectionaries, candies, table sauses, pickles etc. Clove oil is used in aromatherapy and is widely used to treat toothache. It is used in medicine for its antibacterial, antiseptic and antibiotic properties.

Clove yields three types of volatile oil - oil extracted from the leaves, stem and buds. These oils differ considerably in the yield and quality. The yield and composition of the oil obtained are influenced by its origin, season, variety and quality of raw material, maturity at harvest, pre- and post-distillation treatments and method of distillation. The chief component of the oil is eugenol. Good quality clove buds contain 15-20% essential oil which is dominated by eugenol (70-85%), eugenyl acetate (15%) and β-caryophyllene (5-12%), which together make up 99% of the oil. The minor constituents like methyl amyl ketone, methylsalicylate *etc*. are responsible for the characteristic pleasant odour of cloves. Clove leaves yield 3.0-4.8% essential oil in which 94.4% is eugenol followed by β-caryophyllene (2.9%), nerol (0.79%) and β-caryophyllene oxide (0.67%). Clove stem yields 6% volatile oil with 80.2% eugenol and 6.6 % β-caryophyllene besides several minor components

Nutmeg

Nutmeg (*Myristica fragrans* Houtt.) belongs to the family Myristicaceae with about 18 genera and 300 species. The name nutmeg is derived from the Latin word *nux muscatus,* meaning musky nut. Two important spices are derived from the fruit - nutmeg and mace. The spice nutmeg is the dried kernel of the seed and mace is the dried aril surrounding it. Both the spices have similar flavour. However, nutmeg is reported to be slightly sweeter than mace and is more preferred in food.

Fixed oil

Nutmeg contains 25-50% lipids as fixed oil comprising mainly myristic, petroselinic and palmitic acids. The extracted or expressed fixed oil is an orange coloured aromatic semi-solid, also known as concrete or nutmeg butter (because it has the consistency of butter at room temperature). It melts at 45-51°C and has a specific gravity of 0.990. It is completely soluble in hot alcohol, but sparingly soluble in cold. However, it is freely soluble in ether and chloroform. The major component of fixed oil is trimyristin. Nutmeg contained 35.7% total lipids out of which 74.9% is myristic acid. Similarly mace contained 30.4% total lipids in which oleic acid (40.3%) and palmitic acid (31.3%) predominated.

Extensive analyses have been carried out on the volatile oil of nutmeg and these have provided the major classes of compounds constituting the oil as: monoterpene hydrocarbons, 61-88%;

oxygenated monoterpenes, aromatic ethers, sesquiterpenes, aromatic monoterpenes, alkenes, organic acids and miscellaneous compounds. The essential oil content in nutmeg from South India ranged from 3.9- 16.5% whereas in mace it varied from 6-26.1% dimethyl pyrazine, , as also substituted pyrazines 2-alkoxy-3-alkylpyrazines: 2-ethoxy-3-isopropyl pyrazine , 2-methoxy-3-*sec*-butyl pyrazine, 2-methoxy-3-methyl pyrazine , in addition to a sulfur compound, 2-methylthio-3-isopropyl pyrazine.

Oleoresin

The oleoresin of cumin is brownish to yellowish-green in color which tends to darken on aging. It contains 60 ml of volatile oil in 100 g. One kg of the oleoresin is equivalent to 20 kg of freshly ground cumin in aroma and flavor characteristics. Cumin oleoresin or absolute is produced in very small quantities, either by the end-user or made to order.

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DETERMINATION OF TOTAL PHENOLIC CONTENT IN PLANT EXTRACTS

Ann Jasmine

The total phenolics of plant extracts are determined using the Folin and Ciocalteu reagent. The FC reagent contains phosphomolybdic/ phosphotungstic acid complexes. The method relies on the transfer of electrons from phenolic compounds in alkaline medium to form a blue chromophore constituted by phosphomolybdic/ phosphotungstic complex where the maximum absorption depends on the concentration of phenolic compounds. The reduced Folin-Ciocalteu reagent is detectable at absorption maxima of 650 nm using a spectrophotometer.

Reagents and Chemical: Folin-Ciocalteu reagent, Gallic acid, Distilled Water, 20% sodium carbonate

Preparation of standard Gallic acid: weigh out 100 mg Gallic acid and dissolve it in 100 mL water. Pipette out 1mL to a 10mL standard flak and make up to 10 mL with water to get a concentration of 100 □g/mL. Pipette out different aliquots of standard gallic acid (0.2 ,0.4, 0.6, 0.8 and 1mL corresponding to 20,40,60,80,100□□g) to five different test tubes (also prepare a duplicate). Make up the volume in each test tube to 3mL with water. Add 0.5 mL of Folin-Ciocalteu reagent, shake and incubate at RT for 3min. Add 2mL of 20% Na2CO3, shake well and keep the samples in boiling water bath for 30 seconds, cool. Measure the absorbance using a spectrophotometer at 650 nm. A calibration curve was constructed. A blank was concomitantly prepared containing 3mL water, 0.5mL Folin-Ciocalteu reagent and 2 mL of 20% Na_2CO_3.

Sample Preparation. Weigh About 0.5–1g of the sample and grind it using pestle and mortar with 3mL 80% Ethanol. Centrifuge the homogenate at 6,000 rpm for 0 min, save the supernatant. Repeat and make up the volume to 10mL. (Plant extract 5 mg/mL methanol may also be used). From this 0.1mL and 0.2mL was pipetted out and same procedure was repeated as that of the standard. All determinations were carried out in triplicate.

The phenolic content was calculated as Gallic acid equivalents GAE/g of dry plant material on the basis of a standard curve of Gallic acid.

DPPH radical scavenging assay

Blois M S 1958 Antioxidant determinations by the use of a stable free radical. Nat. 181: 1199–1200.

Principle

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a nitrogen centred free radical and a trap ("scavenger") for other radicals. DPPH is used as an indicator of the radical nature of that reaction. The colour of which changes from violet to yellow on reduction by H+/e- donation. Substances able to perform this reaction are antioxidants. The hydrogen atom donating property of the extracts was

determined by the decolourization of the methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Blois 1958).

Reagents:
0.004% DPPH in methanol (freshly prepared)
Standard: BHA- 10mg in 100ml methanol

Procedure
BHA was used as standard. A stock solution BHA is prepared in Methanol to achieve a concentration of 0.1mg/mL. Working standards of varying concentration 2, 5, 10, 15 and 20 g/mL are prepared and used for the assays.1mL of this working standard was added to 1 mL of 0.004% DPPH (in methanol) and the reaction mixture and was made up to 5 mL with methanol. Control sample consists of all the reagents except BHA.

The mixture was incubated in dark at room temperature for about 30 min. After incubation, the decrease in absorbance of the mixture was read at 517 nm and the percent inhibition was calculated using the given equation. Analysis was done in duplicate.
The DPPH radical scavenging activity was expressed as IC50 value, which indicates the effective concentration of the extract for scavenging 50% of DPPH free radicals.

$$\%\text{DPPH Activity} = \frac{\text{Ab of Control} - \text{Ab of Sample}}{\text{Ab of Control}} \times 100$$

ANALYTICAL TECHNIQUES IN PLANT BIOCHEMISTRY

N K Leela

Introduction

Analysis of chemical constituents of spices involves extraction and quantification. Spices contain broadly two types of constituents – those impart aroma/ smell and those contribute to colour and pungency. The constituents that impart aroma are volatile in nature and extracted by hydro-/ steam distillation. The distillate obtained by this process is a mixture of several constituents which are analysed by Gas- chromatography. The non volatile constituents are extracted by solvent extraction using a suitable solvent such as acetone, methanol, ethanol etc. The solvent extract upon removal of solvent yields a residue that contains pigments and pungent constituents. The analytical techniques used for quantification of constituents of spices involve UV-Vis spectrophotometry, HPLC and GC.

UV/Visible spectroscopy

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds and biological macromolecules. The analysis is commonly carried out in solutions in the ultraviolet-visible spectral region of the electromagnetic spectrum, ie, 200-800nm. For example, curcuminoids content in turmeric powder is determined by extraction with acetone and the absorbance of the solution is measured at 342nm.

Principle of UV spectroscopy: When a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease in intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution. According to the Beer-Lambert law the absorbance A of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length.

Thus, for a fixed path length, absorbance $A = Log_{10} (I_0/ I) = Ecl$, where I_0 = the intensity of the incident radiation, I = intensity of transmitted radiation, c = molar concentration of solute, l = length of sample cell (cm.) and E = molar absorptivity.

UV spectrophotometer consists of a light source, mono chromator, sample and reference cells, detector, amplifier and recording devices. Tungsten filament and deuterium lamps are widely used as sources of energy. Monochromator enables separation and passage of required wavelength of light through the sample which is captured by the detector and amplified and recorded.

Uses

In addition to qualitative and quantitative analysis, UV spectroscopy has several applications such as to detect the presence or absence of chromophore in the compound, to detect the extent

of conjugation in the polyenes, to identify an unknown compound, to determine the purity of a substance, to determine configurations of geometrical isomers etc.

Table 1. UV- Vis. absorption maxima of analytes

Compound	Wavelength
Piperine	342 nm
Curcuminoids	425 nm
Phenols	650 nm
Starch	630nm
Proteins	660nm
Reducing sugar	620nm
Glucose	540nm
Aminoacid	570nm

Chromatography: Chromatography is a technique which is used to separate different components of a mixture. The use of chromatography started way back in 19th century. It was first employed by the Italian-born Russian scientist Mikhail Tsvet in 1900 to separate plant pigments. During the 1940s and 1950s chromatographic technique developed substantially as a result of the work of Archer John Porter Martin and Richard Lawrence Millington Synge and now we have HPTLC, GC and HPLC coupled with other detection techniques for quantification of wide range of constituents.

The basic principle of TLC, GC and HPLC methods is the same. It involves a stationary phase and a mobile phase. Stationary phase is immobilized on the support particles or on the inner wall of the column tubing. Mobile phase is the phase that moves in a definite direction.

Depending on stationary and mobile phases various combinations of chromatographic techniques are available of which Gas-liquid and liquid- liquid chromatography are most common. In the case of GC, the mobile phase is a gas where as in HPLC it is a liquid/ solvent or a mixture of solvents. GC permits isolation of volatile constituents only. HPLC can be used for the analysis of non volatiles also.

The following terminologies are used in chromatography.

A chromatograph is the equipment that enables separation, e.g. gas chromatograph or high performance liquid chromatograph. Chromatogram in the output from the time-based graphic record of a chromatographic separation.

The eluent is the solvent that carries the analyte; the eluate is the mobile phase leaving the column; eluite is the analyte, the eluted solute. An eluotropic series is a list of solvents ranked according to their eluting power.

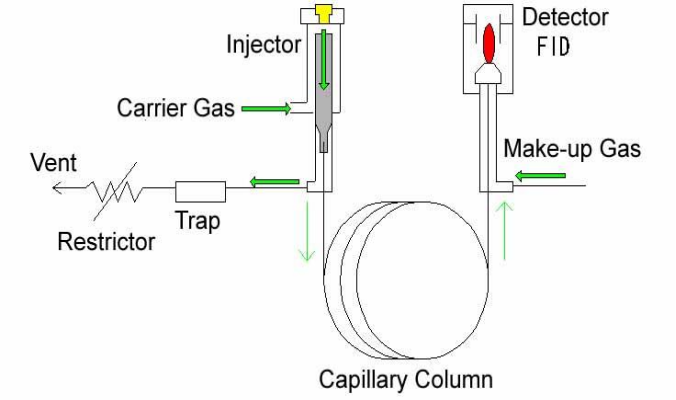


Fig.1. Structure of gas chromatograph

Detector	Application
Thermal conductivity detector/ TCD	Inorganic compounds
Flame ionization detector/ FID	Organic compounds
Electron capture detector/ ECD	Halogenated compounds
Flame photometric detector/ FPD	Phosphorous and Sulphur compounds
Flame Thermionic Detector/ FTD	Nitrogen compounds

Table 2. Different Types of detectors used in gas chromatography

High Performance/Pressure Liquid chromatography

HPLC is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instrument consists of a reservoir of mobile phase, pump, injector, column, and detector. The separation is effected based on differential partitioning of the solute between the mobile and stationary phases. Depending on the nature of the analyte, normal phase or reversed phase columns can be used. Normal phase elution involves use of polar stationary phase and non polar mobile phase; and reversed phase elution is effected with non polar stationary phase and polar mobile phase. In HPLC elution of analyte can be isocratic and gradient based on concentration of mobile phase. In isocratic elution there is no change in the concentration of mobile phase during the run. But the concentration of mobile phase varies during the run in the case of gradient elution.

Detectors: In HPLC UV, photo-diode array, fluorescence, refractive-Index and mass-spectrometric detectors are commonly used. Besides this IR, electrochemical and evaporative light cattering detectors (ELSD) etc. are also available.

Based on the quantity of the analyte and scale of operation, analytical & preparative columns are used. Small quantities of analytes (nanogram) are injected into analytical columns. For purification of analytes in large quantities, preparative columns are used.

In HPLC and GC constituents characterised by comparing the retention time of the analyte with that of an authentic standard. Retention time (RT) is the time taken for a solute to pass through a chromatography column.

Mass Spectrometer

Mass spectrometer is a device used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

The instrument consists of three major components:

1. **Ion Source:** For producing gaseous ions from the substance being studied.
2. **Analyzer:** For resolving the ions into their characteristic mass components according to their mass-to-charge ratio.
3. **Detector System:** For detecting the ions and recording the relative abundance of each of the resolved ionic species.

GC-MS

GC-MS is a combination of GC and MS with the help of proper interface. It is a powerful technique for detection and quantification of volatiles in a mixture where the components in the mixture are separated by gas chromatography and identified by their mass spectra. It is very useful for characterization of constituents of spice volatiles.

Process: During analysis the sample is introduced into the column through injection port and simultaneously carrier gas is passed through the column. Helium or nitrogen is used as carrier gas in GC which carries the analytes from injection port to the detector. During this process the temperature of column compartment is increased gradually to facilitate the separation. The separated components are then directed to the detector unit at their characteristic retention time. The detector captures signals from analytes and is recorded.

In GC- MS the analytes from GC column are directed to Mass spectrometer where the isolated components are subjected to collision with energized electrons and undergo fragmentation. The fragmented moieties are separated based on charge to mass ratio in a strong magnetic field and mass spectrum of each analyte is recorded.InLC-MS, HPLC is coupled with MS enabling separation of analytes and characterization of nonvolatile constituents by their mass spectra.

ADVANCES IN PROTEIN CHEMISTRY: NEW CONCEPT OF PROTEIN FOLDING

Anees K

Chemistry of protein was always been an important aspect of biochemical researchers as the protein enjoys the central role of a functional molecule in the living world. Over the period, since its discovery as a biological catalyst, scientists have attempted to explain the process and forces involved in making of a protein to a functional moiety once it is synthesised inside the cell. The process of making the primary sequence of a protein into a biologically active entity is referred as protein folding. The general understanding of the protein folding can be briefed as follows:

1. The nascent polypeptide synthesised after the protein synthesis, once released to the cytoplasm, gets folded by itself or with the help of chaperons
2. The folding principle is such that the side chain of the each amino acid plays the key role in determining the positioning of the amino acid in the final 3-D structure.
3. The hydrophobic residues, being repellent to water, tend to hide itself from the aqueous environment of the cytoplasm by burying itself deep inside the 3-D structure
4. The hydrophilic residue, being water loving, tends to be on the surface of the protein structure having good contact with the aqueous cellular environment
5. During the process of folding, the protein tries all possible combination wherein the free energy of the state is minimal, due to which many molten globules will be formed with little more stable than the other intermediates, as explained by the energy funnel of protein folding. Proteins keep folding in the correct conformation as it is synthesized in a step wise fashion. Initially the unfolded peptide has highest randomness (entropy) and as it keeps assuming more folded structure the entropy keeps decreasing.
6. Preferential interaction of amino acid side chains arise out of the four well established non-covalent interactions such as hydrogen bond, electrostatic force, hydrophobic interactoins and van der Waal's force

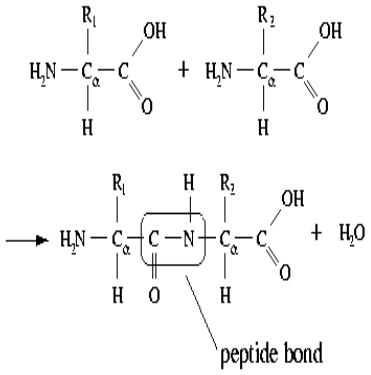
The general notion is that only one native (functional form is present for a protein and all other structures that a poly peptide assumes during folding process are its non-native form.
The classical experiment on renaturation of ribonuclease lead to the formulation of Anfinsen's dogma (also known as the thermodynamic hypothesis) which postulates that at least for small globular proteins, the native structure is determined only by the protein's amino acid sequence. Cyrus Levinthal proposed that for a protein to fold to its native state, a random conformational search does not occur, but the protein fold through a series of meta-stable intermediate-states. The Levinthal paradox forces many researchers to think in a radical manner that the current understanding of more complex nature of protein folding is meaningfully wrong and there lies another way of looking at the protein folding. Under this context it is important to look at a totally different concept of protein folding proposed by Aditya Mittal and his group in 2010.

A newer view of protein folding by Mittal *et al*., which takes into account solvent mediated and amino acid shape and size assisted optimization of the tertiary structure of the polypeptide chain to make a functional protein has been proposed. This will create a new horizon on folding biochemistry of proteins in days to come. The main features of the proposed scheme of the protein folding can be summerised as follows:

1. Rigorous analyses of several thousand crystal structures of folded proteins revealed a surprisingly simple unifying principle of backbone organization in protein folding.
2. Direct consequence of a narrow band of stoichiometric occurrences of amino-acids in primary sequences, regardless of the size and the fold of a protein is attributed for protein folding.
3. "Preferential interactions" between amino-acids do not drive protein folding. All proteins occur in predominantly aqueous environments and water is known to have unique properties that thermodynamically (i.e., given infinite time) exclude any solute, independent of its solubility, given a high enough concentration of the solute.
4. A protein molecule is a large solute, equivalent to a group of highly concentrated small solutes, within the hydration layer of the protein (independent of whether the protein exists as an open chain or in some globular form). Thus, aqueous environment would force the surface-to-volume minimization of the solute within the hydration layer. This would constrain a protein to pack/fold in an "exclusion by water" manner to minimize the overall surface-to-volume ratio, governed by shapes of individual amino-acids. This must be done while satisfying the structural constraints of the primary sequence composition and constitution (i.e., the order in which a given stoichiometry of amino-acids appear). As per the model proposed by Mittal *et al.*, the most important parameters for protein folding (and protein engineering) are (i) exclusion by water and (ii) shape characteristics of individual amino-acids along the sequence that would minimize the surface-to-volume ratio. It can be visualized that protein folds like fitting "Lego Blocks" tied with a thread and packed into the lowest surface-to-volume ratio.

It is very important to note that the solvent accessible area (TSA) analysis of each residue in a protein for all the available crystal structures revealed that irrespective of nature of residues, TSA increases with frequency of occurrence of residues inside the poly peptide. The approach adopted by the authors was as follows: First, they investigated the backbones of several thousands of folded proteins from their published crystal structures in the protein data bank. Assuming protein folding resulted from specific amino-acid interactions, the backbones of folded proteins would be organized within the constraints of defined "neighbourhoods" for Cα atoms of each amino acid. If two amino-acids were to interact with each other (e.g., via side-chains), their respective Cα atoms would be expected to occur in fixed neighbourhood relative to each other, regardless of their actual position in the protein. Coordinates of all atoms in crystal structures of 3718 proteins were taken from the Protein Data Bank and extracted the C_α coordinates for all the amino-acids and neighbourhood analysis was done. For each protein, a 20 x 20 matrix of number of "neighbours", within a defined neighbourhood distance, resulted by considering each of the

amino-acids individually. The neighbourhood distances fixed at 0-9 Å, with increments of 1 Å; 10-90 Å, with increments of 5 Å; Distances of 0-3 Å were chosen as an internal check (since zero neighbours were expected at these distances)



Structure of peptide bond showing the C_α atom forming the back bone

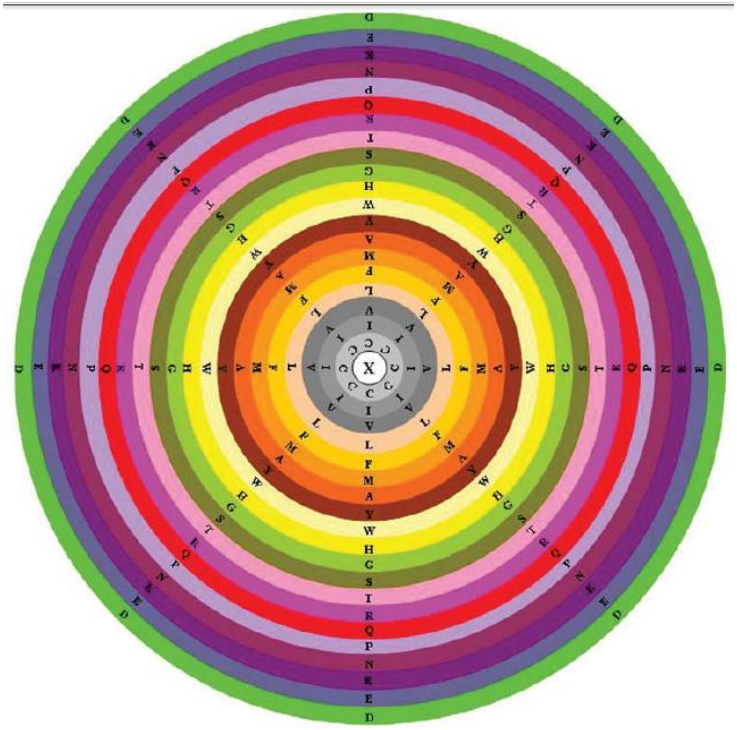
They investigated the presence of preferential neighbourhoods expected to arise out of the four well established non-covalent interactions (hydrogen bonds, electrostatic, hydrophobic and van der Waals) by plotting number of times each of the 20 amino acids appears as a neighbour (no. of contacts) for each amino acid within a defined neighbourhood. A clear sigmoidal trend was observed regardless of the identity of the neighbouring amino acid. These sigmoidal trend's lead to two possible explanations: either it could be due to existence of several spatial distributions, each uniquely defining neighbourhoods of each amino-acid based on its presumed preferences or it could imply a single underlying spatial distribution of neighbourhoods for all amino-acids, regardless of their conventional classification. If the former were true then one would require different equations to fit the different sigmoids. If the latter were true then one would need only one equation that would fit all of the sigmoids for all the amino-acid neighbourhoods. The latter was found true. When the authors plotted the sum of the 20 asymptotes for each amino-acid against the average percentage occurrence of that particular amino-acid in 3718 folded proteins tested, total possible contacts for Cα of a given amino-acid, correlates excellently ($r^2 = 0.99$) with percentage occurrence of that particular amino-acid in 3718 proteins indicating "absence" of any preferential interactions between amino-acids. This argument was supported by low correlation ($r^2 = 0.88$) with percentage occurrence of given amino acid and total possible contacts for Cα of a given amino-acid for unstructured proteins.

To answer the question of protein folding an model-independent analysis of the sigmoid curves were carried out. By drawing a tangent to the steepest part of the sigmoid, authors were able to define three parameters for any given pair of amino acids. "Close-Contacts", "Intermediate-Contacts" and "Long-Contacts". Each of the three parameters for all of the 400 sigmoids were calculated. Since there is amino acid independent spatial distributions, the

frequency distributions for the three parameters extracted out of the 400 sigmoids would show a single uniform distribution. Frequency distributions of each of the three parameters indicated presence of multiple populations rather than a single population. These apparent multiple populations were confirmed to be statistically mutually exclusive by the K-means clustering technique. It would be expected that short-range (occurring within distances of 5-12 Å) pairings of amino acids would show more distinct populations in the presence of specific side-chain interactions. And the medium-range (~20-30 Å) and the long-range pairings (~40-60 Å) of amino acids would be expected to show much lesser number of pairings due to existence of very few known chemical interactions at these distances. If neighborhoods of amino acids were governed by side-chain interactions, then one would expect to see greater "splitting" at short range compared to the Intermediate- and Long ranges. If solvent were playing a major role then the Long and Intermediate range neighborhoods would show greater splitting. At the long-range distances, solvent (predominantly aqueous) is the primary constituent rather than amino acids of a protein. Thus, the results clearly indicate the important, and quite under-rated, role of aqueous surroundings in protein folding. Salient findings of the study can be summerised as follows:

- None of the short range clusters have any common members as nieighbours.
- There was absence of any neighbours in the first cluster for some amino acids (absence of a 'shell' of amino acids immediately around P, Q, N, D, E, R and K).
- Absence of any neighbors for some amino acids in the third cluster(A, V, I, L, Y, F, W, M and C are closely surrounded by a shell of amino acids) was observed
- Regardless of the conventional view of amino acids, the Cα of cysteine is always found in close proximity to all amino acids, especially itself

Accordingly an integrated 'wheel' of neighbourhood was proposed by the authors defining the probability of a given amino acid as a neighbour for any amino acid in folded proteins which is to scale and only the order in which the amino acids appear is important. The wheel is location independent for amino acids.



Integrated ‘wheel’ of neighbourhood of proteins in a folded protein

The major findings and conclusions from the current protein folding model can be summerised as follows:

There is a single spatial distribution of the backbone Cα atoms regardless of the fold and size, with Cysteine-Cysteine pairs being the sole exception.

The number of contacts is a direct result of its frequency of occurrence (stoichiometry) in the primary sequence.

Cysteine, is a “space-filler”. It is found closest to itself, and to all other amino acids.

Exclusion by water is the predominant factor for the protein fold, a “water-centric” view on protein folding rather than a “proteincentric” or a “residue-centric”. Water excludes the polypeptide chain to assume a minimum surface-to-volume ratio. Driving force for surface-to-volume ratio minimisation is the individual shapes of the amino acids.

The conventional classifications of amino acids (e.g. polar, non-polar) and their interactions do not play any significant role in protein folding. Rather, they may be merely post-facto inferences. One can visualize protein folding like fitting “Lego Blocks” tied with a thread and packed into the lowest surface-to-volume ratio.

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AN INTRODUCTION TO CHROMATOGRAPHIC SEPARATIONS

Ann Jasmine

Chromatography is used for separating mixtures of compounds, identifying unknown compounds, establishing the purity or concentration of compounds, monitoring product formation in the pharmaceutical and biotechnology industries. The mixture to be analysed is placed on the stationary phase (solid/liquid) and a mobile phase liquid/gas) is allowed to pass through the system. The molecules present in the mixture get distributed between a stationary and a mobile phase. Chromatography is classified according to the type of equilibration process involved and equilibration process controlled by the type of stationary phase

Adsorption Chromatography

The stationary phase is a solid. Sample components are adsorbed on the solid stationary phase. The mobile phase may be a liquid or a gas. The sample components are distributed between the two phases Example: Thin layer chromatography.

Partition Chromatography

The stationary phase is a liquid supported on an inert solid. The mobile phase may be a liquid or a gas.

Classification based on Mobile Phase

Liquid chromatography: mobile phase is a liquid. (LLC, LSC).
Gas chromatography: mobile phase is a gas. (GSC, GLC).

Liquid chromatography (LC)

Thin layer chromatography (TLC): the solid stationary phase (silica/alumina) supported on glass, plastic or aluminium plates.
Paper chromatography (PC): the stationary phase is a thin film of water molecules H-bonded to surface of Paper/Cellulose
Column chromatography (CC): stationary phase is packed in a column.

Thin layer chromatography (TLC)

TLC is a simple method for testing the purity and identifying compounds. It is a useful technique because it is relatively quick and requires very small amounts of material. Hydroxyl-terminated Silica $[SiO2]_n$ supported on plate (aluminium foil) is used as the stationary phase and the mobile phase (eluent) is a developing liquid which travels up the stationary phase, carrying the samples with it; e.g. hexane, ethyl acetate, alcohol, ether etc. Components of the samples will separate on the stationary phase according to their affinity to adsorb on the stationary phase versus how much they dissolve in the mobile phase. Separation of compounds in a mixture is possible because compounds have different polarities. Polar compounds will adhere more strongly to

silica gel than non-polar compounds due to intermolecular attractive forces. The non-polar compounds will travel faster through the stationary phase with the help of mobile phase. Non-polar compounds will elute first and polar compounds will elute last.
The most common visualization technique is to hold the plate under a UV lamp (Commonly 254 nm). Not all spots are visible under UV. So, Iodine, $KMnO_4$ solution, vanillin solution, ninhydrin, $FeCl_3$ solution, Ceric Ammonium Molybdate (CAM), Sulfuric acid etc. may aid in the visualisation.

Retention factor

$$R_f = \frac{\text{distance moved by substance}}{\text{distance moved by solvent front}}$$

It is characteristic for any given compound on the same stationary phase using the same mobile phase. For substances that are highly non-polar and are very soluble in the liquid R_f will be close to 1.

Column Chromatography

Column Chromatography works on the same principle of TLC (differential migration of components of the mixture with the mobile phase and the stationary phase) except that the sample solution flows down a column of silica gel or alumina filled with solvent
Stationary phase is held in a narrow tube through which the mobile phase elutes under the effect of gravity. Powdered silica/alumina is used as the solid adsorbent and packed in a glass column. Cellulose, CaCO3, starch etc. may also be used. Two techniques used are used for filling the column - dry & slurry methods. In all cases, care should be taken to avoid inclusion of air bubbles

Sample Loading

Wet application: Dissolve the sample in the initial mobile phase and apply by pipette to the top of the column. This is very good method but in most of cases the samples are not soluble in the initial mobile phase.
Dry loading: Dissolve sample in any volatile solvent. The sample solution is then adsorbed on small weight of adsorbent and the solvent is allowed to evaporate. The dry adsorbent loaded with the sample is then applied to the column.

Mobile phase

Solvent plays important role. The choice of the column mobile phase is decided after TLC study in different solvent systems. Good solvent system must produce R_f value less than **0.6** for all materials to be separated by the column. If the system moves them more and produces higher R_f no separation will occur. Systems that do not move spots at all on TLC are not good for column separation. Polarity of the solvent affect the relative rate at which the compound moves through the column. Common Eluent Systems

- Hexane & Diethyl Ether – Low polarity compounds
- Hexane & Ethyl Acetate – Mid polarity compounds
- Toluene & Acetone – Aromatic compounds
- DCM & Methanol – High polarity compounds

Elution Techniques

Isocratic elution- Addition of solvent mixture of fixed composition during the whole process.

Gradient elution-The polarity of the system increased gradually during separation by increasing the proportion of the more polar solvent. A typical gradient may be start with. $CHCl_3$, followed by $CHCl_3$/MeOH mixtures with gradual increase in % of MeOH till all spots are eluted from the system

Polar components adsorb more strongly to the polar silica and elute after the less polar components which move more quickly with the non-polar (relative to silica) solvent. Fractions of certain volume are collected evaporated to small volume and spotted on TLC. Similar fractions are collected together for more purification or crystallization

Differential migration

Different compounds move through the system with different rates of movement this is called "differential migration". The speed of any compound in the mixture is determined by the number of molecules of that compound in the mobile phase.

Suppose we have mixture of materials “A” and “B”, where A has more affinity to mobile phase i.e. large number of molecules are present in the mobile phase and B has more affinity to stationary phase i.e. small number of molecules are present in the mobile phase.

Mathematical representation of differential migration:

Ux: velocity of material X.

U: velocity of solvent (mobile phase).

R: fraction of material X in the mobile phase.

$$\mathbf{Ux = UR}$$

If R = 1 i.e. all the compound molecules are present in the mobile phase.

$$Ux = U * 1$$

$$Ux = U$$

:. Matenial X will move with the solvent velocity.

if R = 0.0 i.e. all the compound molecules are present in the stationary phase.

$$Ux = U * 0$$

$$:. Ux = 0$$

Material X will not move at all. For any material to be separated through the system it must be distributed between the mobile phase and stationary phase.

Distribution coefficient

The factor that control the distribution of any material between the two competitive phases is called "Distribution coefficient" or "Capacity factor" or "Mass distribution ratio" - K

$$K = Cs/Cm$$

Cs : Is the total number of moles of the compound (concentration) in the stationary phase.

Cm : Is the total number of moles of the compound in the mobile phase.

Larger K means that the material retained longer on the column i.e. move slowly. Smaller K means faster movement.

Factors affecting separation:

Factors due to Stationary Phase:

Particle size of the stationary phase: Reducing the particle size increases the surface area and improves separation. However, reduction of the particle size will decrease the flow rate of the mobile phase. In HPLC - very fine particles are used to get very good separation. The flow rate problem is solved by the use high pressure pumps to push the mobile phase through the stationary phase. Columns are made of stainless steel to withstand the high pressure.

Adsorbent activity: The choice of the suitable adsorbent is very important.

Uniformity in packing of the column: If the stationary phase is not packed uniformly then the bands will be irregular and less uniform resulting in poor separation.

Concentration of the mixture: the proper ratio between sample to be separated and the amount of stationary phase is very important too much samples resulted in bad separation.

Factors due to Mobile Phase:

Selection of the proper mobile phase: Very polar mobile phase will wash out all components without any separation. On the other hand very non polar mobile phase will result in broad band and poor separation.

Rate of flow: Slower flow rate usually resulted in a better separation and narrower bands.

Consistency of flow: A continuous flow of the mobile phase during the whole experiment give better separation than interrupting the flow then continue it later.

Factors due to Columns:

Column dimensions: Increasing the length of the column improve separation. However, that usually leads to slower flow rate. Also increasing the column length sometimes is impractical.

Column temperature: Increasing the temperature usually reduces the adsorption power of the stationary phase and increase elution speed. This may leads to decrease in the efficiency separation.

PLANT GROWTH REGULATORS

K.S. Krishnamurthy

Plant Growth Regulators are defined as small, simple chemicals produced naturally by plants to regulate their growth and development. They significantly influence the growth and differentiation of plant cells, tissues and organs. They are chemical messengers that are synthesised in one part of the plant and act in a totally different plant part. There are five groups of plant hormones viz. auxins, gibberellins, cytokinins, abscisic acid and ethylene. Among these ethylene is termed as a gaseous hormone. Lately, brassinosteroids and strigolactones are also considered as plant hormones. Some even consider jasmonic acid also as hormone.

Auxins

Auxins (indole 3-acetic acid) were the first plant growth hormone to be discovered. Auxins include both natural and synthetic compounds which are having growth regulating properties. Plants produce Natural auxins that are synthesised by plants are indole-3-acetic acid (IAA) and indole butyric acid (IBA). Indole acetic acid is predominantly synthesized in the stem apices. Root apices also synthesise indole acetic acid, but to a lesser extent compared to stem apices. Naphthalene acetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) are examples of synthetic auxins. Auxin is transported from the site of synthesis to the site of action, which is not far away. Auxin is also translocated to other plant parts. Auxin is translocated from apex to the base (polar transport which is called basipetal movement), though acropetal movement (base to apex) has also been observed, the amount transported through base to apex is almost negligible. The ratio between basipetal and acropetal movement is approximately 3:1.

Auxins perform many functions in plants which are known as formative effects. These are,

Induces cell enlargement: It is the main action of auxins. Cell enlargement is caused by solubilisation of carbohydrates, loosening of wall micro-fibrils, synthesis of more wall materials, increased membrane permeability and respiration.

Promotes root formation: Promotes initiation and growth of adventitious roots in cuttings at concentration which is inhibitory for growth of intact root.

Induces apical dominance: Apical dominance is the phenomenon by which presence of apical bud does not allow the nearby lateral buds to grow. When the apical bud is removed, the lateral buds sprout. Auxin maintains apical dominance indirectly by stimulating the production of ethylene, which directly inhibits lateral bud growth.

Inhibits abscission: Auxin suppresses abscission of young leaves and fruits. Lower levels of auxins is directly correlated with the formation of abscission zone. However, auxin promotes the abscission of mature or older leaves and fruits.

Enhances cambial activity: Cambial activity is directly influenced by auxin concentration. Auxin also controls xylem differentiation. Auxin initiates division of the vascular cambium in the spring.

Promotes development and differentiation of callus: In tissue culture, the development of callus or mass of undifferentiated cells is promoted by auxin. Differentiation of callus takes place only if both auxin and cytokinin are present in the medium.

Flowering and sex expression: Auxins promote flowering in plants like pineapple but inhibits flowering in many plants. Auxins induce femaleness in some plants.

Seedless fruits: As the seeds mature, they release auxin to the surrounding flower parts, which develop into the fruit that covers the seeds. The carpels producing seedless or parthenocarpic fruits have a higher internal production of auxin that supports the development of fruits as in banana.

Activates tropic responses: Differential distribution of indole 3-acetic acid produces tropical plant responses like phototropism and geotropism.

Induces ethylene synthesis : Higher concentration of IAA induces the synthesis of ethylene.

Agricultural/Horticultural applications of auxins

- Induction of parthenocarpy i.e. development of fruits without fertilization
- IBA is extensively used for plant propagation i.e. for rooting of stem cuttings
- For weed control, 2, 4-D is commonly used as a herbicide (to control dicot weeds).
- In tissue culture, auxins are used to induce callus from explants and for morphogenesis

Gibberellins

Gibberellin was discovered from the fungus *Gibberella fujikuroi* which causes bakane' disease of rice seedlings where the most notable feature is the huge stem elongation. Thereafter, more than 100 gibberellins have been identified from fungi to higher plants. and have been designated as GA_1, GA_2, GA_3 etc. among which GA_3 commonly referred as gibberellic acid was the first to be discovered and is the most studied gibberellic acid. Developing seeds and fruits, young leaves of developing apical buds and elongating shoots are the sites of synthesis of gibberellins. GA moves inside the plant in both acropetal and basipetal directions and the efficiency is more when the movement is acropetal.

Formative effects of gibberellins

Induce stem elongation and growth of other aerial parts: Gibberellins induce stem growth and internode elongation especially in genetically dwarf varieties in maize, peas etc. They also help in cell growth of leaves and other aerial parts. Hence, it can be said that they increase the size of aerial parts like stem, leaves, flowers and fruits

Induce bolting and vernalization: Gibberellins induce sub-apical meristem to develop faster. This causes elongation of reduced stem or bolting in case of rosette plants such as in cabbage and

beet root. Vernalization or low temperature requirement of some plants can be replaced by gibberellins.

Break seed dormancy and promote seed germination: Gibberellins help in breaking the dormancy of seeds, buds, tubers etc. Gibberellins stimulate the production of some messenger RNAs and also hydrolytic enzymes such as amylases, lipases etc. which solubilize the reserve food of the seed and is transferred to embryo which helps in its growth.

Induce parthenocarpy: They can induce development of seedless fruits from unfertilized pistils in fruits like grapes, apple, etc. Along with auxin, gibberellins control fruit growth and development. They help fruits like apples and grapes to elongate and improve their shape.

Flowering and sex expression: Gibberellin treatment induces flowering in long day plants during non-inductive periods. Gibberllins induce maleness in plants like cannabis where they promote formation of male flowers on female plants. In monoecious plants of cucurbits, they can replace female flowers with male flowers

Agricultural /horticultural applications

- The brewing industry uses GA_3 to speed the malting process.
- Spraying gibberellins increase sugarcane yield by lengthening the stem.
- Used to hasten the maturity period in young conifers and promote early seed production.
- Help to promote bolting (i.e. sudden growth of a plant just before flowering) in cabbages and beet.
- Used to increase fruit size in grapes and apples.
- In tissue culture, gibberellins are used to induce organogenesis, particularly adventitious root formation.

Cytokinins

Named because of their discovered role in cell division (cytokinesis), the cytokinins have a molecular structure similar to adenine. F. Skoog and his co-workers observed a mass of cells called ‘callus’ in tobacco plants. These cells proliferated only when the nutrient medium contained auxins along with yeast extract or extracts of vascular tissue. Skoog and Miller later identified the active substance responsible for proliferation and called it kinetin which is a cytokinin. Kinetin does not occur naturally but scientists later discovered several natural (zeatin) and synthetic (benzyl adenine) cytokinins. Natural cytokinins exist in cells where rapid cell division occurs such as in root apices and developing shoot buds. Most of the cytokinins required for the plant body are synthesized in the root tip meristems, and then they are translocated to different regions particularly to meristematic region and expanding tissues; transportation is through xylem stream. Now it is known that a good source for cytokinin is coconut liquid endosperm and milky endosperm of sweet corns.

Formative effects

Promote cell division, cell elongation and morphogenesis: Cytokinins are essential for cytokinesis though chromosome doubling can occur in their absence. In the presence of auxin, cytokinins bring about division even in permanent cells. For cell division in callus and for differentiation of tissues and organs, both auxins and cytokinins are required. Cytokinins also promote cell elongation.

Delay senescence: Cytokinins delay the senescence of leaves and other organs by mobilisation of nutrients.

Suppress apical dominance: Presence of cytokinin in an area causes preferential movement of nutrients towards it. When applied to lateral buds, they help in their growth despite the presence of apical bud. They thus act antagonistically to auxin which promotes apical dominance.

Break seed dormancy: Cytokinins break seed dormancy of various types, including red light requirement of tobacco and lettuce seeds.

Help in differentiation: Cytokinins induce formation of new leaves, chloroplasts in leaves, lateral shoot formation and adventitious shoot formation. They also bring about lignification and differentiation of inter-fascicular cambium. They also promote phloem transport.

Increase resistance to biotic and abiotic stresses: Cytokinins increase resistance to abiotic (high or low temperature) and biotic (disease) stresses.

Flowering and sex expression: Cytokinins induce flowering by replacing photoperiodic requirement in certain plants. Cytokinins induce femaleness in some plants. They also help in parthenocarpy in certain cases.

Agricultural/Horticultural applications

- Cytokinins are essential component of tissue culture for morphogenesis
- Shelf life of cut shoots and flowers can be prolonged by employing cytokinins.
- Helpful in developing abiotic and biotic stress resistance
- Cytokinins application can delay the senescence of intact plant parts.

Abscisic Acid (ABA)

Three independent researchers reported the purification and characterization of three different inhibitors – Inhibitor B, Abscission II and Dormin. Later, it was found that all three inhibitors were chemically identical and were, therefore, together were given the name abscisic acid. It is generally reported that the hormone was first isolated by Addicott (1963) from cotton bolls. It is produced in many parts of the plants but more abundantly inside the chloroplasts of green cells. The hormone is formed from mevalonic acid or xanthophyll. It is transported to all parts of the plant through diffusion as well as transport channels (phloem and xylem).

Formative effects

Induces bud and seed dormancy: ABA induces dormancy of buds during winter especially in temperate regions. ABA induces seed dormancy in many species. This helps the seeds to tolerate

desiccation and high and low temperatures. The sprouting occurs when gibberellins level increases and ABA level decreases.

Promotes abscission and senescence: ABA promotes abscission of flowers and fruits especially young ones. ABA also stimulates leaf fall through prevention of RNA and protein synthesis.

Induces stomatal closure: During abiotic stress, ABA is synthesised de novo rapidly which induces the closure of stomata and hence prevents transpiration. This helps the plants to tolerate abiotic stresses. Hence, ABA is also known as stress hormone.

Stops cambium activity: ABA stops mitosis in vascular cambium during winter.

Inhibits starch hydrolysis: ABA inhibits gibberellin mediated amylase formation during germination of cereal grains.

Promotes flowering: ABA, in small quantities promotes flowering in some short day plants like strawberry, black currant etc.

Induces rooting of stem cuttings: ABA induces rooting of stem cuttings in poinsettia, bean etc.

Agricultural/Horticultural applications

- ABA is used as an antitranspirant (to induce stomatal closure) under water limited conditions
- Can be used for inducing flowering in some short day plants in un-favourable photoperiods.
- Can be used for inducing rooting in many stem cuttings.
- ABA can be used in prolonging dormancy of buds, storage organs and seeds under unfavourable conditions so that they can be made to sprout once the conditions are favourable.

Ethylene

It is a gaseous hormone which stimulates transverse or isodiametric growth but retards the longitudinal one. Businessmen dealing with storing and shipping of fruits had known quite early that a rotten or ripe fruit could trigger early ripening of other fruits present nearby. Cousins found that ripe oranges produced a volatile substance that hastened ripening of unripe bananas nearby. With the help of gas chromatography, Gane identified the ripening causing volatile substance as ethylene. Ethylene was recognised as a plant hormone by Crocker. Ethylene is produced in plants from the amino acid methionine. It is synthesised in almost all plant parts viz. roots, leaves, flowers, fruits and seeds.

Formative effects of ethylene

Flowering and sex expression: Ethylene induces synchronous flowering in plants like pineapple and mango leading to synchronous fruit set. It helps in the production of female flowers in plants which are genetically male plants (eg. cannabis). It also enhances the number of female flowers and hence the fruit set in cucurbits.

Promotes fruit ripening: It promotes ripening of climacteric fruits such as apple, banana, mango etc. and dehiscence of dry fruits

Growth: Ethylene inhibits longitudinal growth but stimulates transverse or horizontal growth and swelling of axis.

Gravity: It decreases the sensitivity to gravity. Roots become Apo-geotropic while stems turn positively geotropic. Leaves and flowers undergo drooping. The phenomenon is called epinasty. Seedlings develop tight epicotyl hook.

Senescence and abscission: It hastens the senescence and abscission of leaves, flowers and fruits.

Induces apical dominance: Ethylene promotes apical dominance in plants

Breaks dormancy: It breaks the dormancy of seeds, buds and storage organs but prolongs dormancy of lateral buds

Promotes root initiation: In low concentration ethylene helps in root initiation, growth of lateral roots and root hairs. This increases the absorption surface of the plant roots.

Agricultural/Horticultural applications

- It is widely used for stimulating colour development and ripening of some fleshy fruits like banana, mango, apple, tomato etc.
- Used for enhancing female flowers and hence fruiting in cucumber.
- Used for promoting flowering and also synchronization of flowering in pineapple
- It is used to stimulate sprouting of storage organs like rhizomes, corms, tubers and seeds

Brassinosteroids

Brassinosteroids are a group of some 40 different steroids that are synthesized by plants and are potent hormones affecting many aspects of plant growth. The most abundant one is named **brassinolide**. It was first isolated from the pollen of *Brassica napus*, hence the name. These hormones act synergistically, or at least additively, with several other hormones such as auxin and the gibberellins. However, they are far more potent than the other hormones as they act at much lower concentrations than the others.

Reported functions of brassinosteroids

- Promote cell elongation
- Promote apical dominance
- Promote leaf senescence
- Enhance seed germination
- Enhance gravitropism
- Increase the production of ethylene
- Inhibit root growth
- Promote the formation of xylem
- Prevent premature abscission of fruit

Strigolactones

Strigolactones are a group of chemical compounds produced by plant's roots. Strigolactones were first isolated in 1966 from cotton plants, specifically from the roots. Due its mechanism of action, these molecules have been classified as plant hormones.

Functions of strigolactones

- Promote the germination of parasitic organisms that grow in the host plant's roots (*Striga* sp or witchweed)
- Signal mycorrhizal fungi , especially arbuscular mycorrhizal fungi, to connect to the root system forming a mutualistic relationship and provide nutrients mainly phosphate.
- Inhibit branching in plants

Detection of plant hormones

There are specific bioassays for each hormone and they can be quantified through these bioassays. The well known bioassay for auxins is Avena coleoptile curvature bioassay and dwarf pea epicotyl elongation for gibberellins. Cucumber cotyledon expansion is a classic bioassay for cytokinins and cucumber hypocotyl elongation inhibition is a simple bioassay for abscisic acid. Pea stem swelling test is used as a bioassay for ethylene. But the bioassays are not sensitive as the compounds are not in purified form and the observed response may not be solely due to the hormone alone as some other compounds in the plant extract used for bioassay may also be contributing. But there are very sensitive detection techniques for each hormone and these sensitive techniques involve elaborate purification procedures. Such techniques are routinely followed by researchers to detect hormones in pico gram levels.

GC/MS and LC/MS provide the separation and sensitivity required for accurate quantification of compounds present in trace amounts in complex matrices, such as auxins and cytokinins. ELISA is still commonly applied to auxin quantification. Immuno assay (using monoclonal antibodies) has also been used to quantify cytokinins and abscisic acid. HPLC is also commonly used to quantify hormones such as auxins, cytokinins and ABA. Immuno assay, GC/MS/MS and HPLC-LC/MS are used for accurate quantification of gibberellins. Gas chromatography is commonly employed to quantify the amount of ethylene present in plant tissues.

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DECIPHERING BIOSYNTHETIC PATHWAY: TOOLS AND TECHNIQUES

Anees K

Life in biochemical terms can be expressed as series of biochemical reaction that occur in an orchestrated manner under an aqueous environment. A slight deviation from this will result in sickness of ill health of a living organism. Major deviation or deviation in vital part of the biochemical reaction results in death of an organism. Therefore the boundary between being alive and dead is very thin from biochemical perspective. The orchestrated manner of biochemical reactions are arranged in the form of biosynthetic pathways.

The cell, being the factory of life operations, therefore plays a vital role in existence of life. Four attributes of cell such as compartmentalisation, pH and temperature maintenance, existence of micro gradient and stream flow of cytoplasm are greatest example of beauty of nature for creation of life. Cell has got specific compartments to perform specific duties due to which the possibility of cross reaction of biochemical activities are minimised and efficiency of bio-process is enhanced. The pH of cell in its compartments and intra compartments are fine tuned such that the specific requirement for optimal enzymatic activity is maintained. If we consider the cell as a engine which works continuously, and maintaining its temperature, one can predict that living world comprises millions of isothermal engines which works with most possible efficiency. The difference in concentration gradient of a particular ion in micro meter level distance acts as signal to initiate a new life process many times. All these factors points to the fact that the life itself as a phenomenon which is most complex and yet to be scientifically explained.

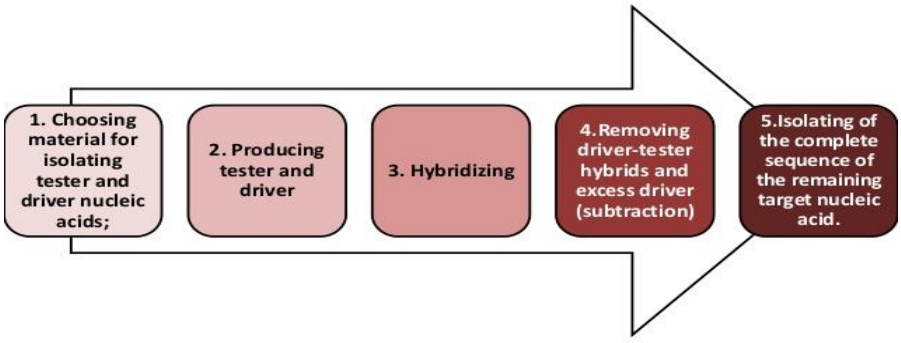
All living forms has to follows law of thermodynamics. According to the first law of thermodynamics, the energy of universe is constant. Energy can be transferred and transformed, but it cannot be created or destroyed. This is also known as law of conservation of energy. Every energy transfer or transformation makes universe more disordered popularly known as the concept of entropy. One of the unique property living world is to create and maintain the order. Therefore more amount of disorderness has to be made outside the living world to follow thermodyanamics principle. The chemical reactions occurring in every cells require boost in reactivity of reactants and precise chemical control. This is exhibited through enzymes and its interdependence. As a whole, metabolism is concerned with managing the material and energy resources of the cell. Some metabolic pathways release energy by breaking down complex molecules into simpler compounds (Catabolic). There are also anabolic pathways or biosynthesis, which consumes energy to build complicated molecules from simpler ones. It is very important to know the 'how' part of the discovery of each biosynthetic pathway to appreciate the beauty of the same. Generally biosynthetic pathways are discovered for its physiological significance, economic importance of the compound, disease management and diagnostics tool development and most importantly for man's curiosity. For a beginner, to know

te art of biosynthetic pathway discovery, it is of utmost important to know from where to start the discovery process.

The two major sources of biosynthetic pathway discovery are the mutants and the contrasting phenotype. Mutants are the main source through which biosynthetic discovery has been made possible with. Many of the mutations are lethal and therefore very tough to use it as a source for study. The conditional mutants are required to study the biosynthetic pathway discovery related to house-keeping genes. Some mutation leads to contrasting phenotype, without causing lethality. Contrasts can be expressed in various forms viz. Phenotype/chemotype, difference in two life stages of an organism, difference in organs/tissues of an organism. In all these cases, the differential expression of the genes/proteins/metabolite of these contrasting objects are studied for unravelling a pathway. These can be detected using various biological tools depending upon the at what level one attempts to explore.

Techniques at RNA level

If one want to know the differential expression in two contrasting phenotype (for eg. Between white and red coulour mutant) at RNA level, two techniques can be mainly adopted namely, Suppression subtractive hybridisation (SSH) and comparative transcriptome analysis. SSH is a technique that enables to compare two populations of mRNA and isolate differentially expressed genes. It requires no prior knowledge of genome and does not preclude any novel gene discovery. Moreover, it enriches differentially expressed genes during the process. The overall outline of the proves can be summarised as follows:



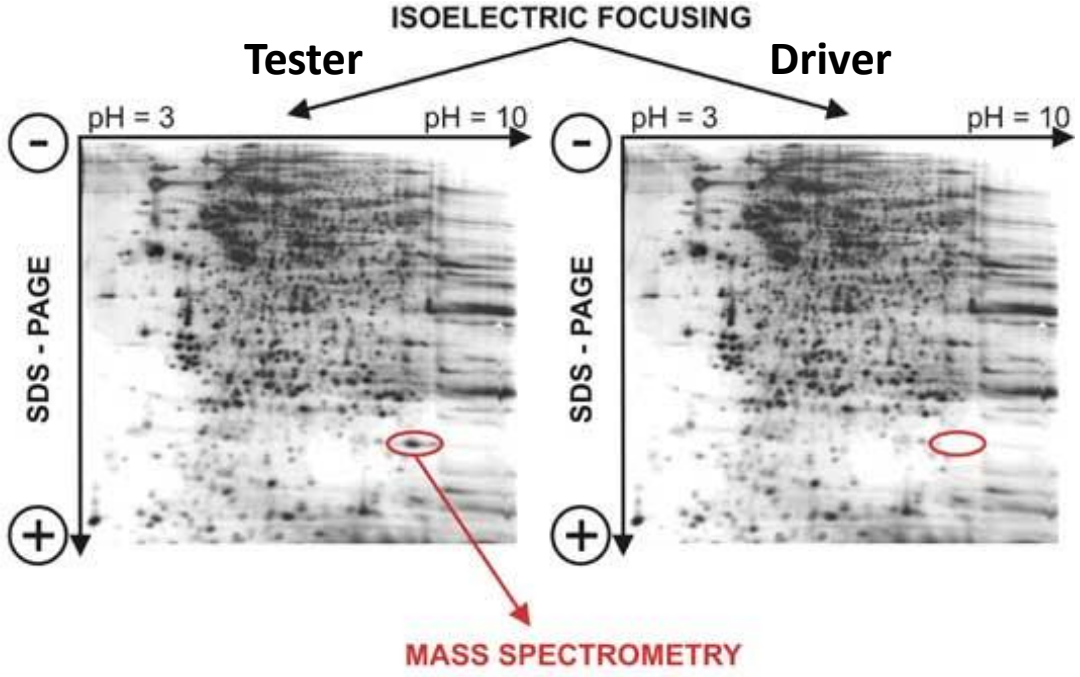
The principle of SSH in brief is that, the cDNA generated out of the two mRNA population of contrasting phenotype (tester and driver) is hybridised by combining two samples, from which the tester-driver hybrids and excess driver cDNA are removed wherein only those single stranded tester specific cDNAs are retained. These tester-specific cDNAs are enriched by PCR and sequenced for its identity.

Comparative trasnscriptomics study involves the comparison of two transcriptome from two contrasting phenotype. Here the transcriptome of two populations are sequences by next generation sequencing method and are annotated individually to know its functional genes. These two data sets are compared for finding differentially expressed genes in two samples. Here

one will get four classes of genes between two samples. 1. Genes which are common in both forms 2. Genes which re specific to tester population 3. Genes which are specific to driver population 4. Genes which are over/under expressed in tester population with respect to driver population. The advantage of transcriptome analysis is that, unlike SSH wherein only tester-specific genes were isolated, all genes are categorically analysed and necessary inferences can be drawn as the cause of contrasting phenotype. Once the gene information is obtained, it has to be put in to the form of biosynthetic pathway of our interest and need to be proven using various approaches to avoid any confusion.

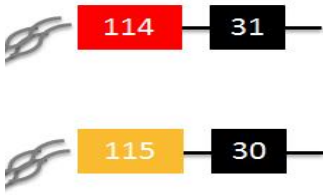
Techniques at protein level

To detect the difference at protein level, two techniques namely 2D gel electrophoresis followed by protein sequencing of differentially expressed proteins and Isobaric tags for relative and absolute quantitation of proteins (iTRAQ) are used. 2D gel electrophoresis approach involves isolation of protein from two different samples, its 2D gel run and spot picking of the differentially expressed protein for its sequencing and identification. In this approach the practical difficulty is to get reproducible gels due to the chance of variation in in sample loading during gel electrophoresis. The gel image of the two samples are superimposed *in silico* to obtain differentially expressed proteins and are sequenced using mass spectrometric technique such as MALDI-TOF-TOF to obtain its identity (sample image is given below).

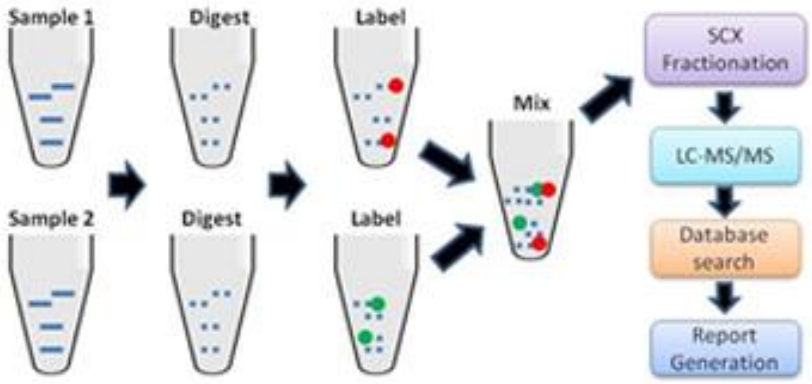


iTRAQ analysis is more advanced techniques for not only detection, but also relative quantification of whole proteome. Here sequence identity of the whole proteome is also revealed. Moreover, one can compare up to eight different samples in one go. ie., multiplexing is possible. Here the protein samples from each population are isolated separately and are subjected to trypsin digestion. Equal quantities of protein samples are taken from each samples and are

subjected to tryspin digesion. The trypsin digests are subjected to tagging by specially designed isobaric (same molecular weight) tags. Each sample is attged with different types of tags and shown below.



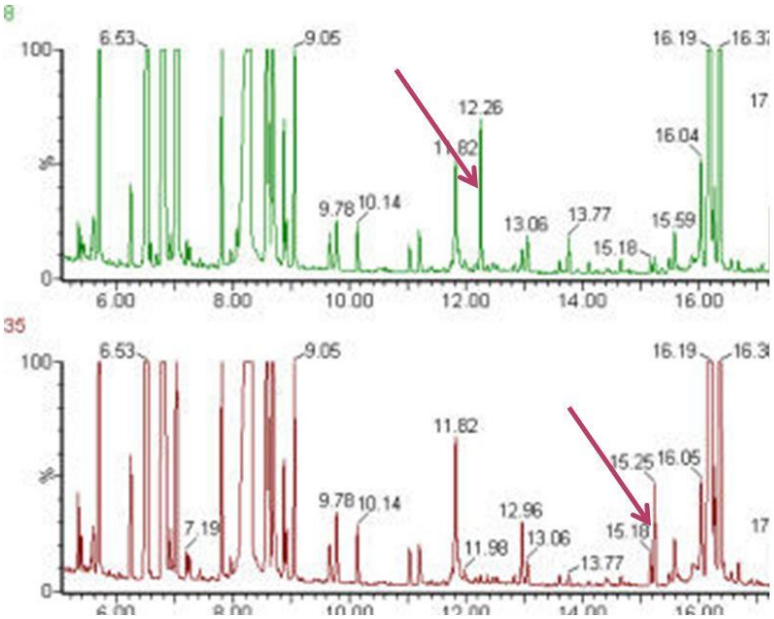
It is important to note that these tags are having a molecular weight of 145 kDa. But the tags are designed in such a manner that there is one kDa difference between them with respect to linker region ranging from 28 to 31 kDa. The other part of the tag is having specific flourophore (red, yellow, green and blue) attached to it so that one can distinguish the identity of protein by checking the type of flurascence it emits. Once the protein samples are tagged, all samples are mixed in one vial and are subjected to LC-MS-MS after fractionation. After first roud of MS we get the relative quantitaion of each peptide among the samples by measuring the relative intensity of each m/z peak in the mass spectra. Then each peach is subjected to further mass spectrometry to get the sequence identity of each peptide. One has to understand that each peak correspond to one peptide attached with the tag and has got only a partial sequence of the whole protein as it has been digested with trypsin earlier. None the less, the whole sequence of the protein can be generated by overlapping the sequences of same protein aided by software. Once we get protein sequences, databse search is carried out to get the functional information of the protein and we look for the proteins of our interest or novel proteins which are necessary for the biosynthetic pathway discovery. The overall process of the iTRAQ can be illustrated as below:



Techniques at metabolite level

Sometimes, it may be easy to detect the difference between two contrasting forms at metabolite level as the two forms are noticeably different with respect to some metabolite. In such cases, whole metabolome of the two forms are studied and compared. It can be done by using LC-MS or GC-MS depending upon the nature of the compound we are looking for, if the compound of

interest is volatile in nature, then GC-MS can be adopted. In both the approaches, the chemical identity of the metabolite is obtained by searching with the available library and the chemicals which are specific to any form are identified as the possible reasons for its difference in phenotype. Example gas chromatogramme is shown below with differentially expressed compounds in two forms.



Then these chemical are searched for its probality of being different intermediate in a common pathway and a probale pathway can be proposed. Once it is done, each and every step of the pathway is attached with probable enzymatic reaction. Then each of these reactions have to be proved unequivocally by means of various other techniques such as enzymatic analysis, western blotting, quantitative PCR, use of specific inhibitors for specific enzymes etc.

In short, all these approaches listed are only the beginning of a long journey towards the discovery of a pathway. No pathway discovery is easy as it has been stated before. It involves lots of trial and error operations which ultimately lead to the final biosynthetic pathway.

PLANT SECONDARY METABOLITES AND ITS SYNTHESIS

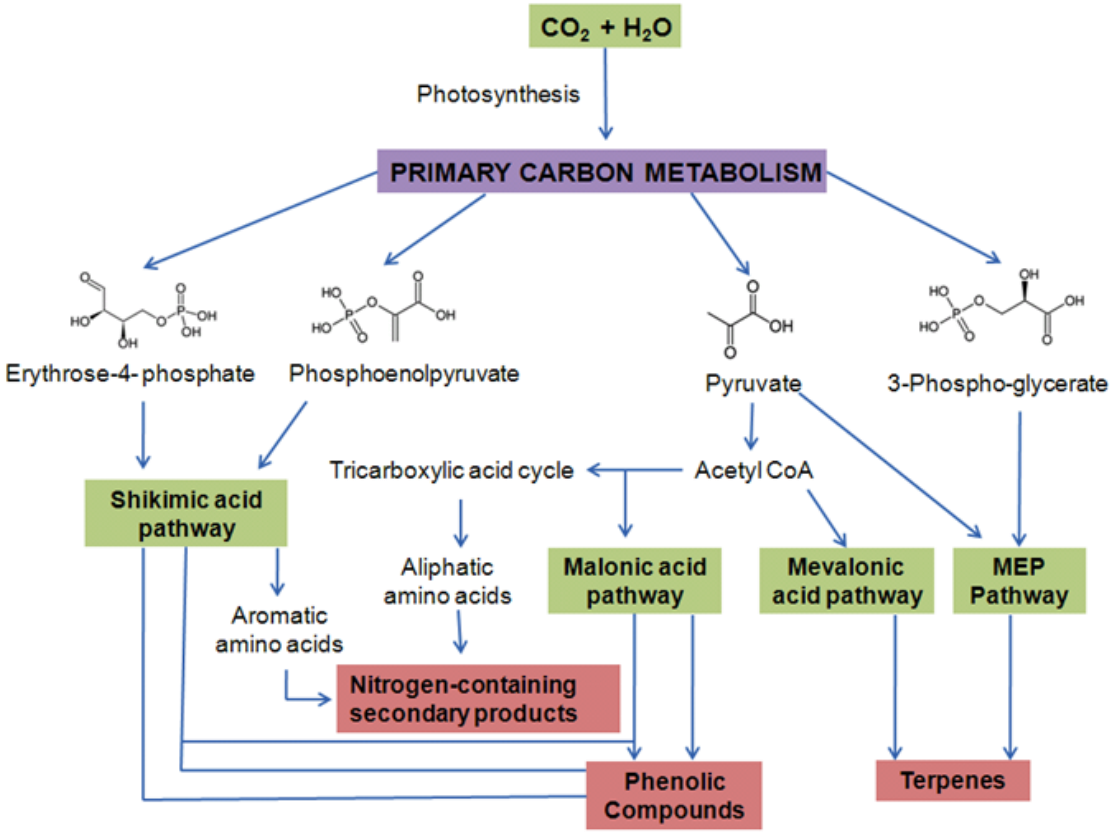
Sivaranjani R

Plant metabolism is divided mainly into primary and secondary. Primary metabolism mainly consists of synthesis of proteins, carbohydrates and nucleic acids which are the building blocks of cellular system. Secondary metabolism occurs mainly in plants and some lower organisms and it consists of the synthesis of compounds which impart characteristic quality to the plant like colour and fragrance of the flower, pungency of spices, etc., The compounds produced through this metabolism are generally referred as secondary metabolites. By definition, secondary metabolites are not essential for the growth and development of a plant, but rather are required for the interaction of plants with their environment. Secondary metabolites from plants are extremely diverse in nature and most of them are species specific. Each family, genus of the plant produces different mix of these compounds and sometimes they are utilized as taxonomic characters to identify and classify plants (Chemotaxonomy). Secondary metabolites are not involved in the primary growth and development processes but act as reinforcement against pest and disease attack and are play a major role in ecology.

The biosynthetic pathway of secondary metabolites has shared compounds between primary and secondary metabolism. The intermediates from carbohydrates and amino acid synthesis are utilized by specific enzymes to divert its pool to secondary metabolite synthesis. Fig 1 depicts the flow of metabolites from primary metabolism to provide substrates for secondary metabolites synthesis. The important building blocks employed in the biosynthesis of secondary metabolites are derived from acetyl-CoA, Shikimic acid, 1-deoxylulose 5- phosphate and mevalonic acid. Shikimic acid pathway leads to the synthesis of three important amino acids namely phenylalanine, tyrosine and tryptophan. From this, phenylalanine and tyrosine are utilized for the synthesis of an important class of secondary metabolite named phenolics. Secondary metabolites are classified into terpenoids, alkaloids and phenolics based on their chemical structure and route of biosynthesis.

Terpenoids

Among secondary metabolites, terpenoids constitute a large family of diverse compounds. Steroids, carotenoids, and gibberellic acid are just some of its members. They are composed by the most important group of active compounds in plants with over than 23,000 known structures. They are polymeric isoprene derivatives and synthesized from acetate via the mevalonic acid pathway. Biosynthesis of terpenoids is made from simple isoprene derivatives which are synthesized from acetate that are linked in head and tail fashion. Isoprene units are synthesized via mevalonic acid pathway. The number of isopreneunits incorporated into a particular terpene serves as a basis for their classification.



General pathway of plant secondary metabolite synthesis (Taiz and Ziger, 2003)

Important molecules of terpenoids (Kogan *et al*, 2006)		
Carbon No	Name	Example
C5	Hemiterpene	Isoprene, prenol, isovaleric acid
C10	Monoterpene	Limonene, eucalyptol, pinene
C15	Sesquiterpene	Abscisic acid
C20	Diterpene	Gibberellin
C30	Triterpene	Squalene, lanosterol, Brassinosteroid
C40	Tetraterpene	Carotenoids
C>40	Polyterpene	Rubber, Vitamin E

Many of the terpenoids compounds have pharmacological activity and are used for diseases treatment both in humans and animals. Some of the terpenoids compounds are used in the industry as flavours, fragrances and spices. Plant essential oil components are fall in the category of monoterpenes and sesquiterpenes imparting volatility to the essential oil.

Alkaloids

As the name suggests, alkaloids are alkaline compounds that contain basic nitrogen atoms. In addition to carbon, hydrogen and nitrogen, this group may also contain oxygen, sulfur and rarely other element such as chlorine, bromine and phosphorus. Alkaloids are synthesized by a large

variety of organisms, such as bacteria, fungi, animals but mostly by plants as secondary metabolites. Most of them are toxic to other organisms and can be extracted by acid-base. Alkaloids have a long history in medication and found to have numerous pharmacological effects. Classification of alkaloids is difficult owing to its structural diversity, yet some are classified based on similarity of carbon skeleton.

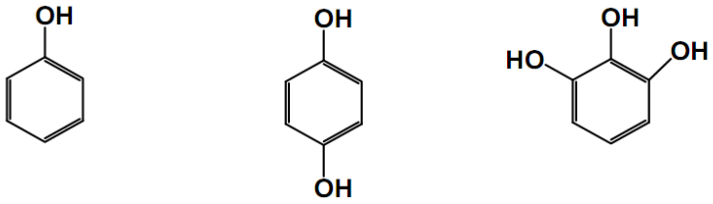
Alkaloids are biosynthesized from aminoacids such as tyrosine and lysine. Although some moieties from other pathways such as terpenoids are often combined to produce structural diversity in alkaloids pool.

Pharmacological effects of some well known alkaloids (Kabera *et al*, 2014)		
Alkaloid name	Sourcew	Pharmacological activity
Atropine	*Atropa belladonna, Darura stramonium, Mandragora officinarum*	Competitive antagonist of muscarinic acetylcholinereceptors, anti cholinergic, anti myopia effects
Berberine	*Berberis sp, Coptis chinensis, Tinospora cordifolia, Argemone mexicana and Eschscholzia californica*	Anti inflammatory, anti bacterial/viral, recently experiments showed anti diabetic and beneficial effects on cardiovascular system and anti cancer and others disorders such as intestinal
Codeine/Morphine	*Papavar somniferum*	Analgesic, antitussive, anti diarrheal, antidepressant, sedative and hypnotic properties
Nicotine	Solanaceae family	Stimulant, antiherbivore, insectide, anti inflammatory
Quinine	*Cinchona succirubra, C. calisya, c. ledgeriana*, plants of Rubiaceae family	Antimalarial, antpyretic, analgesic, anti-inflammatory, antiarrhythmic, bacteriostatic

Phenolics

Phenolic compounds from plants are one of largest group of secondary plants constituents synthesized by fruits, vegetables, teas, cocoa and other plants that possess certain health benefits. They have one or more phenolic group with structural variation. They are characterized by the antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties, and may protect from oxidative stress and some diseases (Park *et al*, 2001). Simple phenolics are bactericidal, antiseptic and antihleminthic. They are distributed in almost all plants and subject to a great number of chemical, biological, agricultural, and medical studies. They are diverse in structure, and present in common the hydroxylated aromatic rings (e.g., flavan-3-ols). Hydroxybenzoic and hydroxycinnamic acids present two main simple phenolic compounds

found in plants. In tea, coffee, berries and fruits, the total phenolic compounds could reach up to 103 mg/100 g fresh weight.



Examples of simple phenolics (phenol, hydroquinone, pyrogallol acid)

Phenolic compounds containing more than one OH-group in aromatic ring are polyphenols. Based on substitution in carbon atoms, phenolic compounds are divided into four main groups: phenolics with one aromatic ring, with two aromatic rings, quinones and polymers. Phenolic compounds with one aromatic ring: a large number of compounds, among them are simple phenols (C6), phenol with attached one (C6-C1), two (C6-C2) and three (C6-C3) carbon atoms.

Phenolic compounds with two aromatic rings: this group includes benzoquinones and xanthones (C6-C1-C6) containing two aromatic rings which are linked by one carbon atom; stylbenes (C6-C2-C6) which are linked by two carbon atoms; and flavonoids, containing three carbon atoms (C6-C3-C6). Flavonoids are further classified into isoflavonoids and neoflavonoids depending on the structure of propane unit and an attaching place of side chain B. Polyphenolics are more than 8,000 different compounds identified to date. That is why the terminology and classification of polyphenols is complex and confusing. Although all polyphenols have similar chemical structures, there are some distinctive differences. Based on these differences, polyphenols can be subdivided into two classes: flavoinoids and non flavonoids, like tannins. The contribution of flavonoids to the total antioxidant activity of components in food can be very high; for instance red wine contains high levels of flavonoids, mainly quercetin and rutin. The high intake of it by the French might explain why they suffer less from coronary heart disease than other Europeans, although their consumption of cholesterol rich foods is higher (French paradox). The tannins also constitute the active principles of plant-based medicines. According the literature, the tannins containing plants are used as astringents against diarrhea, adiuretic against stomach and duodenal tumors.

POST HARVEST MANAGEMENT AND VALUE ADDITION IN SPICES

E Jayashree

Spices have been treasured for the flavor, pungency, aroma and color they impart to food. The delightful flavour and pungency of spices make them indispensable in the preparation of palatable dishes. Over the years, Indian spices have carved out a niche of its medicinal and pharmacological properties in the world of spices. There are 109 spices listed by International standards Organisation (ISO) and the Spices Board, Government of India has listed 52 major spices of commercial importance. India produces around 3.8 million tonnes of spices annually, of this about 10% of the total produce is exported to over 150 countries. The USA, Europe, Australia, Japan, the Middle East and Oceanic countries are the major importers of Indian spices.

General Trend in the World Consumption of Spices

In Asia Pacific, spice consumption will increase at the rate of 4% in the coming years. It is predicted that ready to eat meat products will consume 40,000 tonnes of spices in the next five years. Consumption of spices is also expected through savory, snacks, flavors, bakery (ginger, cinnamon, nutmeg), soups and sauces. Demand for spices in the form of pastes, pulps and wet seasonings are gaining fresh market considering the vast growing economies of many developing countries.

World Awareness of the Potential of Spices

Today there is greater scientifically validated knowledge on spices phytochemistry, therapeutic effects of their bioactive principles and mechanism of action. Health benefits include carminative action, hypolipidemic effect, antidiabetic property, antilithogenic property, antioxidant potential, anti-inflammatory property, antimutagenic and anticarcinogenic potential. Of these, the hypocholesterolemic and antioxidant properties have far-reaching nutraceutical and therapeutic value. Most of the medicinal properties are attributed to the secondary metabolites – the essential oils and oleoresins – present in spices, a large number of which have been identified. Important flavour compounds of spices are listed in Table 1.

Table 1: Important flavour compounds in spices

Spice	Important flavor compounds
Allspice	Eugenol, β-caryophyllene
Anise	(E)-anethole, methyl chavicol
Black pepper	Piperine, S-3-Carene, β-caryophyllene
Cardamom	a-terpinyl acetate, 1-8-cineole, linalool
Turmeric	Turmerone, Zingeberene, 1,8-cineole
Ginger	Gingerol, Shogaol, neral, geranial
Mace	a-pinene, sabinene, 1-terpenin-4-ol
Nutmeg	Sabinine, a-pinene, myristicin
Cumin	Cuminaldehyde, p-l,3-mentha-dienal

Fennel	(E)-anethole, fenchone
Saffron	Safranol
Vanilla	Vanillin, p-OH-benzyl-methyl ether

Medicinal and Pharmacological Properties of Spices

Spices are reputed to possess several medicinal and pharmacological properties and hence find position in the preparation of a number of medicines. Thus, in the indigenous system of Indian medicine (Ayurveda and Unani), spices have a vital role to play. Information regarding the medicinal aspects of spices, which are acceptable in the light of modern knowledge, is described with pharmaceutical codex. In Ayurveda, about 25 spices are used for various herbal preparations and the annual consumption by the Ayurvedic firms in Kerala alone is approximately 3.2 tonnes. Spices which possess some of the important medicinal properties are listed in Table 2.

Table 2: Major spices and their medicinal properties

Medicinal property	**Spices**
Cancer Preventive	Ginger, Black pepper, Nutmeg, Cinnamon, Clove, Turmeric, Cardamom, Vanilla, Allspice, Mace
Antimicrobial	Ginger, Nutmeg, Black pepper, Cinnamon, Vanilla, Turmeric, Clove, Allspice, Cardamom, Mace
Anti-Inflammatory	Black pepper, Cinnamon, Clove, Turmeric, Allspice, Cardamom
Spasmolytic	Cinnamon, Black pepper, Clove, Ginger, Nutmeg, Turmeric
Antioxidant	Vanilla, Ginger, Black pepper, Clove, Turmeric
Antiulcer	Ginger, Black pepper, Turmeric, Cinnamon, Clove, Nutmeg, Vanilla, Allspice, Mace
Hypoglycemic	Cardamom
Antihepatotoxic	Vanilla
Antiallergic	Allspice
Antimigraine	Turmeric, Allspice, Cardamom, Mace
Antiosteoporotic	Black pepper, Allspice, Clove, Cardamom, Mace
Estrogenic/ Androgenic	Cardamom
Immunostimulant	Turmeric, Mace
Antilithic	Allspice
Anti-insomniac	Allspice, Clove, Mace
Antiedemic	Vanilla

Processing and Value Addition in Spices

Value addition in spices is yet another area of activity in which India is moving forward. The consistent effort by various agencies during the last one decade has improved the share of the value added products in the export basket to more than 53%. India can now boast as the monopoly supplier of spice oils and oleoresins the world over. During the year 2009-2010, India exported 6,750 tonnes of spice oils and oleoresins worth 708.75 crores. In the case of curry powders, spice powders, spice mixtures and spices in consumer packs, India is in a formidable position.

Spices thus open ample opportunity for entrepreneurship. To achieve this one of the key requirement is to diversify the products from spices. Secondary agriculture is the watch word for development for both farmers and primary processors of spices. Even though India produces a good quantity of black pepper, ginger, turmeric and cardamom more than 85% of it is consumed within the country itself. Value addition throw open ample opportunity in export. The present scenario in processing and value added products obtained from important spices are discussed.

i. Black pepper

Black pepper, *Piper nigrum* L**.** known as the 'King of Spices' takes about 7-8 months after flowering to reach full maturity. In India, the crop is harvested during December –January in plains and January-April in the high ranges of Western Ghats. It is important to harvest pepper at the proper stage of maturity in order to achieve a dried product of good colour and appearance. Recent advances in product diversification have necessitated harvesting of the berries at different stages of maturity which has to be regulated depending on the various end uses (Table 3).

Table 3: Optimum maturity at harvest for different pepper products

Product	**Stage maturity at harvest**
Canned Pepper	4-5 months
Oleoresin and essential oil	15-20 days before maturity
Dehydrated green pepper	10-15 days before maturity
Pepper powder	Fully mature with maximum starch
Black pepper	Fully mature and 1-2 berries start turning from yellow to red in each spike
White pepper	Fully ripe

Post harvest processing

The primary processing in black pepper involves threshing, blanching, drying, grading and packing. The berries are separated from the spike usually by trampling with human legs. Mechanical threshers with capacities varying from 200 to 1200 kg//h are available which can thresh quickly and provide cleaner products. Apart from the major quality attributes such as pungency and aroma, the appearance with respect to its colour (brown/ black) is of importance

for use of black pepper as a spice in the whole or ground form. Dipping harvested green pepper for a minute in boiling water enhances the enzymatic oxidation and provides a shining black colour to the produce.

At harvest, pepper has moisture content of 60 to 70%, which should be brought to safer levels of 10-11% by adequate drying. The dry recovery varies from 29 to 43% depending on the variety. Sun drying is the conventional method followed for drying of black pepper. Driers developed by various agencies such as solar and mechanical dryers are highly efficient for drying pepper.

Cleaning of dried black pepper removes extraneous matter like spent spikes, pinheads, stones, soil particles etc., enhance the value of the produce and help to get higher returns. On a small scale, winnowing and hand picking remove most of these impurities. The cleaned pepper, is sifted into different grades based on size using sieves.
Black pepper is hygroscopic in nature and absorption of moisture from air, notably during rainy season with high humidity may result in mould and insect infestation. Before storage it is to be dried to around 10% moisture. The dried whole pepper is packed and stored in double burlap bags with polythene liners of 0.076 mm or more in thickness.

ii. Cardamom

Cardamom, known as the 'queen of spices', which belongs to the family of *Zingiberaceae*, is a rich spice obtained from the seeds of a perennial plant, *Elettaria cardamomum* M. Cardamom fruits mature in about 120 days after flowering. Due to prolonged flowering period, cardamom capsules ripen successively at 10-15 days intervals over an extended period of 8 months (from August to March). Generally harvesting is carried out at an interval of 15-30 days and completed in 8-9 rounds by hand picking.

Post harvest processing

The harvested capsules carry soil or dirt on their surface and hence they are washed thoroughly in water. The capsules are then treated with 2% sodium carbonate solution for 10 minutes which enables to retain green colour and prevent mould growth. The alkali treated cardamom capsules are spread in trays and cured in curing houses or mechanical driers. Curing in cardamom is as the process in which the moisture content of freshly harvested cardamom capsules is reduced from 70-80% to 11-12% at an optimum temperature of 45-55^{o}C so as to retain its green colour and volatile oil to a maximum extent. The cured capsules are graded using sieves of 8, 7.5,7 and 6 mm. The graded cardamom is stored over a period of time, in double lined polythene bags.

iii. Ginger

India and China are the world's largest producer and exporter of ginger *(Zingiber officianale*). In India normally harvesting of ginger is done from January to April, varying with the locations. The crop is ready for harvest in about 8 months after planting when the leaves turn yellow and

start drying up gradually. The clumps are lifted carefully with spade or digging fork and the rhizomes are separated from the dried up leaves, roots and adhering soil. Harvesting is to be done from the 6th month onwards when used as green ginger. The quality of ginger is affected by the stage of the harvest and needs to be scheduled for various end uses (Table 4).

Table 4: Stage of harvest of ginger for various end uses

End use	**Stage of harvest (months after planting)**
Vegetable purpose and preparation of ginger preserve, candy, soft drinks, pickles and alcoholic beverages	4 – 5
Dried ginger and preparation of ginger oil, oleoresin, dehydrated and bleached ginger	8-10
Green ginger, oleoresin and volatile oil High dry ginger & starch and low crude fibre Dry ginger	7 8 8 – 9
Salted ginger High essential oil High oleoresin High essential oil & oleoresin	4 – 5 7 7½ - 8 8
High oleoresin and oil content	9
High crude fibre & Low protein and fat Low crude fibre Less fibre & mild pungency	6½ - 7 7 < 7

Post harvest processing

The post harvest processes involved in the processing of matured fresh ginger to dry ginger involves peeling, drying and polishing. Peeling hastens the process of drying and maintains the epidermal cells of the rhizomes, which contain essential oil responsible for aroma of ginger. Indigenously, peeling is performed by partially scraping the peel of ginger rhizomes with sharpened bamboo splinters. The scrapped or partially peeled rhizomes are put for drying on clean drying yard. Traditionally ginger is sun dried in a single layer in open yard. The cleaned and partially peeled ginger with moisture content of about 80% is spread thinly under sun and the moisture content is brought down to 10% or even less for safe storage. It takes about 10-15 days for complete drying. The dried ginger presents a brown, irregular wrinkled surface and when broken shows a dark brownish colour. The dry ginger so obtained is known as rough or unbleached ginger. The yield of dry ginger is 19-25% of fresh ginger depending on the variety and the location where it is grown.

iv. Turmeric

India is the largest producer and exporter of turmeric (*Curcuma longa*) in the world. It is estimated officially that about 80% of the world production of turmeric is from India alone. The

turmeric crop is ready for harvesting in about 7 to 9 months after sowing depending upon the variety. In India, sowing takes place between June and July and harvesting is done from February to April. Before harvest, the dry leaves and stem are cut close to the ground. The rhizome bunches are carefully lifted and soil adhering to the rhizome surface is removed manually and further cleaned to remove roots and scales before they are collected in the curing yard.

Post harvest processing

The traditional method of turmeric processing consists of the following steps washing, boiling/curing, drying, polishing, colouring, packaging and marketing. Traditionally boiling is done in metal or mud pots with (three fourth capacity) water for 1 hr to 1.5 h. Top of the pots are covered with a lid or dry leaves. Boiling process is continued till foams and white foams start coming out. These come out with a special quality of flavour. Rhizomes are tested by pressing with fingers depending on the quantity. The rhizomes as wholes or cut longitudinally into halves and the fingers are generally cured separately, as cooking time varies with difference in thickness. Boiling considerably reduces the drying time and helps in producing a product of fairly uniform color. The cooked rhizomes are spread in open yard for drying and it takes about 10-15 days for complete drying. When dried properly, the rhizomes became hard, almost horny, brittle and of uniform yellow colour. Completely dried turmeric holds 8-10% moisture content. The dried rhizomes are hand polishing by rubbing dried rhizomes against a hard surface. By this process colour of turmeric becomes bright or shining. The product is known in trade as 'polished turmeric'. Mechanical polishing helps to remove scales, roots and some of the epidermal layer through the sieve mesh surrounding the polishing drums.

iv. Chilli and Paprika

Chilli (C*apsicum annum)* is the most widely cultivated crop among the spices grown in India. It is harvested when the pods are well ripened and partially wither at the plant itself. At this stage they would have superior pungency and colour.

Post harvest processing

The harvested pods are kept in heaps either indoor or in shade away from direct sun light for 2-3 days so as to develop uniform red colour. Subsequent to this, the pods are dried under the sun by spreading them out on clean, dry mat, cemented or concrete surface. The harvested chillies in ripe condition have moisture content of 70-80% and need to be dried for 13-15 days for the reduction of moisture to a safe moisture content of 10% and then stored. In mechanical drying, the chillies are dried at a temperature of 50^{o}C and at air velocity of 1.5 m/s. Solar cabinet driers and waste fired driers are also developed for drying of chillies. Packing of dry chillies is done using jute cloth, paper or paper cartons with polythene lining of 300 gauges.

The colour of chilli spice powder is due to the presence of red-pigmented carotenoids. The main pigments are capsanthin, capsorubin, zeaxanthin and cryptoxanthin. Carotenoids are very stable in intact plant tissue. However, when chillies are processed by drying and grinding into spice powder, the carotenoids easily auto-oxidise due to effects of heat, light and oxygen. This leads to a more orange and less intense colouration.

Quality profile of important spice varieties

The medicinal property and aroma quality of spices are attributed to the volatile oils present in spices. The non-volatile part of spice extracted using organic solvents is called the oleoresin. The high yielding varieties of black pepper, cardamom, ginger, turmeric, nutmeg and cinnamon released by Indian Institute of Spices Research along with the quality profile is tabulated in Tables 5, 6, 7 and 8.

Table 5. Quality profile of high quality black pepper varieties

Variety	Yield (dry) (kg/ha)	Oleoresin (%)	Piperine (%)	Essential oil (%)
Subhakara	2352.0	12.4	3.4	6.0
Sreekara	2677.0	13.0	4.1	5.0
Panchami	2828.0	12.5	3.7	3.4
Pournami	2333.0	13.8	3.1	3.4
IISR Thevam	1787.0	8.15	1.6	3.1
IISR Malabar Excel	1065.0	13.5	2.96	3.2
IISR Girimunda	2112.0	9.65	2.2	3.4

Table 6. Quality profile of important cardamom varieties

Variety	Average yield (dry), kg/ha	Essential oil %	1,8- Ciniole %	Terpinyl acetate, %
IISR Suvasini	1322	8.7	42.0	37.0
IISR Vijetha	643	7.9	45.0	23.4
IISR Avinash	847	6.7	30.4	35.5

Table 7. Quality profile of high quality ginger varieties

Variety	Average yield (fresh) t/ha	Dry recovery (%)	Crude fibre (%)	Oleoresin (%)	Essential oil (%)
IISR Varada	22.6	19.5	3.2	6.7	1.8
IISR Mahima	23.2	23.0	3.9	4.5	1.7

IISR Rejatha	22.4	20.8	4.0	6.3	2.4

Table 8. Quality profile of high quality turmeric varieties

Name	Av. yield (fresh) t/ha	Dry Recovery (%)	Curcumin (%)	Oleoresin (%)	Essential oil (%)
Suvarna	17.4	20.0	4.3	13.5	7.0
Suguna	29.3	12.0	7.0	13.5	6.0
Sudarsana	28.8	12.0	5.3	15.0	7.0
IISR Prabha	37.5	19.5	6.5	15.0	6.5
IISR Prathiba	39.1	18.5	6.2	16.2	6.2
IISR Kedaram	34.5	18.9	5.5	13.6	-
IISR Alleppey Supreme	35.4	19.3	6.0	16.0	-

Value added products from spices

A variety of products that can be made from spices are tabulated in Table 9.

Table 9 Value added products from spices

Spice	Value added product
Black pepper	
Green pepper based products	Canned green pepper, green pepper in brine, cured green pepper, frozen green pepper, freeze dried green pepper, dehydrated green pepper, green pepper oil, green pepper pickle, mixed green pepper pickle, green pepper sauce and green pepper-flavoured products.
Black pepper based products	Whole black pepper, sterilized black pepper, ground black pepper, cryoground black pepper powder, pepper oil and oleoresin.
White pepper based products	Whole white pepper, white pepper powder.
Other products of black pepper	Pepper mayonnaise, pepper cookies and pepper tofu. Pepper extract is a valuable adjunct in the flavouring of sausages, canned meat, soups, table sauces and certain beverages and liquor. Pepper oil is used in perfumery and also for manufacturing soaps. Products like lemon pepper, garlic pepper, sauces and marinades have pepper as major ingredient. Curry powders and spice blends have pepper as one of the major ingredient.
Cardamom	Bleached cardamom, decorticated seeds and seed powder, cardamom volatile oil, cardamom oleoresin.

	Other products include encapsulated cardamom which is free flowing and having uniform flavor, cardamom tea, cardamom coffee, cardamom soft drink mix.
Ginger	Ginger powder, salted ginger, ginger oil, ginger oleoresin, ginger-based beverages like ginger beer and ginger ale, ginger candy, ginger paste, salted ginger, salted ginger, crystallized ginger.
Turmeric	Ground turmeric, turmeric oil, turmeric oleoresin, curcumin
Chillies	Paprika oleoresin, chilli colour, chilli pungency, dehydrated chilli, canned chilli, brined/pickled chill fermented chilli, brined/pickled chilli, fermented chillies.
Nutmeg	Nutmeg powder, nutmeg oleoresin, nutmegs butter, mace oleoresin mace oil
Cinnamon	Cinnamon bark oil, cinnamon oleoresin, cinnamon leaf oil, cinnamon powder, cinnamon root bark oil
Clove	Clove powder, clove oil

SPICE PROCESSING FACILITY

E Jayashree

Spice Processing Facility at ICAR-Indian Institute of Spices Research, Peruvannamuzhi, Kozhikode, Kerala is setup with the objectives of encouraging research and entrepreneurship development in spice processing for product and process development. This facility is established to attract entrepreneurs in spice sector by developing integrated processing capabilities, hand holding entrepreneurs, providing training and technical guidance on quality maintenance as well as creating conducive environment for the growth of spices. The processing unit is equipped with state of the art facility for primary as well as secondary processing of spices. The facility has three units for cleaning and grading of black pepper, curry powder production and white pepper production.

1. BLACK PEPPER CLEANING AND GRADING UNIT

Black pepper, the king of spices, is the whole dried fruit of the vine *Piper nigrum*. Black pepper is widely used as a condiment due to its characteristics aroma, pungency and biting taste. Two primary products of *P. nigrum* that are internationally traded are black pepper and white pepper. Fully matured green pepper spikes when one or two berries turn yellow are harvested, threshed and sun dried in open cemented yard to obtain dry black pepper. In this process, there are chances of dust, leaves, sticks and other foreign matters which may contaminate the spice. It is therefore necessary to clean black pepper before it is packaged and used for consumption. The pre cleaning equipments installed in the black pepper cleaning cum grading unit includes a black pepper cleaner cum grader, spiral separator and a metal detector. Once the black pepper is cleaned, it is graded according to size and then packaged in clean gunny bags.

CLEANER CUM GRADER

Function: Removal of dust and stones from black pepper and grading them according to size

Capacity: 200 kg/h

Salient features: It consists of a bucket elevator to lift black pepper, aspirator to suck dust, a vibratory shaker to remove stones and a grader to grade black pepper. The power required to operate the unit is 2.21 kW.

SPIRAL SEPARATOR

Function: Separation of sticks and dry leaves from black pepper

Capacity: 200 kg/h

Salient features: The unit separates black pepper from impurities like stick and dry leaves using gravity and centrifugal force. The unit consists of a bucket elevator to lift black pepper to the feed hopper. The power required to operate the unit is 0.37 kW.

METAL DETECTOR

Function: Removal of metallic impurities from black pepper

Capacity: 300 kg/h

Salient features: The unit is capable of detecting ferrous, non ferrous and stainless steel contaminants from black pepper. The contaminants are sucked out and removed by air compressor. The power required to operate the unit is 1.47 kW.

GRADER

Function: Grading of black/white pepper according to size

Capacity: 100 kg/h

Salient features: The unit is provided with rectangular stainless steel sieves with perforations of 3mm, 4 mm, 5mm and 6mm for grading black/white pepper. The power required to operate the unit is 0.74 kW.

2. CURRY POWDER PRODUCTION UNIT

The curry powder production unit is equipped with facilities for powdering and packaging spices or spice blends. The spices brought to the unit are first checked for its moisture content and if the moisture content was found above 10 per cent, the spices are dried in the solar tunnel drier. The rotary drier can also be used for drying spices. Once the spices are dried they are roasted in the drum roaster before powdering for flavour enhancement. The spices are then crushed in the plate crusher or finely powdered in the micro pulverizer as per the requirement. The powdered spices are sieved in the vibro sifter and is filled in packets and sealed. In case of curry powder production, different spices are blended in the required ratio and powdered. The powder is sieved and put into the blender for the production of homogeneous spice mix. The curry powder is then weighed automatically and filled in packets and sealed.

SOLAR TUNNEL DRIER

Function: Drying of spices

Capacity: 500 kg/batch

Salient features: The drying yard of size 12 × 4 m and centre height of 2.5 m is provided with arches made of galvanized iron pipes which are covered with UV stabilized polyfilm. The unit is provided with biomass furnace as backup and can be used even during rainy season. The electrical equipments provided inside the drier like exhaust fans, controller etc. are operated by solar powered system consisting of photovoltaic cells, inverter, batteries etc

ROTARY DRIER

Function: Drying of spices

Capacity: 50-100 kg/batch

Salient features: Horizontal rotary drier provided with LPG firing system for drying spices.The power required to operate the unit is 0.37 kW and LPG requirement is 1.75kg/h.

ROASTER

Function: Roasting of spices before powdering

Capacity: 25-50 kg/batch

Salient features: Provided with a stainless steel drum for roasting spices and operated using LPG firing system. The power required to operate the unit is 0.37 kW and LPG requirement is 1.05 kg/h.

PLATE CRUSHER

Function: Crushing of spices
Capacity: 100 kg/h
Salient features: Used for crushing spices between 800-1000 microns. The power required to operate the unit is 5.51 kW.

MICRO PULVERISER

Function: Fine grinding of spices
Capacity: 100 kg/h
Salient features: Powdering of spices to 800 microns or below. The unit consists of a hammer mill, blower and an air lock valve**.** The power required to operate the unit is 15.1 kW.

SIFTER

Function: Sieving of spice powders
Capacity: 50 kg/h
Salient features: The unit is a double deck model provided with a circular stainless steel vibro sifter. The power required to operate the unit is 0.37 kW.

BLENDER

Function: Homogeneous mixing of spice powders
Capacity: 50 kg/batch
Salient features: The unit consists of a **s**tainless steel vessel provided with ribbon blender to mix spice powders homogeneously. The power required to operate the unit is 0.74 kW.

FILLING MACHINE

Function: Continuous filling of packets with spice powder
Capacity: 200kg/h.
Salient features: The unit consists of a cup type dozer for automatic measuring and filling of spice powder into packets (50-100 g). The power required to operate the unit is 0.74 kW.

SEALING MACHINE

Function: Sealing of packets
Capacity: 400 packets/h
Salient features: Continuous sealing of spice powder packets. The equipment is provided with a mini band sealer for horizontal/vertical type sealing.

3. WHITE PEPPER PRODUCTION UNIT

White pepper is produced from fully matured freshly harvested green pepper or from black pepper. The freshly harvested green pepper spikes are despiked/ threshed using a pepper thresher and the berries are graded in a rotary grader. Berries of size 4.0 mm and above are used for white pepper production. The fresh berries are washed in the drum washer and introduced into the fermentation tank where the pepper is fermented, with daily change of water in the tank. After required days of fermentation, the fermented pepper is fed into the pulper-cum-washer for the removal of outer skin. The white pepper so obtained is washed and dried for a period of 2-3 days in the solar tunnel drier. The dried white pepper is cleaned, graded and packaged for commercial use.

GRADER FOR FRESH PEPPER

Function: Grading fresh pepper to four grades
Capacity: 150 kg/h
Salient features: Rotary grader provided with stainless steel sieves with perforation of 2.5, 4.5 and 6.5 mm diameter for grading fresh green pepper. The power required to operate the unit is 0.37 kW.

WASHER

Function: Soft washing in water/steam
Capacity: 25 kg/batch
Salient features: The stainless steel washer drum is provided with a brushing unit and tilting arrangement for discharging washed produce. The power required to operate the unit is 0.37 kW.

FERMENTATION TANK

Function: Blanching and fermentation of pepper
Capacity: 250 kg/batch
Salient features: The stainless steel fermentation tank is provided with a perforated basket for holding fresh/dry pepper for blanching as well as for white pepper production. The tank is provided with a circulatory coil for steam circulation.

PULPER CUM WASHER

Function: Washing and removing the skin of fermented green/black pepper for white pepper production
Capacity: 200 kg/h
Salient features: The stainless steel pulper drum is provided with nylon brushes and paddles which facilitates the removal of outer skin of fermented pepper for white pepper production. The power required to operate the unit is 2.21 kW.

NUTRACEUTICALS AND FUNCTIONAL FOOD

R Sivaranjani

The term "Nutraceutical" was coined in 1989 by Stephen De Felice, founder and chairman of the Foundation for Innovation in Medicine, an American organization which encourages medical health research. He defined nutraceutical as a "food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease". Today, nutraceutical is a term commonly used in marketing to describe any product derived from food sources that provide extra health benefits in addition to the basic nutritional value found in food. Such products typically will claim to prevent chronic diseases, improve health, delay the aging process and/or increase life expectancy. Due to minimal international regulation in the area, various types of products can fall under the nutraceutical category and there may be overlap between these and herbals, dietary supplements and functional foods.

Nutraceuticals provide dietary supplementation. They are also intended to provide health benefits apart from fulfilling daily nutrient requirements. Tens of billions of dollars are spent annually on these supplements in worldwide. Nutraceuticals are classified as traditional or natural nutraceuticals (e.g., nutrients, herbals, phytochemicals, probiotic microorganisms, nutraceutical enzymes) and non-traditional or artificial nutraceuticals (e.g., fortified and recombinant nutraceuticals).

Herbal nutraceutical is useful in maintaining health, and it works against nutritionally induced acute and chronic diseases and promotes optimal health, longevity, and quality of life. Vitamins will fall in the category of food supplements whereas antioxidants and fats and oils will form the part of nutraceutical products. Antioxidants, are compounds that scavenge free radicals produced by cellular metabolism in your body. These free radicals damage DNA, RNA, proteins, and lipids. Antioxidants also regulate enzymes that produce free radicals. Oils and fatty acids are another type of nutraceuticals. These are typically polyunsaturated fatty acids. Omega-3 fatty acids are an example of this class of nutraceuticals. The body does not produce these compounds, so all omega-3 fatty acids must be attained from diet or supplementation. Omega-3 fatty acids are found in leafy green vegetables, vegetable oils, and fish oils. Benefits associated with omega-3 fatty acids include decreased triglyceride and very low density lipoprotein levels. They may also improve brain performance and cardiovascular health.

Examples of naturally occurring nutraceuticals

Naturally occurring substances	Select foods containing the substance	Areas with established or emerging evidence of benefit	
Dietary fiber	Fruits, grains, legumes, vegetables	• Lipid control • Arterial hypertension • Glucose control	• Weight control • Intestinal motility
Probiotics (for example, lactobacilli, gram-positive cocci, bifidobacteria)	Many naturally fermented foods (kefir, unpasteurized sauerkraut, soft cheeses, pickled cucumbers)	• Gastrointestinal disorders • Allergies	• Asthma • Cancer • Infections
Prebiotics	Chicory roots, bananas, tomatoes	• Lipid control • Gastrointestinal disorders	• Cancer
Polyunsaturated fats	Fatty fishes	• Cardiovascular disease • Asthma	• Mental health • Diabetes
Antioxidant vitamins (vitamin C, vitamin E, carotenoids)	Citrus fruits, peppers, nuts, seeds, cantaloupe, carrots	• Degenerative disease	
Polyphenols	Tea, dry legumes, berries	• Microbial infection • Neurodegenerative disease	• Diabetes • Cancer • Cardiovascular disease

Note: Inclusion in this figure does not imply that A.T. Kearney endorses the use or potential benefits of any of the substances listed.

Sources: Lipi Das, Eshani Bhaumik, Utpal Raychaudhuri, and Runu Chakraborty, "Role of Nutraceuticals in Human Health," *Journal of Food Science and Technology* 49, no. 2 (2012): 173–183; Office of Dietary Supplements, National Institutes of Health; WebMD

Fig 1: Naturally occurring substances show potential health benefits

Table 1: Classification of nutraceuticals based on food source

Food source	Examples
Plants	β-Glucan, Ascorbic acid, γ-Tocotrienol, Quercetin, Luteolin, Cellulose, Lutein, Gallic acid, Perillyl alcohol, Indole-3-carbonol, Pectin, Daidzein, Glutathione, Potassium, Allicin, δ-Limonene, Genestein, Lycopene, Hemicellulose, Lignin, Capsaicin, Geraniol, β-Ionone, α-Tocopherol, β-Carotene, Nordihydrocapsaicin, Selenium, Zeaxanthin, Minerals
Animals	Conjugated Linoleic Acid (CLA), Eicosapentaenoic acid (EPA), Docosahexenoic acid (DHA), Spingolipids, Choline, Lecithin, Calcium, Coenzyme Q10, Selenium, Zinc, Creatine, Minerals
Microbes	*Saccharomyces boulardii* (yeast), *Bifidobacterium bifidum, B. longum, B. infantis, Lactobacillus acidophilus* (LC1), *L. acidophilus* (NCFB 1748), *Streptococcus salvarius* (subs. Thermophilus)

Table 2: Classification of nutraceuticals based on mechanism of action

Anticancer	Positive Influence on Blood Lipid Profile	Antioxidant Activity	Anti-inflammatory	Osteogenetic or Bone Protective
Capsaicin	β-Glucan	CLA	Linolenic acid	CLA
Genestein	γ-Tocotrienol	Ascorbic acid	EPA	Soy protein
Daidzein	δ-Tocotrienol	β-Carotene	DHA	Genestein
α-Tocotrienol	MUFA	Polyphenolics	GLA	Daidzein
γ-Tocotrienol	Quercetin	Tocopherols	(gamma-linolenic acid)	Calcium
CLA	ω-3 PUFAs	Tocotrienols	Capsaicin	Casein phosphopeptides
Lactobacillus acidophilus	Resveratrol	Indole-3-carbonol	Quercetin	FOS
Sphingolipids	Tannins	α-Tocopherol	Curcumin	(fructooligosaccharides)
Limonene	β-Sitosterol	Ellagic acid		Inulin
Diallyl sulfide	Saponins	Lycopene		
Ajoene	Guar	Lutein		
α-Tocopherol	Pectin	Glutathione		
Enterolactone		Hydroxytyrosol		
Glycyrrhizin		Luteolin		
Equol		Oleuropein		
Curcumin		Catechins		
Ellagic acid		Gingerol		
Lutein		Chlorogenic acid		
Carnosol		Tannins		
L. bulgaricus				

Table 3: Classification of nutraceuticals based on chemical nature

Class/Component	Source	Potential Benefit
Carotenoids	Carrots	Neutralizes free radicals which may cause damage to cells
Alpha-carotene		
Beta-carotene	Various fruits, vegetables	Neutralizes free radicals
Lutein	Green vegetables	Contributes to maintenance of healthy vision
Lycopene	Tomatoes and tomato products (ketchup, sauces, etc.)	May reduce the risk of prostate cancer
Zeaxanthin	Eggs, citrus, corn	Contributes to the maintenance of healthy vision
Collagen Hydrolysate		
Collagen Hydrolysate	Gelatine	May help improve some symptoms associated with osteoarthritis
Dietary Fibre		
Insoluble fibre	Wheat bran	May reduce risk of breast and/or colon cancer
Beta glucan	Oats	Reduces risk of cardiovascular disease (CVD)
Soluble fibre	Psyllium	Reduces risk of CVD
Whole Grains	Cereal grains	Reduces risk of CVD
Fatty Acids		
Omega-3 fatty acids-DHA/EPA	Tuna; fish and marine oils	May reduce the risk of CVD & improve mental, visual functions

There is growing awareness on the importance of nutrition and diet for long-term good health. These have contributed to favourable market conditions for the nutraceutical industry in India. Apart from this, India has other advantages like well-qualified and intelligent human resources for setting up R&D facilities of international standards.

The country is also a cost-effective source of sophisticated raw materials, due to technological advances in areas like fermentation processes, plant extraction and chemical synthesis. These converging economic and demographic trends in India have laid the groundwork for opportunities in the nutraceutical industry.

NANOTECHNOLOGY AND ITS USE IN AGRICULTURE

A Jeevalatha

Nanotechnology is engineering of functional systems at the molecular level and is focused on controlling and exploiting the structure of matter on a large scale below 100 nanometers. Particles whose sizes range from 1-100 nm are called nanoparticles. These are number of atoms or molecules bonded together and intermediate in size between individual atoms and aggregates large enough to be called bulk material.The nanoparticles exhibit various unique features like optical, structural, thermal, mechanical and electromagnetic properties. Once the particle size is reduced below 100 nm, the solid particles begin to demonstrate unusual properties from the bulk material based on Quantum mechanics. Depending on the material used to produce nanoparticles, properties like solubility, transparency, color, absorption or emission wavelength, conductivity, melting point and catalytic behavior are changed only by varying the particle size. Properties like dispersibility, conductivity, catalytic behavior and optical properties alter with different surface properties of the particle. If the surface properties are not controlled, nanoparticles quickly turn into larger particles due to agglomeration and it is therefore crucial to control their agglomeration behavior.

(I) Synthesis of nanoparticles

The methods for making nanoparticles can generally involve either a "top down" approach or a "bottom up" approach (Fig. 1). In top-down synthesis, nanoparticles are produced by size reduction from a suitable starting material. Size reduction is achieved by various physical and chemical treatments (Fig. 2).In bottom up synthesis, the nanoparticles are built from smaller entities, for example by joining atoms, molecules and smaller particles. The bottom up synthesis mostly relies on chemical and biological methods of production.

Biomolecules present in plant extracts can be used to reduce metal ions to nanoparticles in a single-step green synthesis process. Plant extracts may act both as reducing agents and stabilizing agents in the synthesis of nanoparticles. The reducing agents involved include the various water soluble plant metabolites (e.g. alkaloids, phenolic compounds, terpenoids) and co-enzymes. Silver (Ag) and gold (Au) nanoparticles have been the particular focus of plant-based syntheses. Neem, *Aloe vera*, *Catharanthusroseus, Daturametel* and Geranium leaf are some of the plants successfully used for nanoparticle synthesis.

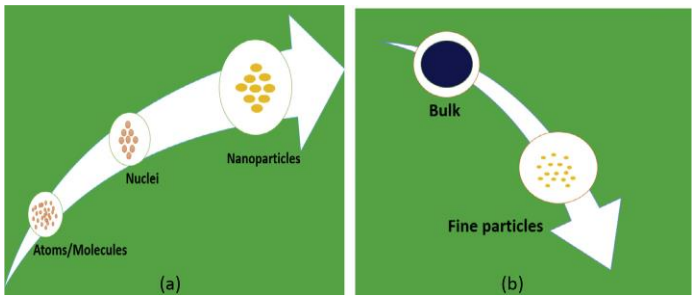


Fig.1. Synthesis of nanoparticles a. Bottom-up approach, b. Top-down approach

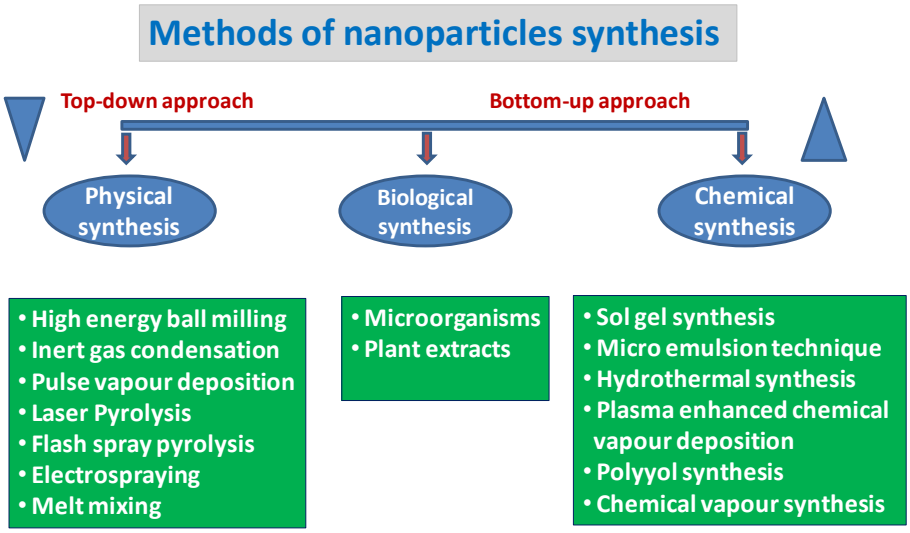


Fig.2. Different methods employed in nanoparticle synthesis

Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymesgenerated by the cell activities. It can be classified into intracellularand extracellular synthesis according to the locationwhere nanoparticles are formed. The intracellularmethod consists of transporting ions into the microbialcell to form nanoparticles in the presence of enzymes. Theextracellular synthesis of nanoparticles involves trapping themetal ions on the surface of the cells and reducing ions in the presence of enzymes.Various microbes are known to reduce the Ag+ ions to form silver nanoparticles and for example *Pseudomonas stutzeri*AG259 isolated from a silver mine, when placed in a concentrated aqueous solution of silver nitrate, played a major role in the reduction of the Ag+ ions and the formation of silver nanoparticles (AgNPs) of well-defined size and distinct topography within the periplasmic space of the bacteria. AgNPs were synthesized in the form of a film or produced in solution or accumulated on the surface of its cell when fungi, *Verticillium*, *Fusariumoxysporum*, or *Aspergillusflavus*, were employed.

(II) Characterization of nanoparticles

Characterizations of nanoparticles are primarily evaluated by the particle size distributionand morphology. With the aid of electron microscopy it's now possible toascertain the morphology as well as the size of nanoparticles. Advanced microscopic techniques as atomic force microscopy(AFM), scanning electron microscopy (SEM) and transmission electron microscopy(TEM) are used for characterization. Scanning electron microscopy determines the size, shape and surfacemorphology with direct visualization of the nanoparticles.TEM operates on different principle than SEM, yet it often brings same type of data. The sample preparation for TEM is complex and time consuming because of its requirement to be ultra thin for the electron transmittance.The AFMis based on a physical scanning of samples at sub-micronlevel using a probe tip of atomic scale and offers ultra-high resolution in particlesize measurement. Stability of colloidal material is usually analyzed through zeta potential ofnanoparticles. Zeta potential is an indirect measure of the net surface charge. As this electric potential closes to zero, particles have tendency to aggregate enormously. So Zeta potential is an indicator of the electrostatic or charge repulsion/attraction between the nanoparticles. For this reason zeta potential can be described as one of the important fundamental parameters to affect stability of the nanoparticles in a great extent.For more stability, the zetapotential should be more than +40 or less than -40. Particle size analyzer is used to measure the size and zeta potential of nanoparticles. The nanoparticles are sonicated using ultra sonicator before going for characterization in particle size analyzer. Raman spectroscopy, UV vis spectroscopy, FITR, Thermal gravity analyzer, X-ray diffraction analysis (XRD) are also used in nanoparticles characterization.

(III) Application in agriculture

(a) Plant disease diagnosis

Nanoparticles such as gold nanoparticles, magnetic nanoparticles and quantum dots, are most widely used for molecular detection purpose. Gold nanoparticles are widely used in rapid immune diagnosis.Lateral flow immuno assay (LFIA) have been developed for detection of many pathogens including pathogens infecting plants.LFIA is based on the interaction between the target virus and immunoreagents (antibodies and their conjugates with colored colloidal particles like gold nanoparticles) applied on the membrane carriers (lateral flow test strips). When the test strip is dipped into the sample being analyzed, the sample liquid flows through membranes and triggers immunochemical interactions resulting in visible coloration in test and reference lines (Fig. 3).

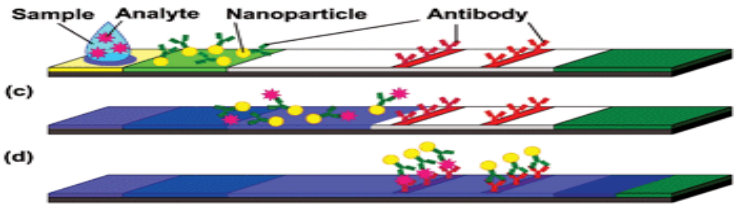


Fig.3. Diagrammatical representation of LFIA strips

Pathogen biosensing strategies are based on biological recognitionusing different receptors such as antibodies, DNA probe, phage

andothers. Antibody based biosensors and DNA biosensors are available for plant pathogen detection. QDs are a category of semiconductor crystals (i.e. CdSe, CdTe, CdS, ZnSe, PbS, and PbTe) with typical diameters that range between 2 nm and 10 nm (without outside coating). QDs provide a bright fluorescence (~10-20 folds higher than an organic fluorophore) and also present a higher photostability against photobleaching than conventional organic dyes. QDs are applied in deep tissue imaging in clinical and life science studies, gene-expression studies, high-throughput screening, and medical diagnosis. QDs have been applied in live cell imaging, *in vitro* imaging, *invivo* and animal imaging and virus tracking. Low wavelength ultraviolet (UV) and violet lasers are typically employed to excite QDs, since they induce maximal fluorescence emission.

(b) Pest management

Nano materials are used for delivery of pheromones for pest control in plants. Ananogel has been prepared from a pheromone, methyl eugenol (ME) using a low-molecular mass gelator for effective management of fruitfly, *Bactroceradorsalis* in Guava. The nanopheromone was found very stable at open ambient conditions and slowed down the vaporation of pheromone significantly (DeepaBhagat et al., 2013).Nano matrix was successfully developed for loading ethyl 4 methyl octanoate pheromone to enhance the longevity of lureto manage Rhinozerous beetle in coconut (K. Subaharan, CPCRI, Kasargod). The recent development of a nanoencapsulated pesticide formulation has slow releasing properties with enhanced solubility, specificity, permeability and stability. These assets are mainly achieved through either protecting the encapsulated active ingredients from premature degradation or increasing their pest control efficacy for a longer period. Formulation of nanoencapsulated pesticides led to reduce the dosage of pesticides and human beings exposure to them which is environmentally friendly for crop protection.

(c) Nanofertilizers

The nano-fertilizers are synthesized by fortifying nutrients singly or in combinations onto the adsorbents with nano-dimension. Both physical (top-down) and chemical (bottom-up) approaches are used to produce nanomaterials, and the targeted nutrients are loaded as it is for cationic nutrients ($NH4^{+}$, K^{+}, Ca^{2+}, Mg^{2+}) and after surface modification for anionic nutrients (NO^{3-}, $PO4^{2-}$, $SO4^{2-}$). Nano-fertilizers are known to release nutrients slowly and steadily for more than 30 days which may assist in improving the nutrient use efficiency without any associated ill-effects. Since the nano-fertilizers are designed to deliver slowly over a long period of time, the loss of nutrients is substantially reduced vis-a-vis environmental safety (Manikandan *et al*., 2015).

(d) Food packaging

Recently, somepackaging materials incorporated with “nanosensors” to detectthe oxidation process in food have been produced and usedin food industry. Other exploitation way isthe

incorporation of NPs in packaging and this technologywill slow down some biochemical processes such as oxidation,degradation, etc. thus it help to extend the shelf-life of foodproducts. In the food packaging industry, the most used materialsare plastic polymers that can be incorporated or coated withnanomaterials for improved mechanical or functional properties. Silver NPs havebeen successfully embedded in the plastic for making food storagebins, and this acts like disinfection of bins, thus minimizingharmful bacterial growth.

(e) Encapsulation of functional foods

Nano-encapsulation is a technique successfully used to improve the survival of microorganisms in dairy products, protect sensitive food components, ensure against nutritional loss and incorporate unusual or time-release mechanisms into the formulation. Liposomes have been used in the food industry to encapsulate functional ingredients, and more recently, they have been explored for their ability to integrate food antimicrobials that could aid in the protection of food products against growth of spoilage and pathogenic microorganisms. Lipid-based nanoencapsulation can potentially improve the solubility, stability, and bioavailability of foods, thus preventing unwanted interactions with other food components. Nanoliposomes are some of the most promising lipid-based carriers for antioxidants. Nanoliposomes also help in controlled and specific delivery of nutraceuticals, nutrients, enzymes, vitamins, antimicrobials, and additives. As the research in nanotechnology increases, it also raises public concerns about safety of the products of nanotechnology for usage. Therefore, a comprehensive assessment of potential risks to human health is essential before the nano products are commercially available. More studies and regulations concerning the impacts of these nanomaterials on human and environmental health need to be conducted and established to assure safety.

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EXTRACTION OF ENZYMES AND PROTEINS FROM PLANTS, PROTEIN ESTIMATION AND ISOZYME ANALYSIS

K S Krishnamurthy

Enzymes are biological catalysts that regulate the rate of a specific biochemical reaction in living organism. When it comes to the hand of a biotechnologist, it becomes the "*tailor's scissors and the surgeon's scalpel*." Enzymes are originally discovered in yeast by Buchner brothers (En= in; zyma=yeast). They are structurally proteins.

How do enzymes work?

The chemicals at which an enzyme operates are called **substrates.** Enzymes are **specific** ie; they catalyse only a few closely related chemical reactions –or in many cases only one particular reaction. They form temporary chemical compounds with their substrates (E-S complex), which then release the products and regenerating the original enzyme molecule for reuse.

The prosthetic group is the non-protein group of the enzyme, which is bound by the chemical bonds. Haem, biotin.pyridoxal phosphate etc. function as prosthetic groups. **Apoenzyme** is the remaining protein part of the enzyme, which is inert. **Coenzyme** is the coworker of prosthetic group and apoenzyme, since without coenzyme, the enzyme is non-reactive. They combine with the enzyme and leave it after catalysis.

Eg; DPN, NAD, ATP, ADP etc

Cofactor: When a prosthetic group consists of single atoms of some metals like Mg^{++}, Fe^{++}, Cu^{++}, Mo^{++} then it s known as **cofactor** and can be easily separated from the protein part.

There are two ways an enzyme can work;

1. Lock and key hypothesis
2. Induced fit model

Lock and Key model

Enzymes have one or more regions called *active sites*, which are located close to one another on the enzyme surface. During the course of a reaction, the substrate molecules occupy the sites and are temporarily brought together. At this the shape of the enzyme molecule changes resulting in strain in critical bonds in the substrate molecule. The new chemical compound produced has little affinity for the substrate and moves away.

Induced fit model

Here the active sites of the enzyme are not rigid. When a substrate binds to the enzyme, it induces a change in the shape of the enzyme molecule resulting in optimum fit of the substrate-enzyme reaction. This puts a strain on the substrate, promoting the reaction.

Regulation of enzyme action

The chemical reactions are controlled by the enzymes. But what controls the enzymes?

1. The mechanism of enzyme regulation depends on the amount of enzyme produced, which is directed by a specific gene, which in turn is switched on by a signal from the hormone or any other cellular product.
2. *Allosteric control*: Enzymes which are present in the inactive form in the cytoplasm may be activated. In the inactive form, the active sites of the enzymes have no shape, so the substrate does not fit. In some cases, active site and substrate site are also present. When a molecular activator or cyclic AMP attaches to the site, the shape of the enzyme molecule changes, making the active site available for substrate combination.

Isozymes and allozymes

Underlying principles

1. Certain enzymes are well-characterized as to their genetic basis and inheritance
2. Individuals of particular plant populations or species may harbor one or more alleles of each enzyme
3. Genetic variation and various parameters of population subdivision, relationships and Mendelian inheritance are calculated based on the proportion of alleles within and among individuals, populations and species

Important assumptions of isozymes and allozymes

1. Enzyme variation is a representative reflection the overall DNA variation
2. Two comigrating "identical" alleles for the same enzyme in two different individuals/ populations/species are coded for by the exact same mutation

What are isozymes?

Isozymes (Isoenzymes) were discovered 30 years ago and since then lot of information has accrued on the subject. These studies have helped us in understanding the metabolic regulation in plants, bacteria and animals and in making available a multitude of highly sensitive markers. Isozymes are also being used in diagnostic clinical biochemistry.

Thus isozymes are ***multiple molecular forms of enzymes, which can be separated from one another using several techniques***. They are also proteins, carrying a net negative charge. Relative migration rate of isozymes under polarised field is a function of their MW,

configuration, Amino acid composition etc. Isozyme structure is the direct expression of genetic information contained in m RNA and provide direct link to DNA. And structural genes encoded therein. Hence isozyme polymorphism is especially rewarding to plant and animal geneticists and breeders.

Nomenclature & terminology

1. In many cases, the normal enzyme name should be used followed by a no. Which is given according to the electrophoretic mobility under defined conditions.
2. Increase of complex enzymes where more than one zone is seen, numbers may be used to designate major groups. Thus, 1a, 1b, 2a, 2b etc.

What are allozymes?

While isozymes refer to *isomers of functional types of enzymes regardless of locus*, allozymes refers to isomers *within a genetic locus.* Starch gel electrophoresis is used to assay the allozymes of numerous gene loci .The enzyme migrate through the gel matrix in response to the electric field at a rate proportional to the net surface charge. After electrophoresis, the gels are cut into thin horizontal slices and each slice is put into an enzyme specific staining solution.

Multi locus enzyme information from a single tree can provide a biochemical fingerprint, a genotype of that tree. Allozyme data from populations of trees can form a powerful database for analysing the genetic characteristics of that tree.

Why enzyme multiplicity does occurs?

1. Genetic or primary causes
2. Post translational or secondary causes

In the former, the organism carries multiple genes, each one encoding for a different enzyme sub unit, while in the latter, homogeneous enzyme sub units are modified differentially from a single gene.

Isozymes as genetic markers

Isozyme analysis is an important experimental technique in Genetics. Each isozyme sub unit, by definition, is the result of a different gene, whether encoded by multiple alleles at a single locus or multiple loci. Use of isoenzymes as markers is wide spread and the hypothesis ‘one-gene one-isozyme sub unit ‘has been proposed in 1980.

Well-set example of isozymes - LDH

In glycolysis, glucose molecule is metabolised to form pyruvate. The final reaction of anaerobic glycolysis is the conversion of pyruvate to lactate calalysed by the enzyme Lactic De Hydrogenase (LDH). In skeletal muscle where oxygen deprivation is common during exercise,

this reaction is efficient and large amount of lactate can be formed. In tissues that preferentially oxidise glucose aerobically to CO_2 and water, such as cardiac muscle the reaction is not efficient and pyruvate is preferentially converted to Acetyl CoA and then enters TCA cycle.

Five major LDH isozymes are found in different vertebrate tissues. Each LDH molecule is composed of 4 polypeptide chains, but the sub unit composition of the 5 LDH isozymes are different. There are two types of polypeptide chains in LDH called **M** (for skeletal muscle) and **H** (for heart muscle), which can be combined in 5 different ways.

Different genes encode the H and M polypeptide chains and the two genes are expressed to different degrees inn different tissues. Thus in heart muscle the gene for the H sub unit is more active than the gene for the M sub unit. Thus **LDH 1** is the predominant one in cardiac muscle, while **LDH5** is more active in skeletal muscle.

Applications

Measurement of LDH isozymes in serum has been extensively used to find the site and nature of tissue injury in humans. Thus when blood supply to the heart muscle is severely reduced as during a heart attack, muscle cells die and liberate **LDH 1** to the blood stream. In contrast, **LDH 5** increase in serum is indicative of muscular dystrophy.

Isozyme markers

Isozyme markers have been developed in several horticultural crops for characterisation, varietal identification, sex determination etc. as follows:

Crop	**Objective**	**Reference**
Amaranthus	Classification	Oken *et al*, 1996
Citrus	Classification	Dequin *et al*, 1994
Tomato	Nematode resistance	Rick and Fobes, 1994
Asparagus	Sex determination	Maestri *et al*; 1991

Applications

1. Widely applicable for population-level and species-level comparisons among closely related organisms
2. Still the "work horse" for population-level studies, although many have switched to DNA-based approaches (restriction site variation, RAPDs and other anonymous markers)

Advantages

1. At least some enzymes will show variation in most plant studies; no need to do extensive screening beyond preliminary tests for successful enzyme/gel/buffer systems
2. Purportedly cheaper to do lots of samples than other methods, including DNA

3. Initial cash outlay for equipment not huge, no particularly expensive pieces of equipment beyond gel rigs and electrophoresis power supplies
4. Disadvantages
5. Must use fresh or recently frozen tissue; cannot store material for long term beyond freezing
6. Relatively few data points per unit of effort--at most a couple of dozen alleles possible even with extensive screening
7. Must have some understanding of genetic situation for each plant group--polyploid taxa will show complex patterns, difficult to interpret
8. Some plant species or species groups will display little or no variation; must use more sensitive, DNA-based methods to access variation in those
9. Availability of limited no of enzyme loci
10. Developmental and season –dependant expression of activity.

Reagents for Native gel electrophoresis:
Acrylamide - bisacrylamide mix
Acryl amide 30%
Bisacryl amide 0.8%
Strore at 4^0C, away from light

1.5M Tris pH 8.8 (4x resolving gel buffer)
pH adjusted to 8.8 with 4N HCl

1.0M Tris pH 6.8
pH adjusted to 6.8 with 4N HCl

10% Ammonium persulphate (APS)
Should be prepared fresh as APS decomposes slowly during storage

10 % Sodium Dodecyl Sulphate (SDS)
(Only for SDS PAGE)
Electrophoresis (Reservoir) buffer (10x) for Native gel
7.56 g TRIS + 38 g glycine, pH ajusted to 8.3, volume made up tp 250 ml with deionised water.

Electrophoresis (Reservoir) buffer (5x) for SDS PAGE
15.1 g TRIS + 94 g glycine, pH adjusted to 8.3, 0.1% SDS., volume made upto 1000 ml with deionised water

Note: It is advisable to use deionised water for the preparation of reagents. In case of non-availability, double distilled water may be used.

Sample buffer for SDS PAGE

4x stacking gel buffer 2.5 ml
10 % SDS 0.4 ml
Glycerol 2 ml
Bromophenol blue 2 mg
Mercapto ethanol 5 % (final conc.)
Make up to 10 ml with deionised water

Ingredients for Native gel (10% gel), Volume 7.5 ml

Acryl amide bisacryl amide mix 2.5 ml
1.5M Tris (8.8 pH) 1.9 ml
Double distilled water 3.025 ml
10 % APS 0.075 ml
TEMED 10 µl

Ingredients for SDS (10% gel), Volume 10 ml

Acryl amide bisacryl amide mix 3.3 ml
1.5M Tris (8.8 pH) 2.5 ml
Double distilled water 4.0 ml
10 % SDS 0.1 ml
10 % APS 0.1 ml
TEMED 10 µl

Ingredients for stacking gel (5 % gel), Volume 6 ml

Acryl amide bisacryl amide mix 1 ml
1.0M Tris (6.8 pH) 0.75 ml
Double distilled water 4.2 ml
10 % APS 0.06 ml
TEMED 10 µl

Gel casting

1. Rub the glass plates thoroughly with ethanol, dry and assemble them. The notched glass plate should be facing out wards.
2. In an erlenmeyer flask, prepare the appropriate volume of the solution containing the desired concentration of acrylamide for the resolving gel, using the values given earlier. Mix the components in the order shown. Polymerization will begin as soon as the TEMED is added. Without delay, swirl the mixture rapidly and proceed to the next step.
3. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel (length of the teeth of the comb plus 1 cm). Using a pasteur pipette, carefully overlay the acrylamide solution with 0.1% SDS (for gels containing $\leq$ 8 % acrylamide) or isobutanol (for gels containing $\geq$ 10 % acrylamide). Place the gel in a

vertical position at room temperature. The overlay prevents oxygen from diffusing into the gel and inhibiting polymerization.

4. After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionised water to remove any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.
5. Prepare the stacking gel in a beaker/flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given earlier. Mix the components in the order shown. Polymerization will begin as soon as the TEMED is added. Without delay, swirl the mixture rapidly and proceed to the next step.
6. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a clean teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Place the gel in a vertical position at room temperature.
7. Teflon comb should be cleaned with water and dried with ethanol before use.
8. After polymerization is complete (30 minutes), remove the Telfon comb carefully. Using a squirt bottle, wash the wells immediately with deionised water to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates using a bent hypodermic needle attached to syringe.
9. Load up to 50 µl of each of the samples in to the bottom of the wells using a micropipette or a syringe. Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom buffer reservoir). Apply current at the rate of 1.5-milli amp per gel. After the dye front has moved into the resolving gel, increase the current to 2-milli amp per gel and run the gel until the bromophenol blue reaches the bottom of the resolving gel. Then turn off the power supply.
10. Remove the glass plates from the electrophoresis apparatus and place them on a paper towel. Using a spatula, pry the plates apart. Mark the orientation of the gel by cutting a corner from the bottom of the gel that is closet to the left most well.
11. The gel now can be fixed and stained.

Staining Procedure (For Isozymes)

1. Conduct electrophoresis in starch or polyacrylamide disc/slab buffer gels (with no SDS) at low temperature
2. Immediately after electrophoresis, incubate the gel in the substrate solution(s). The zones where the enzymes are located in the gel are visualized due to the appearance of coloured reaction products. After sufficient incubation period, stop the reaction by adding

appropriate stop solution and photograph the zymogram. Otherwise, the relative position of each visualized band in the gel may be drawn schematically for easy reference.

3. Enzyme extraction and stain for various isoenzymes are given below:

Esterase

Enzyme Extract

Homogenize the sample material in 5 fold volume of 10 mM sodium phosphate buffer (pH 9.5), 1 mM EDTA Na^{2+}, 1 mM 2-mercaptoethanol. Centrifuge the homogenate at 10,000 x g for 10 min and use the supernatant as enzyme source. All operations are at 0-4ºC. Incubate the gel in a solution given below at 37ºC for 20-30 min, preferable in dark.

Sodium dihydrogen phosphate 2.8 g
Disodium hydrogen phosphate 1.1 g
Fast blue RR salt 0.2 g
Alpha-naphthyl acetate 0.03 g
Water to 200 ml

Stop the enzyme reaction by adding a mixture of methanol: water: acetic acid: ethyl alcohol in the ration 10:10:2:1.

Polyphenol oxidase (PPO)

Enzyme Extract

The PPO is extracted by homogenizing the material in 0.01 M potassium phosphate buffer (pH 7.0) containing 1% non-ionic detergent (Tween 80) at 0ºC for 15 min. Centrifuge the homogenate at 20,000-x g for 15 min at 0ºC in a refrigerated centrifuge. Use the supernatant as enzyme source.

Equilibrate the gel for 30 min in 0.1% p-phenylenediamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer.

Peroxidase

Enzyme Extract: Same as above

Incubate the gel in the following solutions
Benzidine 2.08 g
Acetic acid 18 ml
Hydrogen peroxide (3%) 100 ml
Water 80 ml

Bright blue coloured bands appear in gel. When the bands are stained sufficiently, arrest the reaction by immersing the gel into a large volume of 0.67% sodium hydroxide or 7% acetic acid solution for 10 min.

Staining SDS polyacrylamide gels with Coomassie brilliant blue

1. Dissolve 0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methanol:DD H_2O (1:1 v/v:) and 10 ml of glacial acetic acid. Filter the solution through a whatman No. 1 filter paper to remove any particulate matter.
2. Immerse the gel in at least 5 volumes of staining solution and place on a slowly rotating platform for a minimum of 4 h at room temperature.
3. Remove the stain and save it for future use. Destain the gel by soaking it in the methanol: DD H_2O (1:1 v/v:) and glacial acetic acid as in step 1 without the dye on a slowly rocking platform for 4-8 h, changing the destaining solution 3-4 times.
4. The more thoroughly the gel is destained, the smaller the amount of protein that can be detected by staining with Coomassie Brilliant Blue. Destaining for 24 hr usually allows as little as 0.1 μg of protein to be detected in a single band.
5. Store fixed gels in 7 % acetic acid or in 20 % glycerol. Stained gels should not be stored in destaining buffer, which will cause the stained protein bands to fade.

Protein estimation by Lowry's method

Protein can be estimated by different methods as described by Lowry's and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolyzing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry *et al* is sensitive enough to give a moderately constant value and hence largely followed. Protein content of enzyme extracts is usually determined by this method.

Principle

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein pus the colour developed by the biuret reaction of the protein with thealkaline cupric tartrate are measured in the Lowry's method.

Materials

2% Sodium Carbonate in 0.1 N Sodium Hydroxide (Reagent A)
0.5% Copper Sulphate ($CuSO_4$. $5H_2O$) in 1% potassium sodium tartrate (Reagent B)
Alkaline Copper solution: Mix 50 ml of A and 1 ml of B prior to use (Reagent C)

Folin-Ciocalteau Reagent (Reagent D) - Reflux gently for 10 hrs a mixture consisting of 100 g sodium tungstate ($Na_2WoO_4.2H_2O$), 25 g sodium molybdate ($Na_2MoO_4.2H_2O$), 700 ml water, 50 ml of 85% phosphoric acid and 100 ml of conc. HCl in a 1.5 l flask. Add 150 g lithium sulfate, 50 ml water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1 l and filter. The reagent should have no greenish tint (Determine the acid concentration of the reagent by titration with 1 N NaOH to a phenolphthalein end point).

Protein solution (stock standard): Weigh accurately 50 mg of bovine serum albumin (Fraction V:) and dissolve in distilled water and make up to 50 ml in a standard flask.

Working standard: Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg.

Procedure

Extraction of protein from sample: Extraction is usually carried out with buffers used for the enzymes assay. Weigh 500 mg of the sample and grind well with a pestle and mortar in 5-10 ml of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of proteins

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard into a series of test tubes.
2. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Add 5 ml of reagent C to each tube including the blank. Mix well and allow to stand for 10 min.
5. Then add 0.5 ml of reagent D, mix well and incubate at room temp in the dark for 30 min. Blue colour is developed.
6. Take the readings at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

Calculation

Express the amount of protein mg/g or 100 g sample.

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PLANT TISSUE CULTURE

Sharon Aravind

Plant tissue culture is the culture and maintenance of plant cells or organs in sterile, nutritionally and environmentally supportive conditions (*in vitro*). Plant cell and tissue culture include the cultural techniques for regeneration of functional plants from embryonic tissues, tissue fragments, calli, isolated cells, or protoplasts.

Principle

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones

History of Plant Tissue Culture

G. Haberlandt, a German botanist, in 1902 cultured fully differentiated plant cells isolated from different plants. This was the very first step for the beginning of plant cell and tissue culture. He developed the concept of *in-vitro* culture of plant cells and is aptly regarded as the father of tissue culture. With the identification of a variety of chemicals like cytokinin, auxin, other hormones, vitamins, etc. and their role in affecting cell division and differentiation, the methods of plant tissue culture was developed in a proper manner. Three other scientists Gautheret, White and Nobecourt (1939) also made valuable contributions to the callus culture development of plant tissue culture techniques. The first plant from a mature plant cell was regenerated by Braun in 1959. Foundation of commercial plant tissue culture was laid in 1960 with the discovery for a million fold increase in the multiplication of Cymbidium (an orchid) which was accomplished by G.M.Morel.

In India, the work on tissue culture was initiated during 1950s at University of Delhi. This initiation is credited to Shri Panchanan Maheshwari who was working there in the Department of Botany. Discovery of haploid production was a land-mark in the development of in-vitro culturing of plants. Shri S.C. Maheshwari and Sipra Guha made a remarkable contribution in the development of plant tissue culture in India. Later on the development in the composition of nutrient media and genetic engineering served as a basis for further success in the plant tissue culture techniques.

Micropropagation techniques

These basic steps for *in vitro* culturing of plants are:

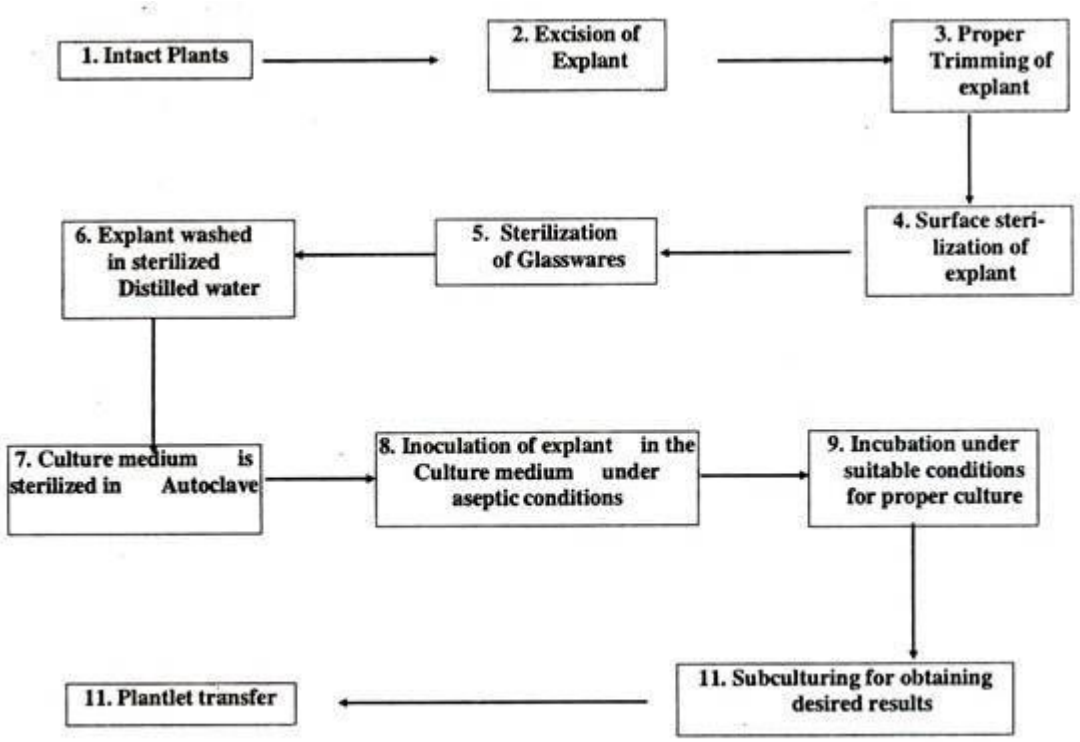


Fig. 1. Steps in general technique of Plant tissue culture.

(a) Selection and Surface Sterilization of Explant

Suitable explant (the plant or plant part excised for the in-vitro cultivation) selected based on age, size, healthy, contaminant free and is then excised from the donor plant. Explant is then surface sterilized to eliminate contaminants using anti-fungal agents (bavistin), sodium hypochlorite at (1-2%) or mercuric chloride (0.1% solution).

(b) Preparation and Sterilization of Culture Medium

A suitable culture medium is prepared with special attention towards the objectives of culture and type of explant to be cultured. Prepared culture medium is transferred into sterilized vessels and then sterilized in autoclave. It is a medium containing salts, trace elements, vitamins, carbon sources and growth regulators. Organic supplements like agar-agar are used. Generally B-5 medium (Gamborg *et al.)* or MS medium (Murashige and Skoog) are used.

(c) Inoculation

Sterilized explant is inoculated (transferred) on the culture medium under aseptic conditions.

(d) Incubation

Cultures are then incubated in the culture room where appropriate conditions of light, temperature and humidity are provided for successful culturing.

(e) Sub culturing

Cultured cells are transferred to a fresh nutrient medium to obtain the plantlets.

Sub culturing:- Transferring of tissue to fresh media after a stipulated time. Usually sub-culturing is done after every 4-6 weeks. However, the suspension cultures are sub-cultured after every 3-14 days.

(f) Transfer of Plantlets:

After the hardening process (i.e., acclimatization of plantlet to the environment), the plantlets are transferred to green house or in pots and finally in soil. Transfer is done when roots and shoots appears.

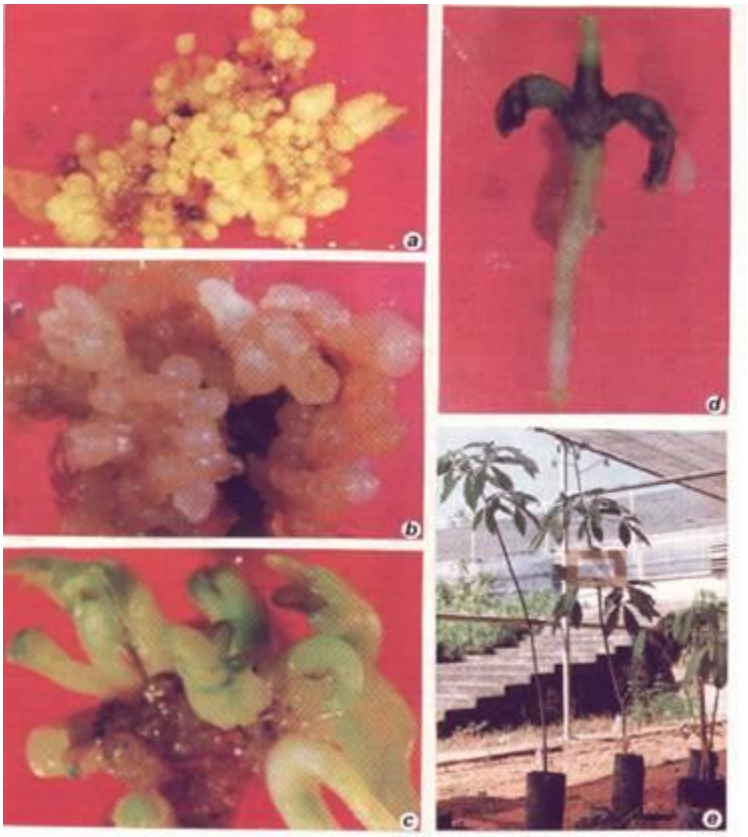
Classification of Tissue Culture Techniques:

Callus Culture: When the cells divide into an undifferentiated mass it is called as callus. Any part of a plant can be used to produce the calli. It may be a stem, leaf, meristem or any other part. It is used to produce variations among the plantlets.

Suspension culture: The callus produced from the explants are grown on nutrient solutions (that are semi solid) for a period of time and they are induced to produce plants with new traits.

Embryo Culture: The method of culturing mature and immature embryos in media is called embryo culture. By this method, it is possible to produce plants from dormant seeds and seeds with metabolites that inhibit germination. This method is very important in crop improvement programs.

Somatic Embryogenesis: When the plants are grown on nutrient media, calli are formed. When these calli are subjected to growth in cytokinin medium, somatic embryos are formed. They are circular, elongated, heart and torpedo shaped. Among that torpedo shaped embryos produce the whole plant that is very robust (Fig: 3).



a. Somatic embryos

b. Heart-shaped somatic embryos

c. Elongated somatic embryos

d. Torpedo shaped somatic embryos
e. Whole plant

Fig: 2. Generation of Plants by Somatic Embryogenesis

Apart from this above mentioned method, the suspensions of the callus are transferred to conical flasks and separate somatic embryos are produced and allowed to mature. These mature embryos when induced with cytokinin at the fourth stage produce the whole plantlets. Moreover these plants are hardened in a green house after rooting occurs. Somatic embryo culture is a very special method in plant tissue culture. Calcium alginate is used to produce artificial seeds from somatic embryos. It is very useful in studying secondary metabolites from the cell and to do research in somatic embryo culturing.

Organogenesis: Any plant part when placed and cultured on a media, tries hard to get back its own life. In this phenomenon, the callus are produced. These callus are subjected to cytokine treatment which produce the shoots. This method of producing plant organs is called as organogenesis. These shoots are transferred to root including auxin contained media and we thus get the whole plantlets.

Embryo Rescue Culture: Some commercially important crops were hindered in germination due to the constraints related to seed anatomy and physiology. Embryo abortion is a major factor to this. These embryos of such seeds are isolated and cultured on suitable nutrient media so as to

regenerate the plants easily. Embryos obtained after incompatible sexual mating also be rescued by this methodology.

Meristem Culture: Disinfected small bits of meristem tips of 0.1 to 0.5mm length are used to produce callus on suitable media to produce whole plants. This is called as meristem -culture and is used to produce pathogen free plantlets**.**

Anther Culture: When the pollen/anthers of the correct stage are collected and grown on suitable media, it is called as anther culture. It is used to produce haploid plants.

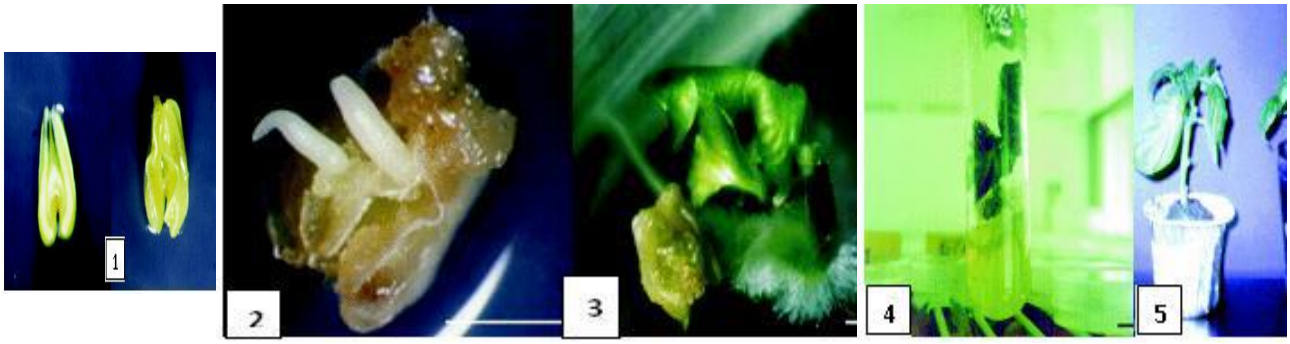


Fig: 4. Production of Plants by means of Anther Culture.

1. Pollen on media 2. Callus formation 3. Shoot initiation 4. Root initiation 5. Whole plant.

Two Stages in Anther /Pollen Culture: The culturing of pollen consists of two stages namely direct and indirect culturing methods. In the direct method, the pollen by themselves produce the plant directly from the medium. In the indirect method, the pollen produces a callus from which haploid plants are produced. Diploid plants are produced from the pollen sacs in a short period of time because the tips of these plants are always pathogen free.

Shoot Tip Culture: The meristem tip of a plant is more efficient in creating a whole plant than its tissues from the stem. This idea is made use of in the shoot tip culture. This method is used to produce plants free from pathogens, for meristems do not support the growth of viral particles. Hence the plants produced by this method can be stored pathogen free for a longer period.

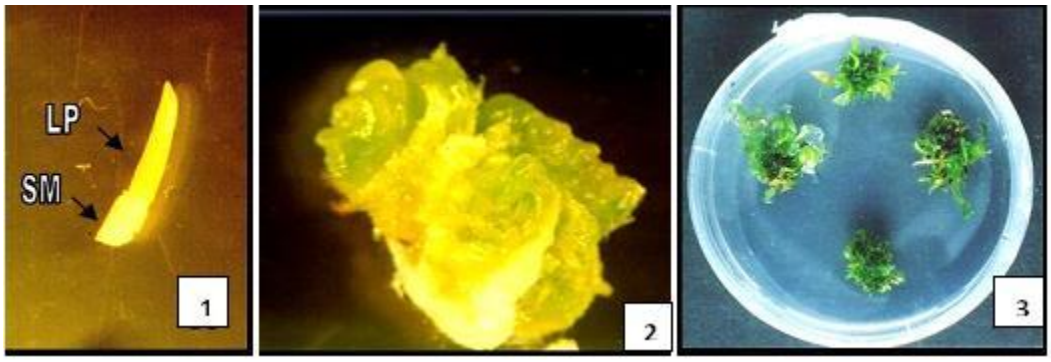


Fig: 3. Methods of Producing Plants from Shoot Tip Culture.

1. Shoot tip in the medium 2. Shoot callus 3.Growth of stem
4. Root initiation 5.Hardening 6.Whole plant

The choice of mother plant for collecting the pollen, proper maintenance in lab, the pH balance of the nutrient medium, incubation period for producing the plant are all considered to be major factors.

Rejuvenation of Plants: It is very feasible to produce the whole plant out of tissues collected from old plants in very short period of time. This is called plant rejuvenation. This method was demonstrated successfully in tapioca.

Hybrid Sorting: It is also possible to produce hybrids from incompatible species (where in the hybrids are hard to be formed) by means of protoplasmic fusion. Hybrid may be formed by culturing the fused protoplasts in suitable nutrient media.

Applications of Plant Tissue Culture:

a. Gemplasm conservation mainly in the form of cryopreservation of somatic embryos or shoot apices- This technique is mainly used to conserve plant which do not produce seeds or which have recalcitrant seeds which cannot be stored under normal storage conditions in seed gene banks. Hence, vegetative propagated crops such as root and tubers, ornamentals, medicinal plants and many other tropical fruits have to be conserved using *in vitro* methods.

b. Large scale production of useful compounds and secondary metabolites like recombinant proteins used as biopharmaceuticals by using genetically engineered plant tissue cultures.

c. Rapid multiplication of genetically uniform plants (clones) that possess desirable traits. A single explant can be multiplied into several thousand plants in a very short time. Once established, actively dividing cultures are a continuous source of micro cuttings which can result in plant production under greenhouse conditions without seasonal interruption.

d. Eradication of systemic diseases in plants and raising disease free plants by using meristem culture.

e. Soma-clonal variations are useful sources of introduction of valuable genetic variations in plants.

f. Helps plants in imparting resistance to antibiotics, drought, salinity, diseases, etc.

g. Somatic hybrids and cybrids overcome species barriers and sexual incompatibility and produce hybrid plants with desired combination of traits.
h. Embryo culture helps in overcoming seed sterility and dormancy.
i. Haploid production in culture helps to solve various problems of genetic studies and thus aids the plant breeders for producing new varieties.
j. Production of synthetic seeds via somatic embryo differentiation for commercially important plants helps to achieve increased agricultural production.
k. Large scale production of biomass energy.
l. Plant tissue culture aids in producing the genetically transformed plants.
m. Early flowering can be induced by in-vitro culturing of plants so as to attain commercial benefits.
n. Triploids as well as polyploid plants can also be produced by tissue culture techniques for uses in plant breeding, horticulture and forestry.
o. Seedless fruits and vegetables can be produced by following the endosperm culture method which add to their commercial values.
p. Increased Nitrogen fixation ability can be achieved through association of tissue culture techniques with genetic engineering.
q. Callus cultures are useful in plant pathology as they act as an effective tool in the study of mechanism of disease resistance and susceptibility.
r. Different tissue culture techniques help us to study various biosynthetic processes, physiological changes and cytogenetic changes.

Setting Up a Tissue Culture Lab

Any laboratory, in which tissue culture techniques are performed, regardless of the specific purpose, must contain a number of basic facilities like

- A general washing area
- A media preparation, sterilization, and storage area
- An aseptic transfer area
- Environmentally controlled incubators or culture rooms
- An observation/data collection area.

Washing Area

The washing area should contain large sinks, some lead-lined to resist acids and alkalis, draining boards, and racks, and should have access to demineralized water, distilled water, and double-distilled water. Space for drying ovens or racks, automated dishwashers, acid baths, pipette washers and driers, and storage cabinets should also be available in the washing area.

Media Preparation Area

The media preparation area should have ample storage space for the chemicals, culture vessels and closures, and glassware required for media preparation and dispensing. Bench space for hot

plates/stirrers, pH meters, balances, water baths, and media-dispensing equipment should be available. Other necessary equipment may include air and vacuum sources, distilled and double-distilled water, Bunsen burners with a gas source, refrigerators and freezers for storing stock solutions and chemicals, a microwave or a convection oven, and an autoclave or domestic pressure cooker for sterilizing media, glassware, and instruments.

The water used in preparing media must be of the utmost purity and highest quality. Tap water is unsuitable because it may contain cations (ammonium, calcium, iron, magnesium, sodium, etc.), anions (bicarbonates, chlorides, fluorides, phosphates, etc.), microorganisms (algae, fungi, bacterial), gases (oxygen, carbon dioxide, nitrogen), and particulate matter (silt, oils, organic matter, etc.) Water used for plant tissue culture should meet, at a minimum, the standards for type II reagent grade water, i.e., be free of pyrogens, gases, and organic matter and have an electrical conductivity less than 1.0 μmho/cm.

The most common and preferred method of purifying water to type II standards is a deionization treatment followed by one or two glass distillations. The deionization treatment removes most ionic impurities, and the distillation process removes large organic molecules, microorganisms, and pyrogens. Three other methods that will produce type II purity water are absorption filtration, which uses activated carbon to remove organic contaminants and free chlorine; membrane filtration, which removes particulate matter and most bacterial contamination; and reverse osmosis, which removes approximately 9% of the bacterial, organic, and particulate matter as well as about 90% of the ionized impurities.

Transfer Area

Under very clean and dry conditions, tissue culture techniques can be successfully performed on an open laboratory bench. However, it is advisable that a laminar flow hood or sterile transfer room be utilized for making transfers. Within the transfer area there should be a source of electricity, gas, compressed air, and vacuum.

The most desirable arrangement is a small dust-free room equipped with an overhead ultraviolet light and a positive-pressure ventilation unit. The ventilation should be equipped with a high-efficiency particulate air (HEPA) filter. A 0.3-μm HEPA filter of 99.97-99.99% efficiency works well. All surfaces in the room should be designed and constructed in such a manner that dust and microorganisms do not accumulate and the surfaces can be thoroughly cleaned and disinfected. A room of such design is particularly useful if large numbers of cultures are being manipulated or large pieces of equipment are being utilized.

Another type of transfer area is a laminar flow hood. Air is forced into the unit through a dust filter then passed through a HEPA filter. The air is then either directed downward (vertical flow unit) or outward (horizontal flow unit) over the working surface. The constant flow of bacteria-

free filtered air prevents nonfiltered air and particulate matter from settling on the working surface.

Culture Room

All types of tissue cultures should be incubated under conditions of well-controlled temperature, humidity, air circulation, and light quality and duration. These environmental factors may influence the growth and differentiation process directly during culture or indirectly by affecting their response in subsequent generations. Protoplast cultures, low-density cell suspension cultures, and anther cultures are particularly sensitive to environmental cultural condition.

Typically, the culture room for growth of plant tissue cultures should have a temperature between 15° and 30° C, with a temperature fluctuation of less than ±0.5°C; however, a wider range in temperature may be required for specific experiments. It is also recommended that the room have an alarm system to indicate when the temperature has reached preset high or low temperature limits, as well as continuous temperature recorder to monitor temperature fluctuations. The temperature should be constant throughout the entire culture room (i.e., no hot or cold spots). The culture room should have enough fluorescent lighting to reach the 10,000 lux; the lighting should be adjustable in terms of quantity and photoperiod duration. Both light and temperature should be programmable for a 24-hr period. The culture room should have fairly uniform forced-air ventilation, and a humidity range of 20-98% controllable to ±3 percent.

CARBON SEQUESTRATION AND ITS POTENTIAL IN AGRICULTURE

V Srinivasan

A range of anthropogenic activities is disrupting the global carbon (C) cycle, leading to an increase in the global temperature over the 20th century by 0.6 ± 0.2ºC at an average rate of increase of 0.17ºC per decade since 1950 (IPCC, 2007). Anthropogenic emissions of green house gases (GHGs) have increased by 70% from 1970 to 2009, and these are estimated to increase further by 25% to 95% by 2030. The emissions of carbon dioxide (CO_2), the most important anthropogenic GHG, have grown by 80% between 1970 and 2009 and represented 77% of total anthropogenic GHG emissions. The increase in nitrous oxide (N_2O) is primarily due to agriculture (IPCC, 2007). Methane (CH_4) concentration (~ 1775 ppb) in 2005 exceeded the natural range over the last 650,000 years (IPCC, 2007). Although fossil fuel combustion contributes the maximum increase in CO_2 emissions, globally about one third of total anthropogenic increase in GHGs is due to agriculture and land-use change.

Carbon sequestration describes long-term storage of carbon dioxide or other forms of carbon to either mitigate or defer global warming. It has been proposed as a way to slow the atmospheric and marine accumulation of greenhouse gases, which are released by burning fossil fuels. A concern for climate change, relevant to GHGs is that if 750 Gt C stored in the atmosphere constitutes a CO_2 concentration of ~406ppmv, what would be the impacts if the C budget undergoes continuous alteration by the destruction of biomass and the emission of C stored in soils, especially the frozen soils of the northern latitudes. Yet, agriculture can be an important part of the solution to climate change by reducing the net GHG emissions from both industrial and agricultural sectors.

United Nations Framework Convention on Climate Change (UNFCCC) allow biospheric carbon sinks and sources to be included in attempts to meet Quantified Emission Limitation or Reduction Commitments (QELRCs) outlined in the Kyoto Protocol include the activities: forest management, cropland management, grazing land management and re-vegetation. Among two possible strategies of mitigating the climate change, reducing emissions and sequestering emissions, C sequestration in terrestrial biosphere (e.g., forests, agricultural soils) is considered a win-win strategy, and a bridge to the future until low-C or no-C fuel sources take effect. In contrast to the engineering techniques of CO_2 capture and injection into geologic or oceanic strata, soil CS is a natural process in which biomass C is humified and incorporated into the soil C pool. The attendant increase in soil C pool through soil CS improves soil quality and set-in-motion land restorative processes, and advances food security.

Sequestration of C in soils is a useful strategy to reduce the rate of increase of atmospheric abundance of CO_2. Soil C sequestration implies enhancing both SOC and SIC pools through

conversion to a restorative land use and adoption of recommended management practices (RMPs) (Lal, 2004). Adoption of RMPs not only helps in increasing the SOC pool but also helps in increasing the agronomic yield. The rate of SOC loss due to conversion of natural ecosystems into agricultural lands usually far exceeds the rate of soil C sequestration achieved through adoption of RMPs. Factors, which determine agricultural contribution to achieve GHG reduction goals, comprise the biological and physical capacity of soil to sequester C along with available technology and economics.

There are five principal global C pools. The oceanic pool is the largest, followed by the geologic, pedologic (soil), biotic and the atmospheric pool. All these pools are inter-connected and C circulates among them. The pedologic or soil C pool comprises two components: SOC and the soil inorganic carbon (SIC) pool. The SIC pool is especially important in soils of the dry regions. The SOC concentration ranges from a low in soils of the arid regions to high in soils of the temperate regions, and extremely high in organic or peat soils. Therefore, the total soil C pool is four times the biotic (trees, etc.) pool and about three times the atmospheric pool.

Soil C sequestration can be attained through two crucial processes:

a. Decreasing anthropological emissions of CO_2, and
b. Increasing C sinks in the atmosphere.

Both SOC and SIC together comprising about 3.3 times of C present in atmospheric pool (750 Pg) and 4.5 times the size of the biotic pool (560-650 Pg). Increasing soil C pool has several ancillary benefits. It enhances soil quality, sustains and increases agronomic/biomass production, denatures and filters pollutants, maintains clean water and most importantly, mitigates atmospheric abundance of CO_2. Of this enormous amount of C loss due to agricultural practices, a large fraction is stored in the atmosphere.

The C storage potential in world cropland is estimated to be around 0.6-1.2 Pg C/yr. The challenge is to develop and implement policies that are conducive to realization of this potential. Potential of C sequestration in soils of India is 39 to 49 (44 ± 5) Tg C/yr. Intensive agriculture is practiced in the soils of the Indo-Gangetic plains in India, of which Punjab forms a part and there is generous usage of fertilizers and manures. Proper SOM management is inevitable for sustainable agriculture, which is essential to meet the growing demand of food and for maintaining the environment.

The sink capacity of SOM for atmospheric CO_2 can be greatly enhanced when degraded soils and ecosystems are restored, marginal agricultural soils are converted to a restorative land use or replanted to perennial vegetation, and RMPs are adopted on agricultural soils. Converting agricultural land to a more natural or restorative land use essentially reverses some of the effects responsible for SOC losses that occurred upon conversion of natural to managed ecosystems.

Applying ecological concepts to the management of natural resources (e.g., nutrient cycling, energy budget, soil engineering by macro–invertebrates and enhanced soil biodiversity) may be an important factor to improving soil quality and SOC sequestration. In addition to the quantity of input, quality of biomass can also be important in determining the SOC pool. Biodiversity is also important to soil C dynamics. It is defined as the variability among living organisms from all sources, including terrestrial, marine ecosystems and other aquatic ecosystems and ecological complexes of which they are part; this includes diversity within species, between species and for ecosystems.

The potential of SOC sequestration is high in the world's degraded soils and ecosystems estimated at 1216 Mha and agricultural soils estimated at 4961 Mha. These soils have the capacity to sequester C by converting to a restorative land use and adopting recommended management practices. All other factors remaining the same, the potential of SOC sequestration is in the following order: degraded soils and desertified ecosystems>cropland>grazing lands>forest lands and permanent crops. All other factors being equal, ecosystems with high biodiversity sequester more C in soil and biota than those with reduced biodiversity. In managed ecosystems, soil biodiversity is likely to increase with conversion to conservation tillage, replacement of toxic chemicals with viable alternatives, substitution of monoculture with mixed crop rotations and complex/ diverse systems, restoration of degraded soils and ecosystems, and conversion of crop or pasture land to a restorative land use.

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Lal, R. 2004a. Soil carbon sequestration impacts on global climate change and food security. *Science* 304:1623–1627.

Carbon Pool estimation

SOC pool $_{(0\text{-}15,\ 15\text{-}30)}$ (Mg ha^{-1}) = [C concentration $_{layer}$ ($kgMg^{-1}$) × (Bulk density) $_{layer}$ (Mgm^{-3}) × Depth (m) × 10^{-3} Mg kg^{-1} × 10^{4} m^{2} ha^{-1}].

Particulate organic matter: that fraction which is more liable for decomposition, as root mass, organic residues/ debris, coarse organic matter associated with the soil (usually > 0.05 mm size)

Non particulate organic matter: that fraction which is stable and associated with silt and clay fractions, not easily accessible for decomposition.

NPOC= TOC – POC

DROUGHT TOLERANCE AND MITIGATION IN CROP PLANTS

M Alagupalamuthirsolai

The occurrence of unfavorable environmental factors such as moisture deficit / excess, high radiation, low and high temperature, salinity of water and soil, nutrient deficiency or toxicity and pollution of atmosphere, soil and water are likely to affect the crop growth in terms of morphology (plant size, architecture, malformation of plant organs, growth (height, volume, weight), physiological and metabolic processes and yield of crop plants.

Stress and strain any environmental factor potentially unfavorable to plant is termed as stress. The effect of stress on plant condition is called strain. According to Newton's law of motion, a force is always accompanied by a counterforce, for an action there is always equal and opposite reaction. Stress is the action and whereas strain is the reaction.

Drought stress is considered to be a moderate loss of water, which leads to stomatal closure and limitation of gas exchange. Desiccation is much more extensive loss of water, which can potentially lead to gross disruption of metabolism and cell structure and eventually to the cessation of enzyme catalyzed reactions.

Drought stress is characterized by reduction of water content, diminished leaf water potential and turgor loss, closure of stomata and decrease in cell enlargement and growth. Severe water stress may result in the arrest of photosynthesis, disturbance of metabolism and finally the death of plant. Water stress inhibits cell enlargement more than cell division. It reduces plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters. In plants, a better understanding of the morpho-anatomical and physiological basis of changes in water stress resistance could be used to select or create new varieties of crops to obtain a better productivity under water stress conditions. The reactions of plants to water stress differ significantly at various organizational levels depending upon intensity and duration of stress as well as plant species and its stage of growth. Understanding plant responses to drought is of great importance and also a fundamental part for making the crops stress.

Adaptation to drought Drought resistance

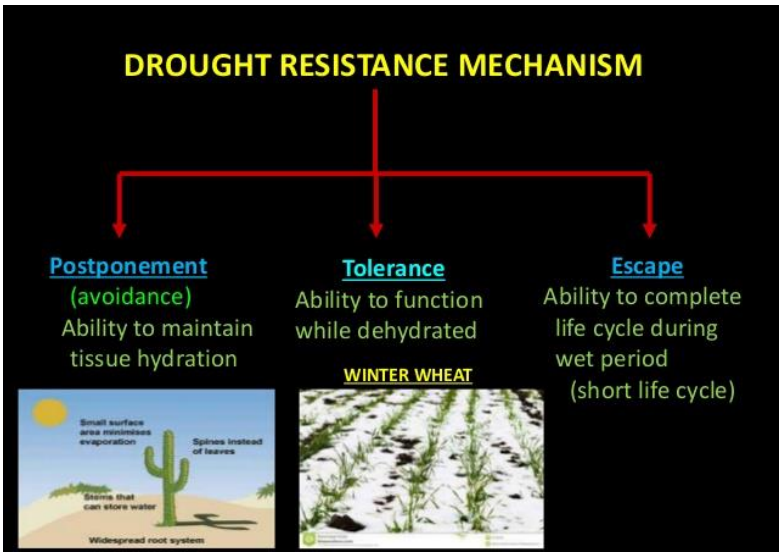
Drought resistance is defined as the capacity of plants to survive during the period of drought with little or no injury. There are three important categories of plants growing in the areas facing drought. They are ephemerals, succulents and non-succulent perennials

1. Ephemerals: These are short lived plants and they complete their life cycle within a short favorable period during rainy season. They pass dry periods in the form of seeds. They are called as drought escaping plants.

2. Succulent plants: These plants accumulate large quantities of water and use it slowly during dry period. Thus, they pass dry periods or drought without facing it. Such plants develop

several morphological adaptations for reducing transpiration such as thick cuticle, reduced leaf area, sunken stomata etc.

3. Non succulent plants: These plants are in fact the real drought enduring (tolerant) plants. They tolerate drought without adapting any mechanism to ensure continuous supply of water. They develop many morphological adaptations which are collectively called xeromorphy. They develop, in general, greyish colour, reflecting surfaces, smaller leaves, extensive root system, leaf fall during dry season, sunken stomata and thick cuticle etc. They develop an elaborated conducting system. The stomata remain closed mostly in dry periods. The plants develop several protoplasmic peculiarities such as cell size, cell structure, increased permeability, increased imbibition power, elasticity, small vacuoles, higher osmotic pressure etc. Osmotic adjustment



The adverse effects of water stress on crop growth can be mitigated by the application of chemicals such as nutrients, anti-transpirants and Plant Growth Regulators (PGRs), which induce the plants to become adaptive to water stress situations for a specified period and the water requirement for such periods can be minimized or saved.

The drought breeding strategies

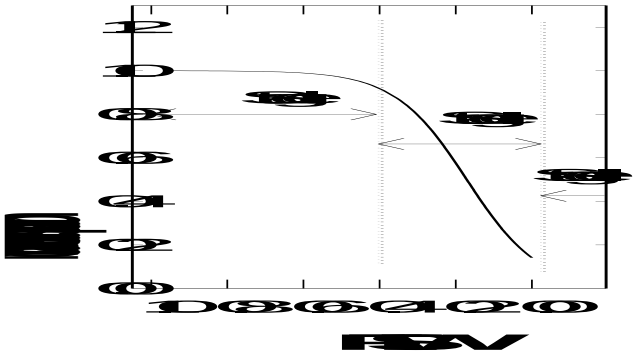
Both conventional and trait-based approaches have been used in breeding programs for drought tolerance. The empirical breeding approach is based on selection for yield and its components in a given drought environment. While such an approach has been partly successful, it requires large investments in land, labor, and capital to screen a large number of progenies plus the difficulty of sampling even a part of the expected range of variability in stress occurrence in the target environment. In addition, there is evidence of increasingly marginal returns from conventional breeding, suggesting a need to seek more efficient methods for genetic enhancement of drought tolerance. On the other hand, the ability to associate drought adaptive responses with the expression of specific physiological mechanisms has the potential to help greatly in establishing screening protocols and permit better management of genotype ×

environment (G × E) interactions. However, it has been argued that a focus on very basic mechanisms is likely to be at the cost of the linkages to final grain yield, and increased measurement costs, thereby complicating conventional and molecular breeding for drought tolerance.

Recent research developments in biotechnology have revived interest in targeted drought tolerance breeding and use of new genomics tools to enhance crop drought resistance. Marker-assisted breeding is making possible the improvement of field crops, particularly for traits where phenotyping is only possible late in the season, is difficult, or is prohibitively expensive. As a complement to the recent rapid progress in genomics, a better understanding of physiological mechanisms of drought response will also contribute to the progress of genetic enhancement of crop drought tolerance. It is now well accepted that the complexity of the drought syndrome can only be tackled with a holistic approach that integrates physiological dissection of crop drought avoidance and tolerance traits using molecular genetic tools such as MAS, microarrays and transgenic crops, with agronomic practices that lead to better conservation and utilization of soil moisture, and better matching of crop genotypes with the environment.

Crop Yield and plant water use

Generally, the response of plants to soil water deficits can be described as a sequence of three successive stages of soil dehydration. Stage I occurs at high soil moisture when water is still freely available from the soil and both stomatal conductance and water vapor loss are not limited by soil water availability. The transpiration rate during this stage is therefore determined by



environmental conditions around the leaves. Stage II starts when the rate of water uptake from the soil cannot match the potential transpiration rate. Stomatal conductance declines, limiting the transpiration rate to a rate similar to that of uptake of soil water, resulting in the maintenance of the water balance of the plant. Finally, stage III begins when the stomata are no longer able to limit the transpiration to that water available from the soil even through stomatal conductance is at a minimum. At this time the plant must resort to other mechanisms of drought adaptation if the plant is to survive.

Virtually all major processes contributing to crop yield including leaf photosynthetic rate, leaf expansion and growth are inhibited late in stage I or in stage II of soil drying. At the end of stage II, these growth-supporting processes have reached zero and no further net growth (i.e. increase in biomass) occurs in the plants. The focus of stage III is survival and water conservation mechanisms which will allow the plant to endure under these severe conditions must be used if available. Plant survival is a critical trait in natural dry-land ecosystems, but for most agricultural situations, stage III often, but not always has little relevance to questions about increasing crop yield. Consequently, the amount of water available up to the end of stage II for all practical purposes determines the cumulative growth and yield on a particular soil. Recovery from stage III can only be of relevance to yield performance if water is added to the system while there is still sufficient time for growth. Therefore, options involving mechanisms to enhance crop survival, thus do not usually mean any increase in crop yield under severe drought stress conditions. Increased crop yields and water use efficiency generally require the optimization of the physiological processes involved in the critical early stages (mainly stage II) of plant response to soil dehydration.

Characterization of drought resistance traits

Several physiological, morphological and phenological traits/mechanisms have been associated with drought stress adaptation, either in stage-I, stage-II or stage III processes of soil drying including:

- Plant emergence characteristics/vigor
- Phenology/ Elasticity of development
- Nutrient acquisition/Uptake efficiency
- Water use efficiency
- Photosynthesis, Radiation Use Efficiency
- Carbon Discrimination (^{13}C □)
- Deep Root development
- Hormonal regulation (ABA, GA, Ethylene)
- Osmotic Adjustment/RWC
- Canopy temperature
- Staygreen/ Delayed senescence
- Grain number maintenance
- Grain fill duration and rate
- HI under Drought
- Yield and its components, Etc.

Despite many decades of research on ‘drought tolerance’ in several crops, little progress has been reported in terms of genetic enhancement of crop productivity under water-deficits environments. Plant physiologists do not always pay enough attention while evaluating the adaptive role of a particular physiological mechanism to grain yield or biomass production under

drought. Also, there is no unanimity in relation to what constitutes drought tolerance. Only limited progress has been reported in developing near iso-genic lines (NIL) or recombinant inbred lines (RILS) for specific traits of drought resistance. Another limitation is that lab physiologists have been reluctant to adapt their trait assessment protocols to allow phenotyping of sufficiently large number of individual plants that progenies can be segregated in sufficient numbers for good screening experiments to demonstrate that their target traits are indeed heritable.

Plant breeders on the other hand are unwilling to invest much of their time and resources pursuing traits without this basic information. Breeders and crop physiologists need to work closely in testing the viability/validity of the trait-based approaches for drought tolerance. This has not happened to any great extent previously, thus missing a good opportunity for advancement. Applied breeding programs always want a clear demonstration of the value of a trait before considering incorporating it as a selection criterion. However, without breeder's participation, development of genetic stocks with specific traits incorporated into locally-adapted genetic backgrounds to test the hypothesis of the value of a trait is very difficult.

Identification of simple to observe morphological and phenological traits, reflective of mechanisms and processes that confer drought tolerance is a priority activity in drought research. An appropriate screening trait for drought stress tolerance should fill the following criteria: (i) a strong link with higher or more stable grain yield in the target stress environment, (ii) a high level of heritability, and (iii) the expression of tolerance must be easily measurable, with adequate replication.

Integrated drought management options

Given the increasing scarcity and competition for water resources, irrigation is generally not a possible option to alleviate drought problems in most rainfed areas. Therefore, drought management strategies, whether agronomic or genetic, therefore need to focus on maximum extraction of available soil moisture and its most efficient use in crop establishment, maximum crop growth, and for increasing biomass and seed yield. In order to plan crop yield improvement programs for a given target drought-prone area the following steps are essential:

- Characterize the major patterns of drought stress and their frequency of occurrence in the target environment.
- Evaluate crop response to the major drought patterns (simulation modeling)
- Match crop phenology (growth period, sowing, flowering, and seed filling) with most favorable period of soil moisture and climatic regimes
- Develop a strategy for the optimal use of supplementary irrigation, when available
- Increase available soil water to crop through agronomic management practices

- Identify plant traits that would maximize (i) use of available soil moisture in transpiration (ii) production of biomass per unit water transpired, and (iii) partitioning into seed, thereby conferring enhanced crop water productivity.

Agronomic and genetic options that do not involve external input of irrigation can only partially alleviate drought effects, because yield is always lower than what can be achieved with irrigation.

Mitigation of water stress

1. Nutritional Management

Among the major nutrients, potassium and magnesium are found to be highly deficient due to water deficit conditions. Therefore, application of potassium enhances the water uptake as well as the water relations in the plant tissues by osmoregulation processes, by acting as a potent osmoregulator (osmolyte), thereby the solute potential is reduced. Besides, potassium nutrition also helps in the favorable stomatal regulatory mechanisms, which regulate the water balance of the plants. This has also resulted in the increased WUE of the plants. Similarly, magnesium is component of chlorophyll, its content and uptake is drastically reduced due to the water stress effect. This is most prominent in Mg-loving crops like cotton.

Besides macronutrients, deficiencies of micronutrients also appear under water deficit situations due to the following reasons:

1. Depletion due to erosion and leaching. In India, annual soil loss is estimated to be about 6000 Metric tons and obviously due to loss through runoff water and soils
2. Continuous use of micronutrients free NPK fertilizers in dryland agriculture and diminishing the use of organic matter, FYM, compost and green / green leaf manures.
3. Use of high-yielding varieties (HYVs), adoption of intensive systems of farming and cropping and use of heavy doses of fertilizers, increased proportionately the mining of micronutrients from the soil
4. Since increased crop production arising from the heavy demand of the nutrients in rapid depletion of macro and micro-nutrients unless regularly replenished. Consequently, the deficiencies of micro-nutrients in general and that of Zn, Fe and B in particular are widely spread under stress conditions.

Therefore, foliar application of the following nutrients depending upon the occurrence of their efficiencies will mitigate the water-stress induced nutritional imbalance in crops.

a. 2 % DAP
b. 0.5 to 1 % Potassium chloride (KCl)
c. 0.5 % Zinc sulphate
d. 0.5 – 1.0 % Ferrous sulphate + 1 % urea
e. 0.3 % Boric acid

2. Use of Antitranspirants

In India, about 90% of the land is under rainfed farming; therefore, it is very essential to manage every drop of water received through rains. Though various measures are adopted to conserve he rain water, yet rainfed farming is often subjected to drought. Transpiration is said to be unavoidable evil but it has several functions to attend in the crop cycle. For producing one tone of food, the crop plant requires varied amount of water as furnished below:

Cereals and legumes: 400 – 500 litres of water / kg of grains

Fruits and vegetable: 1000 litres / kg of food

Water transpired by crops (season / plant):

Maize : 200 litres

Sunhemp : 27 litres

Cotton : 8 – 10 litres / day

Citrus : 100 – 200 litres / day

Trees (9 – 10 m height) : 300 – 800litres / day

Forest trees of 400 – 600 trees: 20,000 barrels / day (1 barrel = 500 litres)

Similarly, the WUE of crops is also different and ranges from 0.24 to 1.75 kg / mm of water / ha. The WUE of sorghum is higher but that of cotton is the lowest. This difference lies with the maturity period and nutritive value of the crop. Cotton grows for six to seven months while sorghum grows for four months. Drought reduces the yield by 0 – 100% depending upon the severity. Prolonged drought can drastically reduce the yield to zero level. But, intermittent drought for 10 – 15 days at early or late stage is common under rainfed conditions. Drought during the critical phenological phase like flowering and grain development is highly detrimental. However, the crop productivity is dependent on how fast a plant can recover after a stress of 6-10 days. The severity of intermittent drought of 6-10 days during critical stages of the crop can reasonably be avoided by the use of antitranspirants and thus crops can be saved. Antitranspirants can effectively be used to the crop under water stress with adverse rainfall.

Classification of Antiranspirants (ATs) and field responses

The ATs are categorically classified on mode of action in the following four types:

I. Materials causing stomatal closure

1. Herbicides like 2, 4 – D, Phosphon D and Atrazine
2. Fungicides like Phenyl Mercuric Acetate (PMA)
3. Metabolic inhibitors like hydroxy sulfonates, potassium metabisulphite *etc.*
4. Growth hormones like ABA, Ethrel, TIBA, succinic acid, ascorbic acid and Cycocel (CCC)

II. Reflectant Types

1. Kaoline, 2. China Clay, 3. Calcium bicarbonate, 4. Lime water

III. Thin-forming chemicals

1. Hexadecanol (Higher alcohols), 2. Cetyl alcohol, 3. Methanol, 4. Paclobutrazol, 5.Brassinolide, 6. Resorcinol

IV. Polyethylene materials forming thick films

1. Mobileaf, 2. Folicot, 3. Waxol, 4. S- 800, 5. Hico-110R

(All the above chemicals are trade names given by the companies)

The purpose of ATs is to maintain the growth and productivity under stress conditions and it is never recommended for high productivity / unit area. It saves the crop and helps to get marginal yield when the expectations are zero.

Role of ATs in Irrigation Water Saving

Some of the ATs can also be used through drip (as **Fertigation**) to save the frequency of irrigation. In this context, the crop productivity could also be increased by 26.2, 23.6 and 15.4 % over unsprayed control with the sprays of Hico-100 R, paclobutrazol and 8– Hydro Quinine respectively under 6 limited irrigations as against 9 irrigations and thus considerably saved irrigation water.

Thus, assured benefits of ATs to the crops can be summarized as below:

1. Optimized yield levels under infrequent rainfall situations
2. Assured better crop growth and yield when no yields are expected using severe drought
3. Getting normal sized grains
4. Improved seed quality (so that produce can be used for seed purpose)
5. Saving of crops with marginal crop productivity under drought
6. Reducing irrigation especially in post-rainy long duration crops like cotton and pigeon pea
7. Minimizing irrigation frequency and saving water through drip irrigation (eg. Cetyl alcohol and / or Hexadecanol)
8. Monitoring crop loss with limited inputs
9. Monitoring / managing drought
10. Arresting fast receding soil moisture for better growth and yield of rabi crops
11. Very useful for farmers with minimum irrigation facilities
12. Saving large nurseries when water is scarce in summer months

3. Use of Plant Growth Regulators (PGRS)

The plants possessing moderate canopy development (moderate values for LAI), less reduction in photosynthesis, deeper root system, higher root / shoot ratio and delayed senescence will perform better under water stress conditions.

Toward this, application of some of the PGRs will prove beneficial for better crop growth and development when grown under water deficit situations. Some of the PGRs and their effects on crops in order to suit to the water stress conditions are:

Cycocel & Mepiquat chloride: For promoting root growth (for more water absorption) and suppressing leaf area development (for reducing transpiration loss of water) and delaying on set of leaf senescence.

Cytokinins and Salicylic acid: They delay the leaf senescence processes and also favour stem reserve utilization by the developing grains especially during the water deficit situations.

Brassinolides: These PGRs increase the photosynthetic activity of the plants.

Ascorbic acid: Ascorbic acid acts as an anti-oxidant agent for scavenging Reactive Oxygen Species (ROS) accumulating under stress and thus avoiding membrane damage.

Pre-sowing Hardening of Seeds / Plants:

Hardening of seeds / plants to required temperature / chemicals enables the plants to overcome the specific stresses. This process actually hardens the protoplasm (by osmoregulation), which enables the seeds to absorb more water under favourable situations to maintain its viability under unfavorable conditions.

4. Chemicals used for seed hardening process especially under rainfed conditions:

1. 1% KCl, 2. 1% KH_2PO_4, 3. 100 ppm Succinic acid, 4. 0.5% NaCl, 5. 100 ppm $ZnSO_4$, 6.100 $MnSO_4$, 7. 100 ppm Ascorbic acid, 8. 250 ppm Cycocel, 9. 0.5% $MgSO_4$

Thus, these chemicals / PGRs could serve as boon to the frustrated farmers of rainfed areas, if rightly adopted with perspective vision to have food security. Adoption of the agro techniques is the only solution for farmers of dryland and water stressed scenario to save millions of world population in millennium especially in the developing countries, like India.

5. Agro-techniques for mitigating Water Stress

1. Foliar spray of 2%c DAP + 1% KCl (MOP) during critical stages of flowering and grain formation
2. 3% Kaoline spray at critical stages of moisture stress
3. Foliar spray of 500 ppm Cycocel (1 ml of commercial product per litre of water)
4. Mulching with 5 tones of sorghum / sugarcane trash, which saves 19-20% of irrigation water by reducing evaporation loss of water
5. Split application of N and K fertilizers as in cotton at 45 and 60 DAS
6. Use of biofertilizers *viz.*, Azospirillum or phosphobacteria @ 10 packets / ha along with 25 kg of soil or FYM
7. Application of 12.5 kg / ha along with 37.5 kg of sand
8. Seed hardening with 1% KH2PO4 and other salts for 6 – 8 hours (depending upon nature of seed coat) soaked in equal volume of water
9. Spray of 40 ppm NAA (4 ml of Planofix in 4.5 litres of water)
10. Seed treatment + soil application + foliar spray of Pink Pigmented Facultative Methanotrophs (PPFM) @ 106 as a source of cytokinin.
11. As in cotton, nipping terminal portion f main stem beyond 15th (at 70 - 80 DAS) and at 20th node (at 90 DAS) in the case of hybrids and varieties respectively for arresting transpiration loss of water)
12. Foliar spray of 0.5% zinc sulphate + 0.3 % boric acid + 0.5 % Ferrous sulphate + 1% urea during critical stages of moisture stress

Conclusions

1. Good knowledge of the target environment is essential in drought breeding: what type of stress is more frequent?
2. Yield stability under drought scenarios and crop water productivity (ratio of yield achieved per water used) should be the target
3. Drought screening systems should be uniform, repeatable and differentiate genotypes
4. Field and irrigation management are critical for drought screening
5. Screening procedure are function of targets, needs and means. Each breeding program should adapt procedures to won environment and conditions.
6. Trait-based improvement needs careful choice and requires integration of breeding and other disciplines

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ESTIMATION OF EPICUTICULAR WAX CONTENT

M Alagupalamuthirsolai

Waxes Plants exhibit diverse adaptive mechanisms to prevent water loss from leaf surfaces, such as leaf movement, leaf rolling, sunken stomata, efficient regulation of stomatal movement, reduction in leaf size, changes in leaf orientation and canopy architecture, and heavy cuticle depositions. Water loss from leaves can be through the stomatal pore or the waxy cuticle. Plant species native to arid lands often have thicker, more waxy cuticles than those from moist habitats, and in many cases, a high wax amount is correlated with drought resistance in crop and tree species. It has often been postulated that heavy wax deposits of plant surfaces limit transpiration and improve water retention. Moreover, many plants possess the ability to respond to water-limited environments by increasing the deposition of epicuticular waxes, which likely increases the cuticle's ability to function as a hydrophobic barrier. In Morus species (mulberry) genotypes having elevated leaf cuticular wax amount and crystal size and density exhibit reduced leaf post harvest water loss, suggesting that surfaces wax is one of the important drought trait (Mamrutha et al., 2010).

Leaf epicuticular wax (EW) content was determined at flowering in trial 1 and 2 in non-stress and 25 to 39 days after with- holding irrigation in stress plots in trial 2 using the method described by Ebercon *et al*. (1977) and the content was expressed in µg dm^{-2}. In non-stress plots, leaves were collected during the flowering stage. The detailed protocol is given below

Preparation of Acidic $K_2Cr_2O_7$ reagent

Reagent was prepared by mixing 20 ml of distilled water with 10 g $K_2Cr_2O_7$ in one litre capacity beaker. The resultant was mixed with 500-ml ice-cold concentrated sulfuric acid with vigorous stirring and heated on a water bath until clear solution was obtained. The final color of the solution was dark brownish golden yellow and the reagent was stored in amber color bottle.

Procedure

The second and third fully expanded leaves just below the flag leaf were sampled at the flowering stage uniformly in all the DH lines for determination of EW content both in control and stress plots. To assess the intensity of the stress in plots, we monitored plants visually under stress treatment and also measured leaf RWC from the stress plots. Samples were collected when the leaf was at 65 – 70 % of RWC. Leaves were collected by giving a cut at collar region and kept in a test tube containing water. The test tubes were placed inside the icebox before transporting to the laboratory. In laboratory, both ends of leaf blade were cut; middle portion alone was taken for wax estimation. Leaf area was determined using Area meter (Licor 3000). After that leaf blade was dipped in 20-ml chloroform for three seconds. The extract was evaporated on a boiling water bath until the smell of chloroform could not be detected. After adding 5 ml of acidic $K_2Cr_2O_7$, samples were placed in boiling water bath for 30 min. The

samples were kept on ice and made up to 17-ml using distilled water. Then the samples were taken out of ice and allowed 30 minutes for color development. After cooling, the optical density of the sample was read at 590 nm.

Preparation of Standards

Polyethylene glycol (PEG)-3000 was used as standard for EW estimation

Preparation of stock solution (250 ppm)

A stock solution of 125 mg of PEG-3000 in 500 ml chloroform was used for preparation of range of concentrations

Standards

Concentration

Blank	:	20 ml chloroform	
50 ppm	:	4 ml of 250 ppm (stock)	+ 16 ml chloroform
100 ppm	:	8ml of 250 ppm (stock)	+ 12 ml chloroform
150 ppm	:	12 ml of 250 ppm (stock)	+ 8 ml chloroform
200 ppm	:	16 ml of 250 ppm (stock)	+ 4 ml chloroform
250 ppm	:	20 ml of 250 ppm (stock)	

Then the estimation of standard solutions proceeded as in the case of sample.

A standard curve was drawn with absorbance versus concentration of standards. The absorbance values were plotted in this curve and the corresponding concentration was noted. The EW content was calculated and expressed as µg dm^{-2}

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ESTIMATION OF PROLINE IN PLANTS

M Alagupalamuthirsolai

Among the amino acids present in the plant system, 20 are present in protein called as protein amino acids. Among the protein amino acids, Glutamic acid and Aspartic acid are very important for synthesizing other amino acids. Proline is a basic amino acid which is accumulates in the plant system during stress condition. For the accumulation of proline, glutamate serves as precursor. There are three ways by which accumulation of proline can occur in plant tissue under stress conditions.

1. Degradation of proteins releasing amino acids into cell sap.
2. *De novo* synthesis of proline.
3. Inhibition of proline oxidation.

Free proline is reported to induce stress tolerance in a variety of plants through dehydration of protoplasm. It acts as storage compound for carbon and nitrogen during moisture stress when both starch and protein synthesis is inhibited. The accumulation of proline in the cells results in increasing osmotic pressure of the cell, thereby enabling the cells to maintain the turgor potential, by the way proline acts as an osmoticum. Quarternary ammonium compounds like glycine betaine, proline betaine and alanine betaine are other osmolytes accumulate during stress condition.

Principle

During the selective extraction with aqueous sulphosalicylic acid, proteins are precipitated as a complex. The extracted proline is made to react with ninhydrin in acid medium conditions to form the chromophore (red colour pigment complex) and read at 520 nm.

Reagents required

1. 3 % aqueous sulphosalicylic acid
2. Acid ninhydrin
3. 6 M Orthophosphoric acid
4. Glacial acetic acid
5. Toluene
6. Pure Proline as standard

Acid Ninhydrin preparation

Weigh 2.5 g of ninhydrin and dissolve in 60 ml glacial acetic acid and 40 ml 6 M orthophosphoric acid and stir well with slight warming in hot water bath until the contents dissolved. Use within 24h.

Procedure

Weigh 500 mg of leaf sample and macerate with 10 ml of 3 % sulphosalicylic acid and centrifuge the contents at 3000 rpm for 10 minutes. Take 2 ml of supernatant solution in a test tube and add 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. Keep the contents of the test tubes in hot water bath for one hour at 100° C and after cool the test tubes under tap water. Transfer the solution to a separating funnel and add 4 ml of toluene. Shake the separating funnel uniformly for 30 seconds and note the formation of two different layers. Discard the colorless bottom layer and collect the upper pink color (chromophase) solution and measure the OD value at 520 nm using toluene as blank

Preparation of Standard

Weigh 10 mg of pure proline and dissolve in 100 ml of distilled water, which will give solution of 100 ppm stock. Prepare different concentrations of proline standard solution Viz., 10, 20, 30, 40, 50, 60, 70, 80 and 90 ppm by diluting the stock solution. Run the series of standard in similar way as that of the leaf sample. Draw a standard graph. Plot your sample OD in the standard graph and find out the corresponding concentration (x μg).

Calculation

$$\text{Amount of proline} = \frac{x}{2} \times \frac{10}{500} \times 1000$$

Amount of proline present in the given sample is expressed in **μg g^{-1}**

FUNCTIONAL SOIL MICROBES

C Sarathambal

Plant associated bacteria

The rhizosphere is the narrow zone of soil specifically influenced by the root system. This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria. This situation is reflected by the number of bacteria that are found around the roots of plants, generally 10 to 100 times higher than that in the bulk soil. The rhizosphere is populated by a diverse range of microorganisms and the bacteria colonizing this habitat are called rhizobacteria. Plant-associated bacteria can be classified into beneficial, deleterious and neutral groups on the basis of their effects on plant growth. Beneficial free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria (PGPR). Independent of the mechanisms of vegetal growth promotion, PGPRs colonize the rhizosphere, the rhizoplane (root surface), or the root itself (within radicular tissues). It is well established that only 1 to 2% of bacteria promote plant growth in the rhizosphere. Bacteria of diverse genera have been identified as PGPR, of which *Bacillus* and *Pseudomonas* spp. are predominant.

PGPR affect plant growth in two different ways, indirectly or directly. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. This can happen by producing antagonistic substances or by inducing resistance to pathogens. A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. PGPR, as biocontrol agents, can act through various mechanisms, regardless of their role in direct growth promotion, such as by known production of auxin phytohormone, decrease of plant ethylene levels or nitrogen fixing associated with roots.

PGPR and their interactions with plants are exploited commercially and hold great promise for sustainable agriculture. Applications of these associations have been investigated in maize, wheat, oat, barley, peas, canola, soy, potatoes, tomatoes, lentils, radicchio and cucumber. In this review, we will consider the mechanisms of action of biocontrol agents and describe some successful examples of these rhizobacteria controlling plant diseases.

Production of plant growth promoting substances

Phytohormones, also called plant growth regulators (PGRs), are well known for their regulatory role in plant growth and development. PGRs are organic substances that influence physiological processes of plants at extremely low concentrations. Because the concentration of hormonal signals is critical to the regulation of various physiological processes in plants, local changes of

phytohormone levels can lead to characteristic changes in plant growth and development. In 1979, production of auxins, cytokinin-like and gibberellin-like substances was proposed for *A. brasilense*, since the increased number of root hairs and of lateral roots observed after inoculation with this bacterium could be mimicked by the application of a mixture of indole- 3–acetic acid, kinetin, and gibberellic acid. Moreover, in several other studies the increased plant growth observed after inoculation with *Azospirillum* was proposed to be due to bacterial phytohormone production. Eighty per cent of microorganisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites. Bacteria belonging to the genera *Azospirillum, Pseudomonas, Xanthomonas, Rhizobium, Alcaligenes, Enterobacter*, *Acetobacter* and *Bradyrhizobium* have been shown to produce auxins that help in stimulating plant growth. Isolates producing IAA have stimulatory effect on the plant growth. When the crop is inoculated with the isolates capable of IAA production significantly increases the plant growth by the N, P, K, Ca and Mg uptake of sweet potato cultivar.

Synthesis of enzymes that can modulate plant growth and development

Ethylene is a potent plant growth regulator that affects many aspects of plant growth, development and senescence In addition to its recognition as a "ripening hormone", ethylene promotes adventitious root and root hair formation, stimulates germination, and breaks the dormancy of the seeds. However, if the ethylene concentration remains high after germination, root elongation (as well as symbiotic N_2 fixation in leguminous plants) is inhibited. It is widely believed that many plant growth promoting bacteria may promote plant growth by lowering the levels of ethylene in plants. This is attributed to the activity of the enzyme 1–aminocyclopropane-1–carboxylate deaminase, which hydrolyzes ACC, the immediate biosynthetic precursor of ethylene in plants. The products of this hydrolysis, ammonia and α-ketobutyrate, can be used by the bacterium as a source of nitrogen and carbon for growth. In this way the bacterium acts as a sink for ACC and as such is lowering the ethylene level in plants, preventing some of the potentially deleterious consequences of high ethylene concentrations.

Antagonistic activity

Rhizobacteria can suppress the growth of various phytopathogens in variety of ways like competing for nutrients and space, limiting available Fe supply through producing siderophores, producing lytic enzymes and antibiosis. Among PGPRs, fluorescent pseudomonads are widely reported for their broad spectrum antagonistic activity against number of phytopathogens. The microbial inoculants when used as composite inoculum exhibited maximum efficiency in the suppression of diseases with the characteristic increase in chlorophyll content, total number of leaves, shoot height and thereby facilitating overall crop yield than when inoculated singly. However, application of these PGPR strains did not affect populations of beneficial indigenous rhizosphere bacteria including the fluorescent pseudomonads and the siderophore-producing bacterial strains.

Mineral solubilization

One of the various mechanisms by which rhizobacteria promote plant growth is by solubilization of insoluble minerals. Phosphorus is the second most important macronutrient next to nitrogen in limiting crop growth. More than 40% of the world soils are deficient in phosphorus and the acid weathered soils of tropical and sub- tropical regions of the world are particularly prone to phosphorus deficiency (Vance, 2001). A survey of Indian soils revealed that 98 per cent of these need phosphorus fertilization either in the form of chemical or biological fertilizer. Application of chemical phosphatic fertilizers is practised though a majority of the soil P reaction products are only sparingly soluble. Under such conditions, microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. P solubilization by plant-associated bacteria has been well documented in a number of studies. This group covers bacteria, fungi and some actinomycetes. These organisms solubilize the unavailable forms of inorganic-P like tricalcium, iron, aluminum and rock phosphates into soluble forms by release of a variety of organic acids like succinic, citric, malic, fumaric, glyoxalic and gluconic acids. Phosphorus solubilizing microorganism include different groups of microorganisms, which not only assimilate phosphorus from insoluble forms of phosphates, but they also cause a large portion.

Apart from phosphorus, micronutrients like Zn, Fe and Mn are found to be deficient in most of the soils with Zn as a foremost nutrient throughout the world (Alloway, 2001). Zinc, the micronutrient required for plant growth, is an essential component of over 300 enzymes and play catalytic, co-catalytic or structural roles in many plant systems (Christie *et al.*, 2004). For alleviation of Zn and other micronutrients important for crops, their application is done mainly in soluble form as zinc sulphate the soluble form of Zn applied to the soil get transformed into different unavailable forms due to the soil reaction. These transformations are based on the type of soil and other nutrients available. Zn is mainly transformed into zinc carbonate in highly calcareous soils, reacts with Fe and Mn oxide minerals, and while converted into zinc phosphate in higher P fertilizing soils. Inclusion of a bacteria solubilizing zinc, as a bioinoculant in crop production technology is really beneficial for a country like India having high incidence of zinc deficiency (more than 70 per cent). A term called zinc solubilizing bacteria (ZSB) was coined for those bacteria that are capable of solubilizing the insoluble zinc compounds / minerals in agar plate as well as in soil. Potassium solubilizing bacteria such as *Bacillus mucilagenosus* and *Bacillus edaphicus* are example of microorganism that used in bio inoculants. Potassium solubilizing bacteria are able to solubilize potassium rock through production and secretion of organic acids.

Potassium solubilizing bacteria is a heterotrophic bacterium which is obtaining all their energy and cellular carbon from pre-existing organic material. Besides, Potassium solubilizing bacteria are aerobic bacteria which play an important role in maintaining soil structure by their contribution in the formation and stabilization of water-stable soil aggregates. In addition, this

Gram positive bacterium can produce substance that stimulate plant growth or inhibit root pathogens investigated silicon and potassium mobilization by silicate mineral solubilizing bacteria, *Bacillus globisporus*.

Siderophore and hydrogen cyanide production

In the case of iron uptake, it was suggested that plants can benefit from the siderophores produced by several plant growth promoting rhizobacteria. Although iron is one of the most abundant minerals on Earth, in the soil it is relatively unavailable for direct assimilation by microorganisms. Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition. Under iron-limiting conditions PGPB produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion. Siderophores (Greek: "iron carrier") are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms. Many siderophores are non-ribosomal peptides, although several are biosynthesised independently. Siderophores are also important for some pathogenic bacteria for their acquisition of iron. Siderophores are amongst the strongest binders to Fe^{3+} known, with enterobactin being one of the strongest of these. Distribution of siderophore producing isolates according to amplified ribosomal DNA restriction analysis (ARDRA) groups, reveals that most of the isolates belong to Gram negative bacteria corresponding to the *Pseudomonas, Enterobacter* genera, *Bacillus* and *Rhodococcus* genera are the Gram-positive bacteria found to produce siderophores.

Cyanide (HCN) production is one of the possible ways by which rhizobacteria may suppress pathogens growth in soil. The plant-parasitic nematodes are among the most destructive plant pests, causing substantial economic losses to agronomic crops worldwide. HCN is potentially an important compound with activity against root knot nematode can act as a useful model system for studying plant-parasitic nematode control using *Pseudomonas*. Multitrophic interactions mediate the ability of fungal pathogens to cause plant disease and the ability of bacterial antagonists to suppress disease. A pathogen metabolite functions as a negative signal for bacterial antibiotic HCN biosynthesis, which can determine the relative importance of biological control mechanisms available to antagonists and which may also influence fungus-bacterium ecological interactions.

ADVANCED TECHNIQUES FOR DETECTION OF FOOD-BORNE PATHOGENS

C Sarathambal

Food-borne pathogens are of diverse in nature and keep causing major health problems in human worldwide. Many pathogens cause diseases in human, which are transmitted through various food items. Microbiological safety of food has become an important concern of consumers, industry, and regulatory agencies. Contamination may occur through pollution of air, water, soil, and also at various points while raising food crop, harvesting stage, processing, and during preparation. The presence of microbes (*Salmonella* spp., *Campylobacter, Listeria monocytogenes, E. coli*., etc.) in food poses food poisoning problem. Assessment of quality and safety of food requires microbiological analysis. Growth of microbes can result in organoleptic changes in food rendering it unacceptable to the consumer. To address this problem, the industry is implementing quality assurance systems such as Hazard Analysis Critical Control Point (HACCP). At present, there are many methods for detecting food-borne pathogens. In this part, we have discussed about various rapid methods employed for identification of food-borne microorganisms.

1. TEMPO filler and reading station

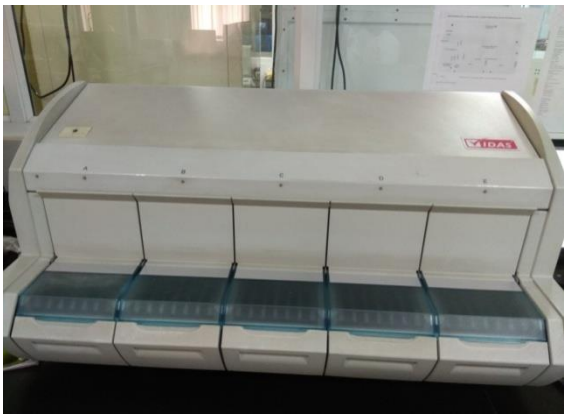
The TEMPO instrument is the food industry's automated quality indicator testing system for the enumeration of quality indicator organisms in food and environmental samples. TEMPO automates food safety testing for total viable counts, coliform counts, generic *E. coli*, and *Enterobacteriaceae*, lactic acid bacteria, *Staphylococcus aureus*, and yeasts/molds. Enumerating these organisms is important to a food quality lab to provide evidence of process control, effectiveness of interventions, and an indication of product spoilage and safety.
bioMérieux innovates by proposing specific culture media which allow rapid bacterial growth. bioMérieux miniaturized the Most Probable Number (MPN) method with 16 tubes in a single consumable, the TEMPO card.

TEMPO features

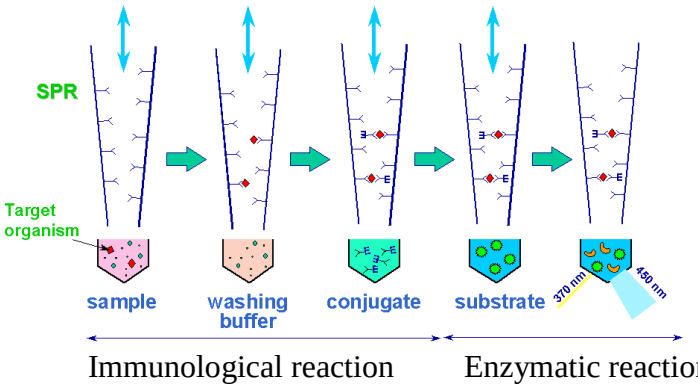
- The card, at the heart of the TEMPO system, offers the precision of the 16-tube MPN method without the usual tedious preparation.

- Each medium contains a fluorescent indicator based on the formula of traditional culture media and bioMérieux's expertise in bacteriology.
- Each well in the card corresponds to a dilution tube and the size of the well corresponds to 1 to 3 levels of dilution.
- After distribution of the culture media-sample mixture in the wells of the card, the bacteria multiply during incubation and metabolize the culture media.
- According to the number and size of the positive wells (fluorescent or non-fluorescent), the TEMPO system uses statistical methods to calculate the number of microorganisms present in the initial sample.
- The final result is expressed in CFU/g.

2. VIDAS



VIDAS® is a **reliable** and **easy-to-use automated benchtop immunoanalyzer**. Based on the Enzyme Linked Fluorescent Assay (ELFA) technology, it provides high quality **on-demand** test results.



Automated from sample insertion to results, with ready-to-use kits and reduced manipulations, the results generated by VIDAS® are objective, and full traceability is ensured.

Advantage of using VIDAS for pathogen screening

- Accuracy- Manual errors are minimal and the system is highly sensitive
- Traceability – Bar code scanner for the strips.

- Time saving – All the pathogen screening can be done within 24 – 48 hrs compared to10 days in conventional method.
- Safety – Closed system with no chances of contamination or contact of pathogen with the user.
- Reduced workload – 1 step procedure for all samples
- Streamlined workflow

3. VITEK compact 2

This format focuses on the industrial microbiology-testing environment while also having application for low to middle volume clinical laboratories. Features specifically developed for industrial microbiology include 21 CFR Part 11 compliance (for electronic records and signatures) and a colorimetric reagent card (BCL) used to identify the spore-forming Gram-positive bacilli (i.e., Bacillus and related genera). The other colorimetric reagent cards (GN, GP, YST) apply to all system formats for both industrial and clinical laboratories

The reagent cards have 64 wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation (described below). Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system.

There are currently four reagent cards available for the identification of different organism classes as follows:

1. GN - Gram-negative fermenting and non-fermenting bacilli
2. GP - Gram-positive cocci and non-spore-forming bacilli
3. YST - yeasts and yeast-like organisms
4. BCL - Gram-positive spore-forming bacilli

4. MALDI –TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight)

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is a high throughput technology based on the comparison of the protein fingerprint obtained by microbial cells with a database of reference spectra by means of the use of various algorithms integrated in systems recently made commercially available. In the last few years this tool has been increasingly studied and applied for the identification and typing of microorganisms. MALDI-TOF MS is referred to as a "soft" ionization technique, because it causes minimal or no fragmentation and allows the molecular ions of analytes to be identified, even in complex mixtures of biopolymer.

SCREENING OF *ASPERGILLUS* SP. ISOLATES FOR AFLATOXIN PRODUCTION

C Sarathambal

Aim

To screen the *Aspergillus* sp. isolates for aflatoxin production.

Methodology

Isolation of *Aspergillus*

Aspergillus strains were isolated from nutmeg samples collected from wholesale markets and farmers of various districts of Kerala and Tamil Nadu. Samples were collected in polypropylene bags and processed for isolation of *Aspergilli* under aseptic conditions using laminar air flow and plated in Czepeck Dox agar and Potato dextrose agar medium containing chloramphenicol (125 mg/L) followed by incubation at 24-28ºC for 5-7 days. The different *Aspergillus* like colonies appeared were subcultured and stored. This isolation procedure was carried out in three replicates for each sample.

Detecting the ability of *Aspergillus flavus* isolates for aflatoxin production

To test the ability of *A. flavus* isolates for aflatoxin production, Aspergillus differentiation medium containing chloramphenicol (ADM) prepared. According to were used. ADM were inoculated isolated *Aspergillus* like colonies and the plates were incubated at 28°C for 5 days. After incubation the plates were examined for development of orange colour colonies in reverse side were indicated for aflatoxin production.

Observation

Isolates of aflatoxin producing *Aspergillus* spp. were differentiated from those of non toxin producing groups according to the colour pigmentation of the undersides after 5 days of incubation. The reverse colour of the ADM plates ranged from weak orange colour to a bright orange colour was observed.

Appendix

Media composition

a) *Aspergillus* Differentiation medium

Peptone	: 10g
Yeast Extract	: 20g
Ferric ammonium sulphate	: 0.50g
Dichloran	: 0.002g
Agar	: 15g

b) Czapeck Dox Agar

Sucrose	: 30g

Sodium nitrate : 20g
Dipottasium phospate : 1.0g
Magnesium sulphate : 0.5g
Pottasium chloride : 0.5g
Ferrous sulphate : 0.01g
Agar : 15.0g

C) Potato Dextrose Agar

Potato : 200g
Dextrose : 20g
Agar : 20g
Distilled Water : 1000ml

ISOLATION OF PATHOGENS FROM SOIL, ROOT AND LEAF

R Praveena

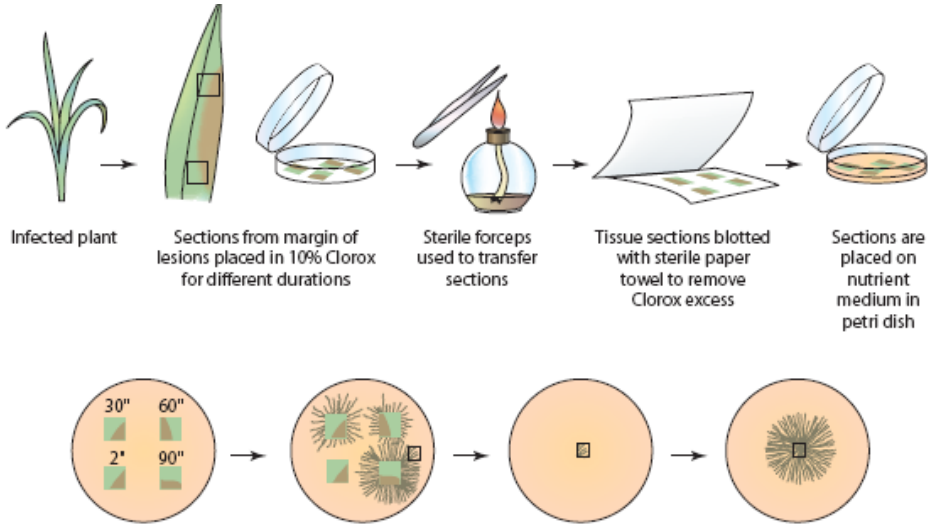
Plants are infected by different kinds of microbial pathogens *viz*., fungi bacteria and viruses. In addition to host plant the required inoculum for infection is present in the soil, water and air. Whatever may be the source of inoculum, the susceptible plant species or crop varieties may exhibit clear visible local symptoms in or on the tissues where infection is initiated. If the pathogen is able to find favourable conditions for further development, systemic symptoms are induced in tissues or organs far away from the point of pathogen entry into the plant. When the symptom of infection is not expressed externally, it is termed latent infection. Some fungal pathogens infecting host plants do not induce any visible symptom, as they remain dormant. Detection of microbial pathogens refers to the process of establishing the consistent presence of a particular target organism(s) within the plant or in its environments, irrespective of the development of visible symptoms in the plant suspected to be infected by the pathogen(s) in question. Diagnosis, on the other hand, relates to the identification of the nature and cause of the disease problem under investigation.

Isolation of fungi from infected plant parts

Leaves

The most common method however for isolating pathogens from infected plant parts involve:

- Cutting several small sections 5 to 10 mm square from the margin of the infected lesion to contain both diseased and healthy–looking tissue.
- These are placed in one of the surface disinfectant solution (0.1% sodium hypochlorite) for 1 minute. Alternatively, Clorox (10%), or hydrogen peroxide (50%) may be used for surface sterilization of plant tissues.
- It is then washed three times in sterilized distilled water in Petri plates and transfer the material with forceps. It can be dried on clean tissue-paper and then plated on nutrient medium
- The sterilized pieces are aseptically transferred to Petri dishes containing standard medium like potato dextrose agar (PDA) supplemented with streptomycin sulfate, at the rate of three to five pieces of tissues per Petri plate.
- The Petri plates were incubated at room temperatures (25–27°C) that may favour the pathogen development and observed daily for mycelium development from the bits.
- A portion of mycelium developing on the nutrient medium is transferred to the agar slants for purification and storage for further examination. Actively growing mycelium from the medium is transferred to agar slants for further studies as mentioned above. Slow growing and difficult-to isolate fungal pathogens may require specific or selective media for their development.



From Stems, Fruits, Seeds, and Other Aerial Plant Parts:

Almost the method described for isolating pathogens from leaves can also be used to isolate these pathogens from superficial infections of stems, fruits, seeds, and other aerial plant parts. In the infected stems, roots or fruits, the fungal pathogens may be present in the deep-seated tissues. In such cases, the infected tissues have to be washed with sterile water thoroughly, followed by swabbing with cotton wool dipped in ethanol (80%) and exposure to an alcohol flame (from spirit lamp) for a few seconds. Using flame-sterilized scalpel, the outer layers of tissues are removed rapidly and small pieces from the central core of tissues in the advancing margin of infection are cut. They are then sterilized by dipping in alcohol (90%) and sterilized again by exposure to alcohol flame for a few seconds. The Petri dishes containing appropriate nutrient medium, after transferring the sterilized infected tissues, are incubated at required temperature and for optimum period.

From Roots, Tubers, Fleshy Roots, and Vegetable Fruits in Contact with Soil

Isolating pathogens from any diseased plant tissue in contact with soil presents the additional problems of numerous saprophytic organisms invading the plant tissue after it has been killed by the pathogen. For this reason, the first step in isolating the pathogen is repeated thorough washing of such diseased tissues to remove all soil and most of the loose, decayed plant tissue in which most of the saprophytes are present. If the diseased root is small, once it is washed thoroughly, pathogens can be isolated from it by following one of the methods described for isolating pathogens from leaves.

Purification of fungal cultures

It is essential to purify the cultures of fungal pathogens isolated from different plant parts and stored in agar slants. Two procedures are commonly followed: (i) single hyphal tip method (ii) single spore isolation method.

Hyphal tip method

A small segment of fungal growth in the agar medium is transferred to the center of Petri dishes containing nutrient medium, using a flame-sterilized inoculation needle and incubated at room temperature or optimum temperature for a few days. As the mycelium grows in the medium, the advancing edge of the mycelium will have hyphal tips well separated from each other and they are marked by a glass marking pencil by observing under the microscope. The marked hyphal tips along with bits of agar are carefully transferred individually to separate agar slants in tubes using sterile inoculation needle. The hyphal tips in tubes will develop into a pure colony of the fungal species under investigation. The fungus can be subcultured at regular intervals to maintain its vigour for various studies.

Single spore isolation method

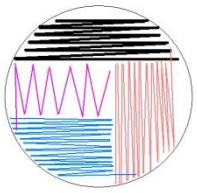
The fungal cultures may be purified alternatively by single spore isolation method. A spore suspension is prepared by transferring the fungal growth in the agar slant to sterile water kept in a sterilized test tube followed by vigorous shaking for a few minutes in order to disperse the spores from the spore-bearing structures. A serial dilution of spores is prepared by transferring serially 1 ml aliquots to a series of tubes containing 9 ml of sterile water. Aliquots (1 ml) of spore suspension at optimal dilutions mixed with melted nutrient agar (at about 45°C) are transferred to sterile petri dishes and the mixture is spread by tilting the dishes gently in different directions for uniformly covering the entire surface of the bottom plate. The petri dishes are then incubated at temperatures that favour spore germination.

The dishes are examined under the microscope at regular intervals and the locations of germinating spores are marked using a glass marking pencil. The marked germinating spores along with a small amount of medium are individually transferred to agar slants for development of colonies from the germinating spores. The fungal cultures contaminated with bacteria and mites may be purified using a simple technique that is based on the observation that the growth of bacteria and movement of mites are confined to the upper surface of the agar medium. The contaminated culture is placed upside down on a clean piece of paper. Then a thin layer of agar with the inverted culture is gently removed and transferred to plates containing sterile medium. Colonies developing on this medium are free of contaminants present in the original culture .

Isolation of bacteria from infected plant parts

- Select a piece of affected plant part/ symptomatic tissue
- Cut along into small pieces and add a small amount of water
- Grind tissue and mix
- Use pestle to inoculate plates in initial streak in first section of nutrient agar plates
- Using inoculating loop and aseptic technique, streak the second quarter of the nutrient plate, crossing over the initial streak area one time (orange streak on picture)
- Heat sterilize the loop between sections

- Repeat on third quarter, crossing over the second quarter only once- being careful not to touch the first or second quarter (blue streak on picture)
- If there is enough room on the plate, repeat for a fourth quarter (purple streak on picture)



- Incubate plates at 30°C for 48 hours and Observe plates for growth
- Choose individual colonies- lightly touch the top of an individual colony with a sterile loop or needle and streak on a fresh nutrient agar plate like above.

ISOLATION, PURIFICATION AND MAINTENANCE OF *PHYTOPHTHORA*

R Praveena

Isolation of *Phytophthora* from soil

Phytophthora was isolated from soil samples by baiting technique (Anandaraj and Sarma, 1990) using *Bauhinia variegata* leaf baits. The soil samples were sieved to <2 mm fractions and about 25 g sample was weighed out of this to a beaker or plastic cup with 100 ml distilled water. The contents were mixed properly using a glass rod to make a soil-water suspension. About 10 *B. variegata* leaf bits were then floated on the surface of this with the adaxial surface in contact with water. The beakers/ plastic cups were then covered with a thin polythene sheet to retain humidity and kept at laboratory conditions of 20-24°C for up to 72 h. Two replicates were kept for each sample. Infected leaf bits were observed for coenocytic mycelia growth with typical sporangia coming out from the edge of the leaf let/disc under the microscope. The leaf bits with *Phytophthora* were surface sterilized and plated on PVPH selective medium.

Baiting (Anandaraj and Sarma, 1990)

Plant material can be used as bait to isolate *Phytophthora* from the soil. Suitable baits include stem, leaves, surface sterilized roots, fruits etc. Baiting techniques are appropriate for zoosporic fungi as the zoospores produced from sporangia can actively move towards and infect the bait. The suitable baits can be applied to soil suspensions or to water in which pieces of diseased roots or leaves have been added in order to trap these fungi.

Procedure

- Collect soil along with roots from the rhizosphere of the infected plant
- Sieve the test soil to < 2 mm fractions.
- Put 25 g of the sieved soil into the beaker or polythene container and add 100 ml distilled water.
- Mix the contents thoroughly using a glass rod to make a soil-water suspension.
- Float 10-20 *Albizzia falcate* leaflets, *Bauhinia variegata* leaf bits or any other suitable baits with the adaxial surface in contact with water.
- Cover the beaker with thin polythene sheet to retain humidity keep under laboratory conditions at 20-24°C.
- Observe the leaves for infection daily for up to 72h.
- Remove the infected leaves and observe for coenocytic mycelia growth with typical sporangia coming out from the edge of the leaf let/disc under the microscope.
- Surface sterilize the infected baits with 0.1% sodium hypochlorite for 3 min.
- Rinse with three changes of sterile water, blot dry on sterile filter paper and plate it on selective medium.

Isolation of *Phytophthora* from aerial plant parts

- Wash the samples thoroughly under tap water.
- Excise small pieces of tissue from the from the advancing edge of lesions.
- Surface sterilize the tissues for 3 min in 0.1% NaOCl and then wash in three changes of sterile distilled water.
- Blot dry on a sterile filter paper.
- Plate it on PVPH selective media (Tsao and Guy, 1977) incubate at 25-28°C.
- In the case of infected stem/berries split open the samples longitudinally using a sterile blade before plating on selective media.

Isolation of *Phytophthora* from roots

The presence of phenolic compounds makes the isolation of *Phytophthora* difficult. So it is necessary to reduce the amount of phenolics in the plant part. Leaching method is found to be helpful in this case.

- Wash the infected roots thoroughly under tap water to remove the soil particles adhering to it.
- Transfer these samples to a 250 ml conical flask covered with a layer of muslin cloth and keep under running tap water for 30 min. (to remove the excess phenolics).
- Take out the sample.
- Surface sterilize the tissues for 3 min in 0.1% NaOCl and wash in three changes of sterile water.
- Blot it dry on a sterile filter paper.
- Transfer it to selective medium for isolation of *Phytophthora.*
- Incubate it at room temperature for 72-96 h.

PURIFICATION

From single spore

Procedure

- Cut out mycelial discs from the margins of the colony and transfer it to a sterile Petri dish.
- Add 15 ml sterile distilled water to it so that the water just touches the surface of the discs.
- Keep the plates for sporangia formation under intense light.
- When sporangia form, induce zoospore differentiation by placing the Petri plate in a refrigerator for 10 min.. Bring back to room temperature.
- Collect the zoospores with a micropipette and place on a selective medium.
- Incubate the plates at 24-28°C.
- The single spore colonies formed may be sub cultured to agar (PDA/CMA/CA) slants and maintained in the BOD at 15^{o}C.

From mycelia

Procedure

- Pour PVPH selective medium to sterile Petri plates.
- Allow it to cool and divide the medium into four pieces using a sterile blade.
- Take out a small mycelial bit from the growing edge of the contaminated culture and place it in a sterile Petri plate.
- Keep one piece of the agar block above this mycelial bit and seal the plate with cling film
- Incubate the plates at 24-28 °C.
- Mycelia will emerge out through the agar block. Take out a small mycelial bit from there and inoculate on carrot agar/PDA/CMA medium.

PRESERVATION & MAINTENANCE

1. Growth on agar slants

Inoculate a myceleial disc on carrot agar slant and incubate at 25 °C for 2 days and transfer to 15 °C for maintenance. These are to subcultured at every six months interval.

2. Preservation in Water

Cut out 5 mm mycelial discs from the margins of 72 h culture of *Phytophthora* and transfer it to sterile 2 ml tubes with ~1.2 ml sterile distilled water. Maintain these tubes at laboratory conditions (25±°C). For retrieving, take out a mycelial disc from the tube and remove the excess water by keeping on a sterile filter paper. Place these discs on Petri plates with carrot agar medium. *Phytophthora* species can be stored in water for a period of 2-3 years without any loss of viability.

DETACHED LEAF ASSAY FOR PATHOGENICITY TESTING OF THE ISOLATES

- Excise second/third leaf (from top) from a susceptible variety.
- Wash the leaves with tap water and blot dry on filter paper.
- Take plastic boxes spread with wet filter paper and place the leaves on it in such a way that the abaxial surface faces up.
- Inoculate the leaves on the abaxial with 5 mm mycelial discs cut out from the margins of a three day old culture.
- Place a bit of moistened cotton wad above the disc in order to prevent dehydration.
- Note down the lesion diameter at right angles at 24, 48 and 72 h.

PREPARATION OF CULTURE MEDIA

POTATO DEXTROSE AGAR (PDA) MEDIUM (FOR MYCELIUM GROWTH)

Potato -200 g
Dextrose – 20 g
Agar – 20 g
Distilled water -1 L

- Peel off the potato and cut it into 1 cm cubes.
- Boil the potato pieces in 500ml of distilled water till a glass rod can easily pierce through the pieces.
- Filter the broth through four layers of cheese cloth and dissolve the weighed dextrose in it.
- Add distilled water to bring up the volume to one liter.
- Add agar to the broth and boil till it dissolves completely.
- Transfer it to conical flasks and sterilize at 15 lb pressure for 20 min.

CARROT AGAR (CA) MEDIUM (FOR MYCELIUM GROWTH)

Carrot – 200 g
Agar- 15 g
Distilled water – 1 l

- Peel off the carrots and blend it in a blender along with 500 ml distilled water.
- Filter through four layers of cheese cloth and squeeze out the juice from the residue.
- Add distilled water to bring up the volume to one liter.
- Add the weighed agar to it.
- Boil the mixture and again filter it through four layers of cheese cloth.
- Transfer it to conical flasks and sterilize at 15 lb pressure for 20 minutes
- Transfer it to conical flasks and autoclave at 15 lb pressure for 20 minutes

ANTIBIOTIC MEDIUM FOR SELECTIVE ISOLATION OF *PHYOTPHTHORA*

PVPH MEDIUM *(Tsao and Guy, 1977)*

PVPH stock solution

Pimaricin solution	0.8ml
Vancomycin	224.5mg
PCNB (Pentachloronitrobenzene)	146.3mg
Hymexazol	55.3mg
Sterile distilled water	95ml

- Add 0.8ml of Pimaricin solution in 95ml sterile distilled water.
- Dissolve the weighed vancomycin, PCNB and hymexazol in 5ml DMSO (Dimethyl Sulfoxide) and add it to the above solution.
- Transfer it to sterile amber coloured bottles and store at $4^{o}C$

PVPH medium

- Prepare 100ml carrot agar medium.
- Allow the medium to cool to bearable warm.
- Add 10ml of this stock solution to 100ml culture media and pour this to Petri plates.
- Store the plates in dark and use within five days of preparation.

INTRODUCTION TO BIOLOGICAL CONTROL & MAINTENANCE AND MASS MULTIPLICATION OF BIOCONTROL AGENT *TRICHODERMA*

R Praveena

Biological control is a method of controlling pests such as insects, mites, weeds and plant diseases using other organisms. It relies on predation, parasitism, herbivory, or other natural mechanisms, but typically also involves an active human management role. It can be an important component of integrated pest management (IPM) programs. Natural enemies of insect pests include predators, parasitoids, pathogens, and competitors whereas biological control agents of plant diseases are most often referred to as antagonists.

Biological control can be categorized into 2 types, namely inundated and classical.

- **Inundative Bio-Control:** This approach uses pathogens, where they are used to apply on a target weed at a very high rate in an aspect that is similar to herbicide application. The most common pathogens used in inundative bio-control include nematodes and nuts. This approach does not prevent the invasive plant from implementing at a later date.
- **Classical biocontrol**: It uses agent populations that would waver in a natural prey and predator relationship. This method adopts natural predators of the invasive plant to create an eternal relationship between a plant and biological control animals.

Biological Control Agents include:-

- Predators: They are mainly free-living species that consume preys in large number during their lifespan. Since the majority of insects constitute crop pests. Some of the predators include Lacewings, Spiders, Flies, Beetles, and dragonflies.
- Pathogens: Virus, Bacteria and fungi are relatively pathogenic micro-organisms that are host specifics or kill their host. Some of the microbial diseases occur naturally but they are used as biological pesticides.
- Bacteria: Bacteria's belonging to coccobacillus group are more pathogenic to insects. They are used for biological control. They infect digestive tract of insects thus limiting the options, for controlling insects with sucking mouthparts namely scale insects and aphids.
- Viruses: The use of insect viruses as a controlling agent is still in inception. Since they are host specific, they turn out to have good potential as biocontrol agents.
- Fungi: The fungi Entomophaga is effective against pests namely green peach aphid.
- Parasitoids: They lay eggs in the body of the host (insect), eventually killing the host. It is later used as a source of food for the developing larva. It is one of the most widely used biological control agents.

Merits and Demerits of Biocontrol Agents:

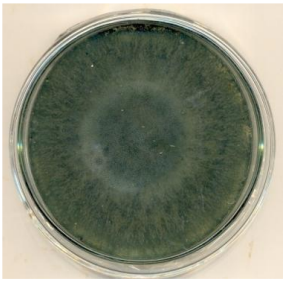
Merits

- The biological control agents are environmentally friendly and cause no side effects.
- Less cost compared to other agrochemicals – pesticides and insecticides.
- Easily available, easy to use and is effective throughout the season.
- Helps in reducing the use of chemicals and other pesticides.

Demerits

- Pest is not completely destroyed by these biological control agents.
- It is effective only for large scale.

Trichoderma is a genus of asexual fungi, frequently present in all types of soil, manure and decaying plant tissues. These fungi are opportunistic, avirulent plant symbionts which act as parasite and antagonists of many disease causing fungi and nematodes. Their dominance in soil may be attributed to their diverse metabolic capability and aggressive competitive nature. The nutritional requirement of *Trichoderma* is minimal and has the mechanism to utilise the cell wall components such as cellulose and chitin. The fungus produces abundant vegetative mycelium and asexual spores (conidia) which grow upon the available nutritional source. Their conidia are well adapted to prevent desiccation than vegetative mycelium. They also form chlamydospores which are the ideal surviving structures to tide over adverse conditions. The different mechanisms of action of *Trichoderma* spp. in their antagonistic interaction with fungal pathogens include: 1. Antibiosis- production of variety of compounds with antibiotic activity which prevent other microbes 2. Competition- by competing for nutrients and space 3. Lysis- production of cell wall dissolving enzymes by which the cell walls of pathogenic fungi are dissolved 4. Mycoparasitism- the fungi coils around the other microbes for its own nutrition 5. Induction of systemic resistance in plants. In addition to the direct action on plant pathogenic fungi, *Trichoderma* spp. are able to colonize plant root surfaces and produce significant changes in plant growth and production. They are able to increase plant nutrition through solubilisation or enhanced uptake of macro and micronutrients and also by the production of plant growth factors. Species such as *T. harzianum, T. hamatum* and *T. lignorum* are widely used for biological control of plant diseases. *T. harzianum* have the adaptation to survive under varying pH and temperature (25 -35^{o}C) conditions.



Trichoderma harzianum

Maintenance of *Trichoderma harzianum* mother culture

- Mother culture of *Trichoderma harzianum* on potato dextrose agar (PDA) slants can be stored under room temperature for three months.
- Under refrigerated condition (4 -7°C), storage period of *Trichoderma harzianum* on PDA slants can be extended for 3 more months.
- The mother culture has to be sub cultured at every three moth interval for one year.

Retrieval of *T. harzianum* from mother culture

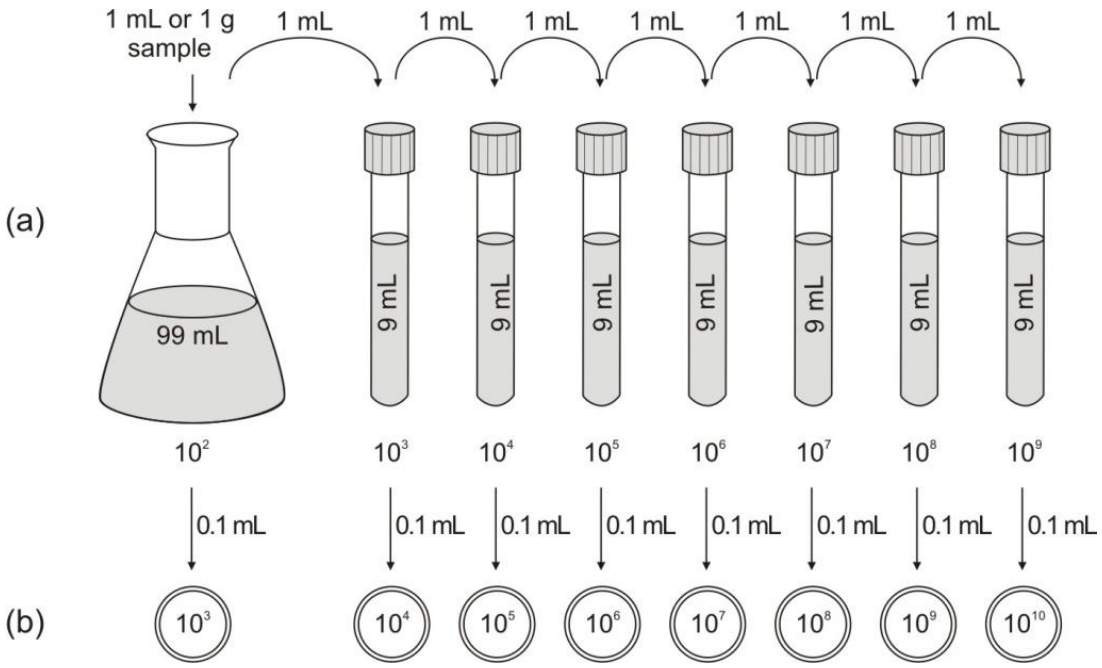
- Prepare PDA and distribute in 250 ml conical flasks @ 50 ml / flask and sterilize at 1.05 Kg /cm^2 (121^o C) for 20 minutes.
- After sterilization, cool the media to 40°C and pour into Petri dishes aseptically @20ml / dish.
- After solidification inoculate a loop full of *T.harzianum* mother culture at the centre of the dish and incubate for 72h at room temperature.
- A white puffy mycelial growth which turns green with the formation and maturing of spores appear in Petri dishes in 72-120h.

Raising *Trichoderma* culture for mass production

- Prepare PDA as above and distribute in 250 ml conical flasks @ 50 ml / flask. Sterilize at 1.05 Kg /cm^2 (121^o C) for 20 minutes.
- After cooling inoculate with 5mm culture discs of *T. harzianum* taken from 72h old culture grown in PDA .
- Incubate the flask for 5-7 days at room temperature to obtain thick green sporulated mass.
- After 5-7 days, add 100ml sterile water to the *T. harzianum* grown flask, a scrape the spore mass and mix thoroughly to distribute the spore mass evenly.
- The prepared spore suspension can be mixed with carrier materials for formulating the products

Quantification

- Prepare water blanks of 99 ml (in 250 ml conical flask) and 9ml in screw cap tubes and sterilize at 1.05 Kg /cm^2 (121^o C) for 20 minutes using an autoclave. (one 99ml water blank and 6-7 nos of 9ml blanks for one sample)
- Cool the water blanks after sterilization.
- Weigh 1 g of the formulated product and add to the 99 ml of sterilized water blank (10^{-2}). Shake the flask constantly to distribute the particles uniformly.
- Pipette out 1ml from this prepared solution to 9ml water blank (dilution 10^{-3}) and distribute the solution by slightly tilting the tubes and this has to be followed in each step and repeat up to 10^{-10} dilution.
- Pour1ml from the last dilution into a Petri dish. Then add molten , cooled TSM to the plate and mix well



Observation

- Specific colonies of *Trichoderma* characterized by white puffy mycelial growth which turns green with the formation of spores appear on Petri plates in 72-96h.
- Count the number of colonies in each dilution and estimate the colony forming units per gram (cfu/g) as number of colonies x dilution factor.

Preparation of media

Potato dextrose agar (PDA) medium

Peeled potatoes	: 200 g
Dextrose	: 20 g
Agar	: 20 g
Distilled water	: 1L
pH	: 5.6 ± 0.2 at 25ºC

- Peel, weigh and boil the potatoes until they become soft with half the quantity of water.
- Crush and strain the starchy decoction of potato through a piece of cheese cloth.
- Add the required quantity of glucose to the potato decoction and stir well.
- Melt agar in the remaining quantity of water.
- Add the solution of potato decoction with sugar to the agar solution.
- Dispense 100ml of the prepared medium into 250ml flasks and plug with cotton. For making plugs roll tightly a small piece of layered cotton.
- Sterilize the prepared medium at 1.05 Kg /cm^2 (121º C) for 20 minutes in an autoclave.

***Trichoderma* Specific medium (TSM)**

$MgSO_4$	: 0.2 g
K_2HPO_4	: 0.9 g
NH_4NO_3	: 1 g
KCl	: 0.15 g
Glucose	: 3 g
Metalaxyl	: 0.3 g
Rose Bengal	: 0.05 g
Chloramphenicol	: 0.25 g
PCNB (Penta chloronitro benzene)	: 0.2 g
Agar	: 15 g
Distilled Water	: 1L
pH	: 5.0 at 25^oC

- Melt agar in water
- Dissolve the weighed constituents except Chloramphenicol to the agar solution.
- Dispense 100ml of the prepared medium into 250ml flasks and plug with cotton. For making plugs roll tightly a small piece of layered cotton.
- Sterilize the prepared medium at 1.05 Kg /cm^2 (121^o C) for 20 minutes in an autoclave.
- Make a stock solution of Chloramphenicol @2.5g in 100ml and add 1ml of this stock to 100ml sterile molten media at the time of pouring into the plates.

MOLECULAR CHARACTERIZATION/DETECTION OF PLANT PATHOGENS

R Suseela Bhai

Accurate diagnosis and identification of plant pathogens is a pre-requisite of disease management to sustain high yield potential of crops. Visual identification (morphological identification) of plant diseases /pathogen, though a very rapid method but hard to perform by inexperienced personnel and is limited particularly to diseases affecting aerial parts of the plants. It cannot be performed effectively in case of soil and seed borne diseases, where several species of pathogens may cause similar symptoms. Microscopic examination of diseased tissues and identification of pathogen on the basis of their morphological characteristics though a preferred method of disease diagnosis, requires highly specialized taxonomists.The characterization of an unknown plant pathogen (i.e. when a new disease symptom is observed and the causative agent needs to be isolated and identified) and the verification of a known pathogen (i.e. when a disease symptom is observed and the causative agent needs verification using pre established data), is based on molecular methods, and focuses on the molecular targets of virulence factors that can identify pathogens.

Some of the major advances occurred in plant pathogen detection during the past decades with the development of monoclonal antibodies and enzyme linked immunosorbant assay. These are rapid methods of viral and bacterial pathogen detection. However, the technique still has certain limitations and sometimes-false epitopic detection leads to erroneous results. Molecular detection and identification of pathogens using nucleic acids based methods have been in use for the past few years. These methods overcome various problems associated with microscopical and immunological detection of plant pathogens. DNA based methods can be used at any developmental stage of the pathogen, since every pathogen propagule contains the entire set of nucleic acids of the organism. Precise detection and identification of plant pathogens can be performed by the use of specific DNA probes in infected tissues and identification at genus/species or even at race level. There are various methods used for making DNA probes.

Molecular methods available for bacterial characterization include: PCR, broad-range PCR, 16S rRNA gene analysis, Multilocus Sequence Typing, Denaturing Gradient Gel Electrophoresis, Restriction Fragment Length Polymorphism, Pulsed Field Gel Electrophoresis and DNA-DNA hybridization. The benefits and limitations of each of the different techniques are compared, and the potential of relative new technologies for characterization are assessed.

PCR based methods

PCR is a very rapid and sensitive method for the molecular diagnostics of plant pathogens, which are otherwise difficult to identify morphologically. Pathogen detection by using PCR is mainly dependent on the design of primers. Specific, semi-specific and arbitrary primers can be used for the PCR. Arbitrary primers are used in RAPD to produce characteristic profiles of amplified

products. However, DNA sequences of plants and other organisms are also amplified with random primers, which lead to ambiguous results. Thus, plant pathogens must be isolated from their hosts or reservoirs and purified before the DNA extraction. Hence, RAPD analysis may not be useful for the direct detection of plant pathogens in infected tissues or for the detection of obligate parasites. However, longer and species-specific PCR primers allow detection of target sequences specimens of infected tissues. Any DNA or RNA sequence that is specific for a particular organism can be used to design specific primers for the detection of that organism using PCR. Primers have also been designed on the basis of pathogen –specific plasmid sequences. Genes controlling the specific properties (e.g.toxinproduction & pathogenicity) of pathogens have also been used as target sequences for specific detection of plant pathogen. For e.g. PCR amplification of Toxgene region (phaseolo toxin producing gene) for the detection of *Pseudomonas syringae* pv. *phaseolicola* and efe (ethylene forming enzyme) gene for the detection of *P.syringae* pvs.*cannabina* and *sesami* have been successfully used.

Subtractive hybridization is another method of pathogen detection, which can be used to select pathogen and even strain specific sequences that are potential targets for designing primers. The technique enriches nucleic acid sequences specific to a particular organism or strain by hybridization and subsequent removal of sequences that are common with other organisms.

Serological techniques can also be combined with PCR e.g. immuno-capture PCR and immuno-PCR. In immuno-capture PCR antigen is concentrated by use of specific antibodies, which is then subjected to PCR, while, immuno-PCR enhances the antigen-antibody reaction. Recent advances in genomics and molecular biology have uncovered the complete genome sequence of two important plant pathogens, *Xyllela fastidiosa* and *Ralstonia solanacearum*. Using the unique sequence data of the pathogens DNA, probes and primers could be designed for the differential detection of pathogen and their characterization at molecular level.

Single nucleotide polymorphisms (SNPs): Molecular characterization and detection of plant pathogens would be better done by the of single nucleotide polymorphisms (SNPs).These markers can detect differences at single base pair level, which is the ultimate limit of molecular detection and has been successfully used for the detection of oomycetous fungi and bacteria.

Characterization of Genetic Variability in plant Pathogens using DNA Markers.

All the disease management strategies based on host resistance require the knowledge of variability in pathogens. Traditional markers used to study the variability in pathogens are based on the use of differential hosts, culture characteristics, morphological markers and biochemical tests. These markers distinguish pathogens on the basis of their physiological characters i.e. pathogenicity and growth behaviours and can group them according to their similarity for these particular characters. However, these markers are highly influenced by the hostage, inoculum quality and environmental conditions. The techniques are time-consuming and laborious.

Moreover, differential hosts are not available in most of the host-pathogen systems, thus variability cannot be assessed. In such cases, molecular markers are used for the characterization of genetic variability in plant pathogens.

The DNA fingerprints (banding patterns) generated by RAPD are compared for their relatedness using genetic similarity coefficients and phylogenetic trees are constructed. PCR based DNA finger printing, particularly with short oligonucleotide primers, has been used for the analysis of genetic variation in plant pathogens.

Molecular identification of plant pathogenic fungi is accomplished by PCR amplification of ITS region followed by either restriction analysis or direct sequencing and BLAST searching against GenBank or other databases (White *et al*., 1990). Identification could be a challenge when using BLAST analysis with ITS sequences because there can be minimal or no differences between some species or, in some cases, intraspecific variation can confuse the boundaries between species (e.g., *P. fragariae* var.*fragariae* and *P. fragariae* var. *rubi* have identical ITS sequences). The ITS region remains an important locus for molecular identification of fungi. However, as more sequence data is collected from a wider range of fungal isolates, the utility of alternative loci for accurate species identification is increasing. The intergenic spacer sequence (IGS) placed between the 28S and 18S rRNA genes are the region with the greatest amount of sequence variation in rDNA. It is frequently used in PCR-based methods when there are not enough differences available across the ITS.

ITS- RFLP PCR to study the diversity of Fungi

Restriction fragment length polymorphism or RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. In RFLP analysis the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting *restriction fragments* are separated according to their lengths by gel electrophoresis. Although now largely obsolete, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. In addition to genetic fingerprinting, RFLP was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing. Here, the ITS region is used to find out the diversity of different *Phytophthora* isolates.

MLST (Multilocus sequence typing) analysis of housekeeping genes with higher variability are being more extensively used to develop diagnostics for fungi, including nuclear genes such as β-tubulin, translation elongation factor 1 alpha (*TEF* 1α), calmodulin, avirulence genes and mitochondrial genes such as the multicopy *cox* I and *cox* II and their intergenic region. Mating type genes also show high diversity and fast evolutionary rate and could be used for inter- and

intra-species differentiation. To enhance the specificity of a diagnostic assay, a combination of multiple diagnostic regions is recommended.

16S rDNA sequencing to study genetic diversity of *R. solanacearum* from different hosts

Genomic DNA isolation from bacteria

Preparation of solutions and buffers

Tris-Cl (1M) Stock 500ml pH 8.0

Tris -60.55g

HCl -21.0ml

Add 300ml of double distilled water to Tris adjust the pH to 8.0 by adding HCl and make up to 500ml. Sterilize by autoclaving.

EDTA (0.5M) Stock 250ml pH 8.0

EDTA 46.54g

NaOH 5.07g

Double Distilled water 222.38 ml

Dissolve EDTA in about 200ml of DD water. Add NaOH pellets to adjust the pH to 8.0. Then made up to 250ml. Sterilized by autoclaving.

TE buffer (10mM) (100ml)

10mM Tris 1ml

0.5mM EDTA 2ml

SDS (10%) 250ml

SDS 25g

DD water 229.5ml

Dissolve SDS was in 200ml of DD water and make up to 250ml.

TAE buffer (50X) 200ml

Tris 48.44g

EDTA 3.723g

Glacial Acetic acid 11.42ml

Dissolve Tris and EDTA in about 150ml of DD water. Add glacial acetic acid to adjust the pH to 8.0 and make up the total volume to 200ml. Sterilize by autoclaving.

NaCl (5M) 200ml

NaCl 58.44g

DD water 178.74ml

NaCl has taken about 6 hours to dissolve by gentle heating. Sterilize by autoclaving.

TE buffer (10:1) 100ml

Tris (1M) 1ml

EDTA (0.5M) 0.2ml

Take 100ml of DD water, from that remove 1.2ml and replace with 1ml of tris and 0.2ml of EDTA. Sterilize by autoclaving.

Streak pure bacterial cultures in CPG agar plates and keep in 30 C for 36-48 hours for incubation.

CTAB/NaCl 100ml

CTAB 10g

NaCl 4.1g

Add about 75ml of DD water, heat to dissolve, volume make up to 100ml. Sterilize by autoclaving.

Proteinase K 25mg/ml

Add 1ml of sterile DD water to 25mg proteinase K tube, mix it well, filter sterilize. Keep 200ml aliquots at -20C. From this 33.33µl is required for one sample of bacteria. It can only be thawed once.

Lysozyme 91mg/ml

Add 2ml of sterile DD water to 182mg lysozyme powder, mix it well, filter sterilize. Keep 200ml aliquots were kept in -20C. From this 20 µl is required for one sample of bacteria.

Procedure

- Inoculate one pure colony of the bacteria from the solid media into appropriate broth and incubate overnight at appropriate temperature
- Adjust bacterial culture to OD 1.0
- Pipette out the quantity of culture needed for making OD 1 in to tubes.
- Centrifuge for 2 min at 14000 rpm at room temperature.
- Discard the supernatant, wash the pellets three times with sterile distilled water.
- To the pellet add 550µl of TE buffer+lysozyme, mixed well and incubate for 30 min at 37C.
- After incubation add 76µl of 10% SDS+Proteinase K .
- Mix the contents by flipping the tube and incubate for 15 min at 65C.
- After incubation add 100µl of 5M NaCl and mix the contents by flipping the tube.
- Then add 80µl of CTAB/NaCl , mix and incubate for 10 min at 65C.
- After incubation add 660µl of Chloroform+isoamyl alcohol.
- Mix the contents by flipping the tube about 30sec.
- Centrifuge for 5 min at 14000 rpm at room temperature.
- After centrifugation, carefully transfer the aqueous layer at the top to a new 1.5ml tube without touching the white middle layer . Repeat this step 2 times.
- Add equal volume of isopropanol and inverted to mix.

- Centrifuge for 15 min at 14000 rpm at room temperature.
- Gently drain the supernatant and add 0.5ml of 70% ice cold ethanol.
- Centrifuged for15min at 14000rpm at 4C
- Remove the supernatant carefully and evaporate the remaining ethanol in the laminar flow about one hour.
- Add 25µl of 10:1 TE to each tube to dissolve the DNA and keep the tubes at 4C for overnight.
- Pool the DNA from the two tubes into one tube so as to get the total volume to 50µl and add RNase to remove the contaminating RNA at a concentration of200µg/ml (if stock was 10mg/ml , then 1µl for each tube).
- Incubate the tubes for 30min at 30C and store the DNA at-20C. The isolated DNA can be visualized in 0.7% agarose gel.

16S rDNA sequencing

For the PCR and PCR product sequencing, use universal primers pAF (5'-AGAGTTTGATCCTGGCTCAG-3') and pHR (5'-AAGGAGGTGATCCAGCCGCA-3')

PCR

Reagent	Reagent stock con.	Required con.	volume for 1 reaction (25µl)
MQ water	1X	1X	15.8
Go Taq buffer	5X	1X	5.0
dNTPs	5mM	200µM	1.0
Mg Cl2	25mM	1.5mM	1.5
pAF	10µM	5pM	0.5
pHR	10µM	5pM	0.5
Taq polymerase	5U/µl	1U	0.2
DNA	200ng	100	0.5

PCR programmes

96˚C 2min 94˚C 30s 60˚C 1min] 35 cycles 72˚C 1min		
72˚C 10min		
8˚C forever		
-end		

The PCR products can be visualized in a 1% agarose gel.

Cut the 1500 bp PCR product, elute and purify using Sigma Gen elution kit
Send the purified PCR product for sequencing
Sequence data on Blast search give the bacterial identity/diversity

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PLANT-PATHOGEN INTERACTIONS

A Jeevalatha

In plants, a complex array of defense response is induced after detection of microorganism via recognition of elicitor molecules released during plant-pathogen interaction. Following elicitor perception, the activation of signal transduction pathways generally lead to the production of active oxygen species (AOS), phytoalexin biosynthesis, reinforcement of plant cell wall associated with phenyl propanoid compounds, deposition of callose, synthesis of defense enzymes, and the accumulation of pathogenesis-related (PR) proteins, some of which possess antimicrobial properties. AOS lead to hypersensitive response (HR) in plants which is a localized or rapid death of one or few cells at the infection site to delimit the pathogen growth. Following the activation of HR, uninfected distal parts of the plant may develop resistance to further infection, by a phenomenon known as systemic acquired resistance (SAR), which is effective against diverse pathogens, including viruses, bacteria, and fungi. According to their lifestyles, plant pathogens are generally divided into necrotrophs and biotrophs and hemi-biotrophs. Necrotrophic pathogens first destroy host cells, often through the production of phytotoxins and cell-wall degrading enzymes, and then feed on the contents. Biotrophic pathogens derive nutrients from living host tissues, commonly through specialized feeding structures. Some plant pathogens display both lifestyles, depending on the stage of their life cycle, and are called hemi-biotrophs. For example, *Phytophthora* spp. are hemi-biotrophic pathogens that feature biotrophy early in infection and necrotrophy in the later stages of host tissue colonization.

Enzymes and toxins in pathogenesis

Most of the fungal and bacterial pathogens produce many enzymes that degrade the plant material in vivo. Enzymes involved in pathogenesis or virulence includes both constitutive and inducible enzymes. Constitutive enzymes are those enzymes which are present all the time in the cells. Inducible enzymes are those which are produced only when they are needed by the cells in response to certain internal or external stimuli. Important enzymes involved in pathogenesis are cutinases, pectinases, cellulases, hemicellulases, ligninases, lipases and proteinases.

Microbial toxins are metabolites produced by plant pathogens (fungi, bacteria), which play a role in host-pathogen interactions and in disease expression. They are low molecular weight substances produced by some pathogens which are capable of reproducing symptoms similar to that found in natural infections in plants. Pathogenic fungi and bacteria often damage their host plants by producing toxins, which cause various symptoms including necrosis, chlorosis, wilting, water soaking and eventually the death of plants. Several phytotoxic metabolites have been found and it includes piricularin from *Piricularia oryzea*, victorin from *Cochliobolus vitoriae*, phaseolotoxin from *Pseudomonas syringae pv phaseolicola*, saccharitoxin from *Helmithosporium sacchari*, cercosporin from *Cercospora* spp..

Phytotoxic metabolites of most of these pathogens have been reported to play a significant role in pathogenesis.

(I) Resistance mechanism in plants

Resistance mechanisms are by far the most important defence mechanisms employed by host plants, including our crops, against pathogens. Avoidance and tolerance play a minor role here. The moment pathogen propagules come in contact with host surface, the plants due to hereditary characters have several naturally occurring physical and chemical barriers (preexisting) resisting penetration, and if at all the penetration occurs, the host reacts by different means resulting in formation of physical and chemical barriers. The first line of defence in plants is present in its surface. Several characters of the plants surface function as barriers to penetration which pathogen must breach to enter the host. The pathogens enter the plant host by penetrating the epidermis along with cuticle and cuticular wax and number of natural openings existing before the onset of the pathogenesis can obstruct penetration. If the pathogen succeeds in penetration; it encounters preexisting internal structural barriers. The external and internal structural barriers existing before pathogen attack is also called Preexisting defence structures or passive/static or anitinfection structures. Plants liberate different chemicals, which interfere with activities of the pathogen and pathogenesis, thereby preventing or reduce infection. These chemicals and the biochemical conditions that develop may act either directly through toxic or lytic effect on the invader or indirectly through stimulating antagonistic plant surface microflora. In many host - parasite interactions, pre-existing toxic substances in the cells form the basis of resistance. In resistant variety, these substances are in abundance while in susceptible variety they may be less or completely absent. Several phenollic compounds, tannins and some fatty acid like compounds such as dienes pre-exist in high concentrations in cells have been implicated for the resistance or young tissues to parasitic fungi such as Botrytis.

a. Types of resistance

Innate resistance is exhibited by the plant in two forms: non-specific (general) resistance, which is effective against several pathogenic species or several strains (races, biotypes, pathovars) of a single pathogen, and specific resistance. In the latter case, one plant cultivar (variety) can resist infection of one or a few pathogenic strains. Extreme resistance is one unique form of specific resistance, which is also called as an immune reaction. This type of specific resistance is not associated with HR, i.e. there are no necrotic spots on the infected resistant plant organs. Another type specific resistance is associated with the gene-for-gene mechanism and the hypersensitive response (HR) of plants. It is the most thoroughly investigated type of plant resistance characterized by the formation of small tissue necroses developing in infected resistant plant parts. The pathogen is arrested and more or less confined to the infection site and is inhibited or even killed in resistant tissues.

b. Gene- for- gene hypothesis

Many major resistance genes operate in a gene-for-gene way. For each resistance gene in the host there is a corresponding avirulence gene in the pathogen and only the corresponding avirulence gene can initiate the hypersensitive reaction (HR) leading to incompatibility. This is called gene for gene hypothesis discovered by Flor in 1971. Resistance and avirulence inherit in most cases in a dominant manner, susceptibility and virulence in a recessive way. The HR is now known to result from the specific interaction at the cellular level of the product of the resistance gene and the product of the avirulence gene. If one of the two products is absent, there is no incompatibility; the normal pathogenicity of the pathogen results in a compatible reaction (the host appears susceptible). What is normally meant with virulence is actually the normal pathogenicity shown in the absence of avirulence.

c. Classes of R gene products

The different isolated R genes have been grouped into five basic classes. Among them, the genes that encode proteins containing a nucleotide-binding site (NBS) and C-terminal leucine-rich repeats (LRRs) represent the largest class of R genes. More than 50 different NBS-LRR R genes have been cloned and characterized from different plant species. These cloned genes provide a lot of information about the structure and function of this class of R genes that exhibit resistance response against a variety of pathogens such as virus, bacteria, fungi, nematodes, and pests.

The widely represented R-protein family (NB-LRR) also contains a nucleotide-binding site (NBS). NB-LRRs are localized in cytoplasm as membrane proteins and contain either a TIR domain (L6, N) homologous to metazoan Toll-interleukin receptor or a putative coiled-coil (RPS2) domain at the N-terminus. A recent modification identified in the TIR-NBS-LRR protein is an additional transcriptional factor WRKY domain towards its C-terminus (RRS1-R). The class II family has extracellular LRRs attached with a transmembrane domain (Cf2/Cf9). The LRR kinase family contains extracytoplamic LRRs fused with cytoplasmic serine-threonine kinase (KIN) domain (Xa21). Another class includes only serine threonine kinase domain either single (Pto) or double (Rpg1). A recently identified R gene (Pi-d2) has intracellular serine-threonine kinase attached with an extracellular binding-lectin (B-lectin). RPW8 (Rpw1) gene encodes a coiled-coil (CC) domain attached to a membrane anchor. A modified eLRR class gene has a transmembrane-LRR fused with a PEST domain for protein degradation (Ve2) (Fig. 1).

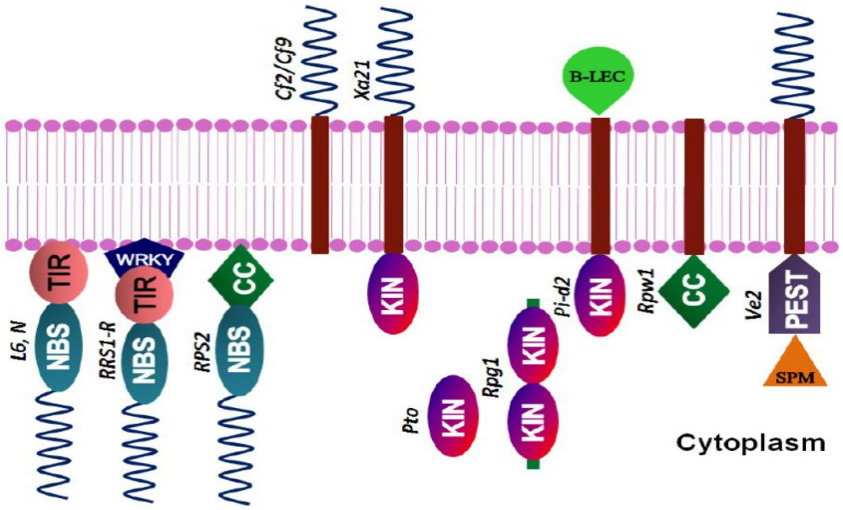

Fig.1. Major classes of R proteins

(II) Molecular basis of plant-pathogen interactions

Plant-pathogen interaction is a multifaceted process, mediated by the pathogen-and plant-derived molecules which mainly include proteins, sugars and lipopolysaccharides. Secreted molecules, derived from the pathogens, are the key factors which determine their pathogenicity and allow their successful colonization inside the host. On the other hand, plant derived molecules are involved in the recognition of the pathogen in order to elicit the defense response. The first interaction between the plants and microbes take place in a apoplast and is mediated by the recognition of microbial elicitors by the receptor protein of the plants. The microbial elicitors, also known as pathogen associated molecular patterns (PAMPs), are recognized by the membrane localized pattern recognition receptors (PRRS) of plants. The bacterial flagellin and elongation factor (EF)-Tupeptide surrogates, flg22 and elf18, and chitin, are common examples of PAMPs, which are recognized by the plant PRRS that include the three receptor-like kinases, flagellin-sensitive22 (FLS2), E-Tu receptor (EFR), and chitin elicitor receptor kinase 1 (CERK1).

The successful recognition of microbial derived PAMPs by PRRs of the plants activates a first line of defense which is known as PAMP-triggered immunity (PTI). To counter attack the PTI, many pathogen deliver various “effector” proteins inside the host cell, which suppress the components of PTI. However, resistance (R) proteins of plants recognize these effector proteins of pathogens and can induce a second line of defense which is known as the effector-triggered immunity (ETI). ETI is quantitatively stronger and faster than PTI and can result in a localized cell death (hypersensitive response) to kill both pathogen and pathogen infected plant cells. PTI and ETI together constitute a major innate immune response, enabling plants to recognize and battle against the pathogen attack (Fig. 2).

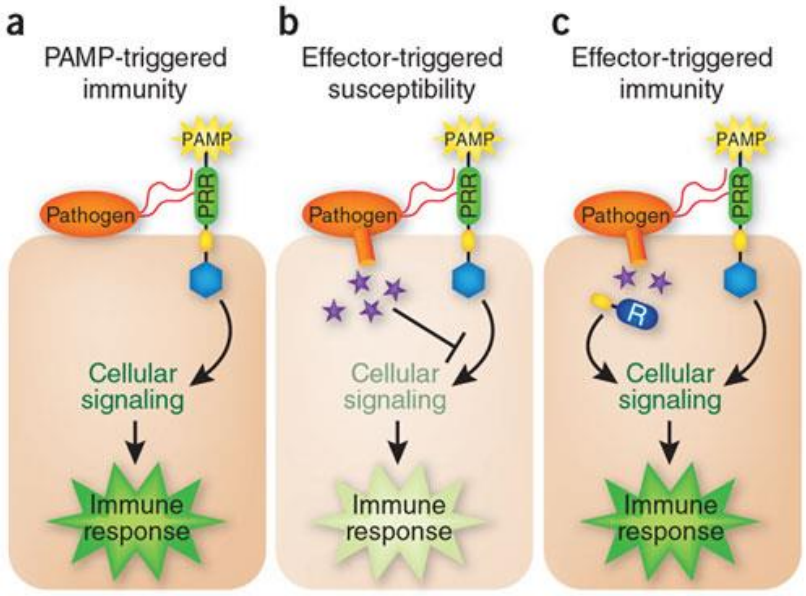

Fig.2. Diagrammatic representation of PTI, ETS and ETI

(a) Effectors

To counter plant defenses, pathogens deploy repertoires of secreted molecules (effectors) that, upon delivery into the host apoplast (extracellular effectors) or cell cytoplasm (intracellular effectors), modify cellular targets to suppress PAMP-triggered immunity (PTI) and enable parasitic infection and reproduction (Fig. 3). In addition, pathogens may also secrete classes of effectors that provoke execution of host cellular processes required for disease development. Consequently, both host and microbe tightly control transcriptional programs that drive responses to external signals. For example, a large number of effectors have been identified in oomycete pathogens. Among them, two classes of cytoplasmic effectors [RxLR and Crinkler (CRN)] are well characterized. RxLR effectors contain the conserved motif RxLR (arginine, any amino acid, leucine, arginine) in their N-termini, which delivers effectors into host cells. CRNs that were firstly reported as crinkling and necrosis-inducing proteins also contribute to virulence by modulating distinct physiological events in host cells. Similar to the RxLR effector, the N terminus of CRN contains a conserved FLAK (phenylalanine, leucine, alanine, lysine) motif that translocates effector protein inside plant cells. Necrosis-inducing proteins (NLPs) are apoplastic effectors that cause cell death and ethylene accumulation in plant cells. The NLP proteins usually possess an N-terminal secretion signal peptide and compose a superfamily of secreted phytotoxic proteins. Gram negative bacterial plant pathogens, such as *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas campestris*, deliver 15 to 30 effectors per strain into host cells using type III secretion systems to suppress PTI and ETI, including the HR. These type III effectors use diverse strategies to alter host immunity. One strategy is to destabilize host protein components, either by direct cleavage or by modulating ubiquitination. Type III effectors can also modify host or RNA metabolism. The third major strategy is to inhibit the kinases involved in plant defense signaling, especially MAPK pathways.

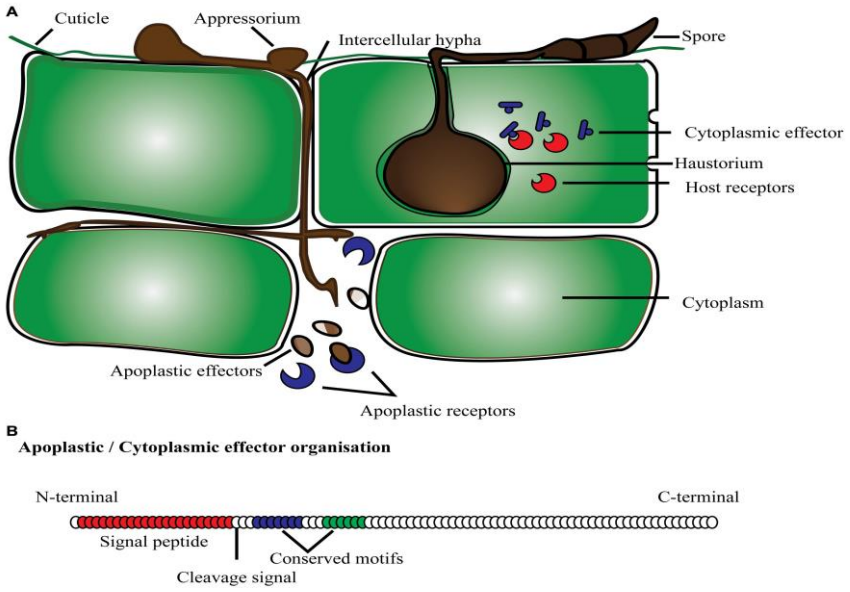

Fig.3. Type of effectors

(b)Hormones in plant immune response

Downstream of PTI or ETI activation, or other early molecular recognition events, diverse plant hormones act as central players in triggering the plant immune signaling network. Salicylic acid (SA) and jasmonic acid (JA) with its derivatives (collectively called jasmonates) are recognized as major defense hormones. However, the hormones ethylene (ET), abscisic acid (ABA), gibberellins (GAs), auxins, cytokinins (CKs), brassinosteroids, and nitric oxide (NO) function as modulators of the plant immune signalling network as well. The composition and timing of the hormonal blend produced can determine whether plant tissues become more susceptible or resistant to the invading organism. Antagonistic and synergistic interactions between diverse hormone signal transduction pathways i.e. cross talk between pathways provides the plant with a powerful capacity to finely regulate its immune response to the invading pathogen.

Salicylic acid pathway

The plant hormone SA plays a major role in disease resistance signalling. The SA response pathway is typically (but not exclusively) effective against microbial biotrophic pathogens. SA is a phenolic compound that can be synthesized from the primary metabolite chorismate via two distinct enzymatic pathways, one involving phenylalanine ammonia lyase (PAL) and the other isochorismate Synthase (ICS/SID2). SA biosynthesis is triggered during PTI and ETI upon recognition of PAMPs or effectors of pathogens. Once the SA pathway is activated at the site of infection, a similar response is often triggered in distal plant parts to protect undamaged tissues against subsequent pathogen invasion. This long-lasting and broad-spectrum induced resistance is referred to as systemic acquired resistance (SAR).

Jasmonic acid pathway

JA and its structurally related metabolites are lipid-derived compounds that are synthesized rapidly via the oxylipin biosynthesis pathway upon pathogen or insect attack. JA biosynthesis starts with the release of α-linolenic acid (α-LA) from membrane lipids. In *Arabidopsis*, two major branches of the JA signaling pathway are recognized: the MYC branch and the ERF branch. In general, the ERF branch of the JA pathway is associated with enhanced resistance to necrotrophic pathogens, whereas the MYC branch of the JA pathway is associated with the wound response and defense against insect herbivores, although MYC2 also plays a role in priming for enhanced pathogen defense.

Systemic Acquired Resistance (SAR)

Host plants can be protected against further pathogen attack if they have survived earlier infection by phytopathogenic viruses, bacteria, or fungi. It appears that the first infecting pathogen immunizes the plant against further infections by homologous pathogens, even though the plant may not carry gene determining cultivar-specific resistance. The readiness of the plant to repel subsequent pathogen attacks spread throughout the whole plant. This response is called systemic acquired resistance (SAR). The development of SAR is often associated with various cellular defense responses, such as synthesis of PR proteins, phytoalexins and accumulation of AOS, rapid alterations in cell wall, and enhanced activity of various defense related enzymes.

Plant-virus interactions

Plant viruses encode relatively few proteins and are exclusively dependent on host cellular metabolism for multiplication and movement. During a viral infection, in a manner similar to non viral infections, an HR response is initiated by Avr/R protein interactions that lead to metabolic changes in defense hormone levels, such as salicylic acid (SA), jasmonic acid (JA), and nitric oxide (NO), and the accumulation of reactive oxygen species (ROS), such as O^{2-} and hydrogen peroxide, both in the infected and non infected tissues. For example, the TMV: N - gene response showed that a gene-for-gene interaction induced a local lesion or HR in the infected plant. Similar to events that occur during bacterial and fungal-triggered immune responses, virus-associated factors, such as virion components or virus-encoded proteins, could be perceived by putative cell surface PRRs or cytosolic NB-LRR receptors (e.g., R proteins) to trigger analogous ETI or susceptible (ETS) responses, culminating in HR, SAR, and/or necrosis phenotypes.

Omics in plant-pathogen interaction study

Plant-pathogen interactions result in the triggering of signalling cascades, which may change the activity of proteins and thus directly result in a phenotypic response. These changes happen on different molecular levels and are quantified using omics technologies (genomics, transcriptomics, proteomics, metabolomics). Microarrays and RNA sequencing (RNA-seq) are the two techniques mainly used for transcriptome wide gene expression analysis. In contrast to

microarrays, RNA-seq has a higher sensitivity in measuring genes with low abundances and allows the identification of novel transcripts as well as sequence variations. The analysis of proteomes at high sensitivity is facilitated by advances in mass spectrometry based methods, which superseded 2-D gel-based proteomics. These methods enable researchers to determine the quantity of proteins and metabolites.

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MOLECULAR IDENTIFICATION OF *PHYTOPHTHORA* SPP. THROUGH PCR ASSAY

A Jeevalatha

The phylogenetic and taxonomic relationships of individual species of *Phytophthora* and their relationships to each other and to other fungal-like organisms can be established using DNA techniques. The nuclear rDNA is a very attractive marker which provides valuable molecular information on fungi and stramenopiles (Chromista). The eukaryotic ribosome comprises three subunits, and these are transcribed from a single gene region in most organisms. Internal transcribed spacer (ITS) refers to the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome (Fig. 1). there are two ITS's in eukaryotes; ITS1 is located between 18S and 5.8S rRNA genes, while ITS2 is between 5.8S and 26S (in plants) (28S in animal and fungus) rRNA genes. Black pepper, being grown mainly in a mixed cropping system where more than one *Phytophthora* species is involved in the disease of one crop or the other, the adoption of molecular tools for the identification of the species associated is highly useful and time saving. Sequence differences in the ITS regions are useful for distinguishing *Phytophthora* species. So the ITS region is amplified using universal ITS primers ITS4 and ITS6 and sequenced for identification upto species level. On the other hand, species specific primers designed from these ITS regions can be used to detect and differentiate *Phytophthora* species using PCR since sequencing involves high cost and time.

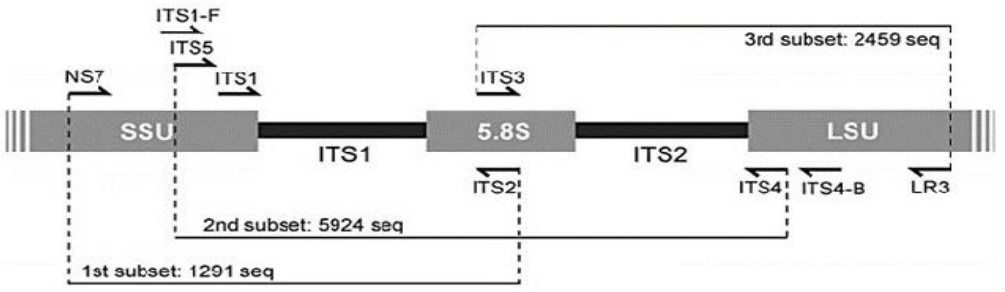


Fig.1. Diagram of the rDNA region of fungi and ITS primer (Source: Bellemain et al., 2010)

Primers:

Forward primer: ITS-6 – 5'- GAAGGTGAAGTCGTAACAAGG-3'

Reverse primer: ITS-4- 5'- TCCTCCGCTTATTGATATG-3'

Product size- approx. 900 bp

PCR reaction mix:

PCR was performed in 20µL reaction volumes. To a 0.2ml thin walled PCR tube, following components were added:

PCR component	Volume (µl)
Nuclease free water (1X)	15.20

PCR buffer with 1.5mM MgCl2 (10X)	2.00
dNTPs (2.5mM)	0.50
ITS - 6	0.50
ITS – 4	0.50
Taq DNA polymerase	0.30
Template DNA	1.00

PCR cycle conditions

Step	Temperature (°C)	Duration	
Initial denaturation	94	5 min	
Denaturation	94	1 min	35 cycles
Annealing	55	1 min	
Extension	72	1 min	
Final extension	72	10 min	
Hold	4		

Gel electrophoresis

The ITS6/ITS4 PCR products were separated by electrophoresis in 1.0 % agarose gel and an expected size of 900 bp fragment was observed.

Purification of PCR product for sequencing

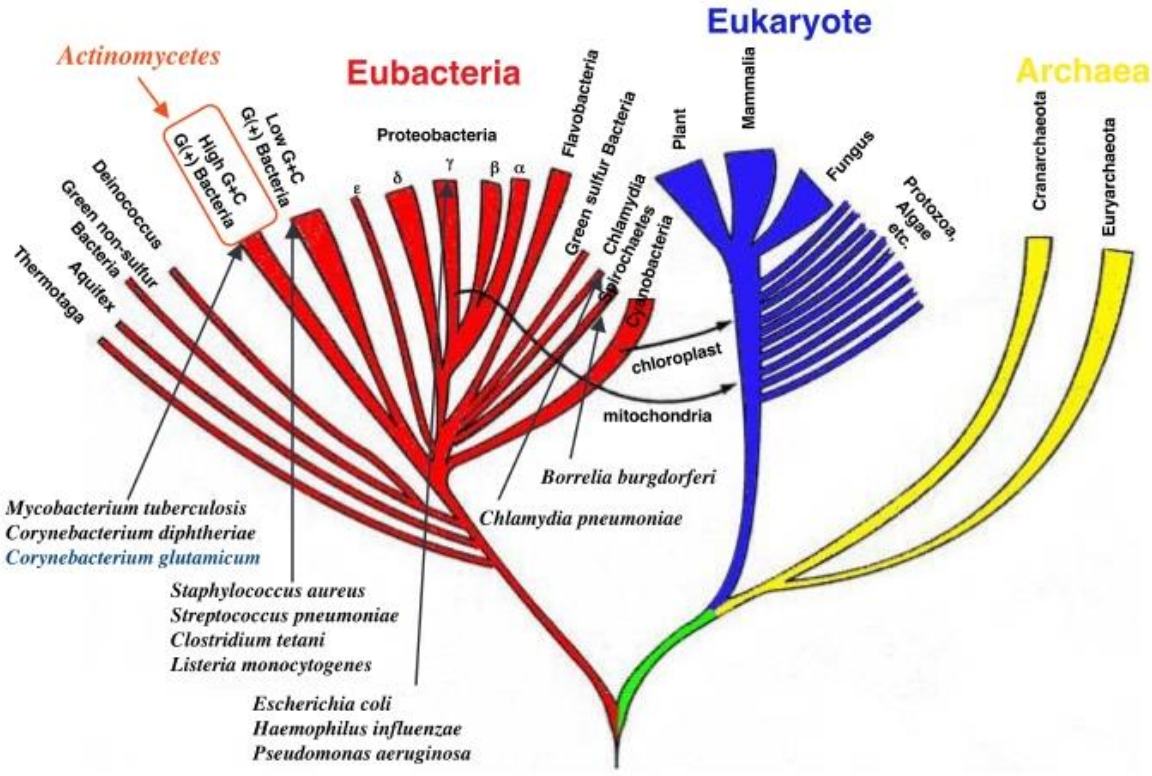
Purification of PCR product was performed using Nucleospin gel and PCR cleanup kit from MN following manufacturer instructions. Two hundred µl of binding buffer per 100 µl of pcr product was added and mixed thoroughly. The spin column was kept in a 2ml collection tube and the sample was added to the column. The mixture was centrifuged for 1 minute at 11,000xg. The flow through was discarded and the column was placed back in the same collection tube. Then, 700µl of diluted wash buffer was added and the tubes were centrifuged for 1 minute. The flow through was discarded and the column was placed back in the same collection tube. The empty column was centrifuged for 2 minutes to ensure complete removal of wash buffer. The spin column was placed in a new 1.5 ml tube and 32µl of elution buffer was added to the centre of the membrane. The column was kept at room temperature for 1 minute and centrifuge d for 1 minute to elute the PCR product.

AN INTRODUCTION TO ACTINOBACTERIA

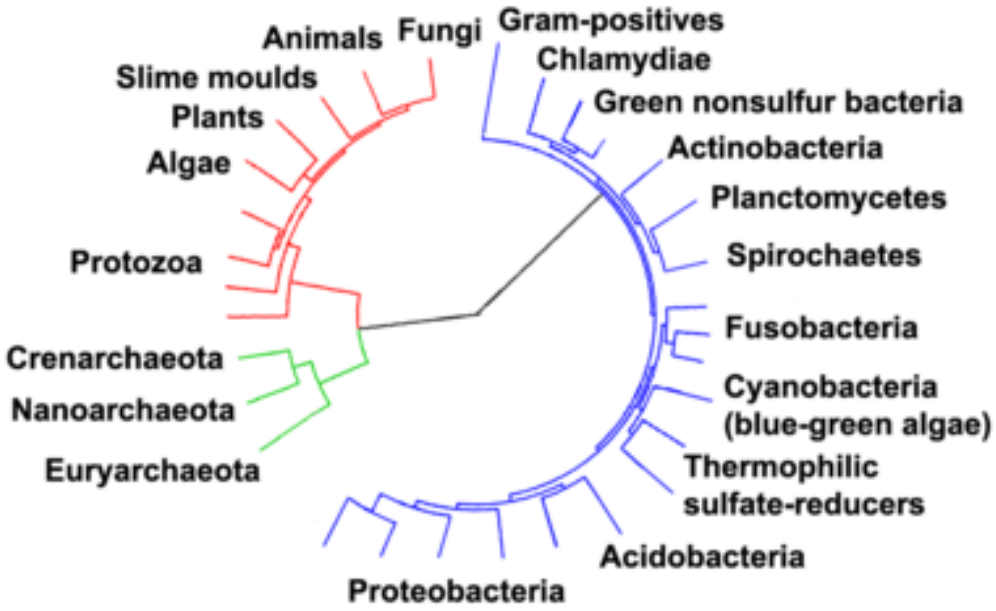
R Suseela Bhai

Actinobacteria are Gram positive prokaryotic organisms with high G+C (Guanine plus Cytosine) content *i.e.* above 55% in their DNA. They are closely related to bacteria, filamentous in nature, belongs to the order *Actinomycetales.* They reproduce either by fission or by means of spores or conidia developed on special spore bearing structures. Actinomycetes (Actinobacteria) are organisms with characteristics common to both bacteria and fungi but possess distinctive features to delimit them into a distinct category. Taxonomically, they are clubbed with bacteria in the same class Schizomycetes and confined to the order Actinomyceta. Actinomycetes are also called as "higher bacteria," / "filamentous bacteria". They possess cell walls containing muramic acid and have prokaryotic nuclei, *Gram-positive*, having a thicker layer of peptidoglycan in their cell. Actinomycetes are also known as Ray fungi. (Akitino= Ray mykes= mushroom/fungi= Ray fungi). Aerial mycelium produces spores-similar to fungi, but differ from fungi because they do not have chitin or cellulose in the cell wall as in fungi. In short Actinomycetes are transitional forms between bacteria and fungi .

The History of actinomycetes goes back to its use as an antibiotic. Its use as an anti fungal agent is mostly responsible for its popularity with antibiotic research today. The first aerobic actinomycete was discovered by Edmund Nocard in 1889 and named as *Nocardia farcinia*. Dr. Selman Waksman is the man to be claimed for the present knowledge of Actinomycetes. Dr. Waksman began researching actinomycetes, at the Rudgers University (State University of New Jersey). Starting with the discovery of actinomycin in 1940 until his retirement in 1958, Dr. Waksman and his students derived 22 different antibiotic compounds from actinomycetes. Three of the antibiotics — actinomycin, neomycin and streptomycin — became commonly used. Actinomycin, the first antibiotic isolated by Dr. Waksman, is used sparingly as an anti-tumor drug which is highly toxic and frequently as an investigative tool for cell biologists. Neomycin is an extremely common antibiotic that is found in many skin ointments such as Neosporin®, as well as numerous treatments for eye and ear infections.



Phylogenetic Tree of Organisms



Antibiotic isolation

Screening of Actinomycetes for antibiotics started in 1937 (Nakhimovskaia, Krassilinikov and Koreniaco) and the first true antibiotic called Actinomycin was isolated in 1940. Antibiotic Streptothricin with chemotherapeutic properties was isolated in 1942. However, the first antibiotic that found application as a chemotherapeutic agent was Streptomycin in 1943. Production of antibiotic is strain characteristic and not a species characteristic. Out of 10,000 known antibiotics, 45-55% is produced by *Streptomyces* sps. Classification/grouping of antibiotics is based on Biological properties, Physical and chemical properties, solubility and utilization

Properties of Actinobacteria

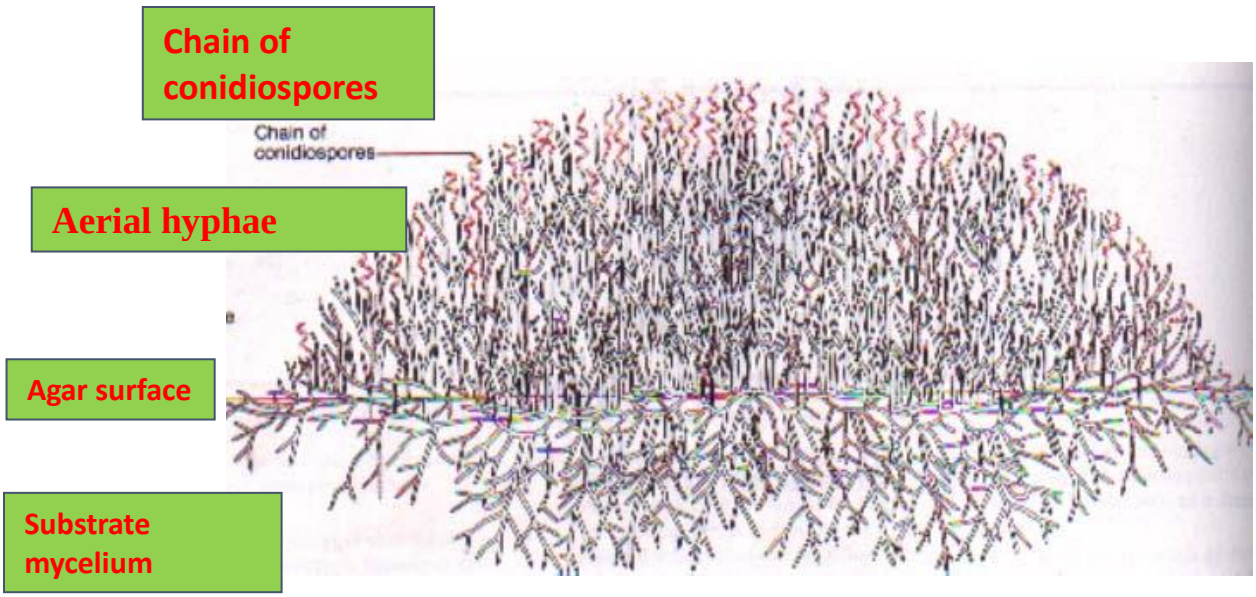
The Strong odor " **earth smell**" that occurs in the air when rain falls after a dry spell of weather is due to a volatile compound called geosmin produced by soil actinomycetes. Some actinobacteria establish symbiotic nitrogen fixing association and some form lichen-like associations with green algae called actinolichens. A number of actinobacteria form close association with plants and act as growth-promoting and biocontrol agents. Actinomycetes also cause diseases in plants (e.g. potato scab, gall, and wilt) and animals (Actinomycosis). Moreover they are the major source of Antibiotics. Among the genera of Actinomycetes, *Streptomyces* sp. is the major component of the total actinomycete population

Classification of Actinobacteria

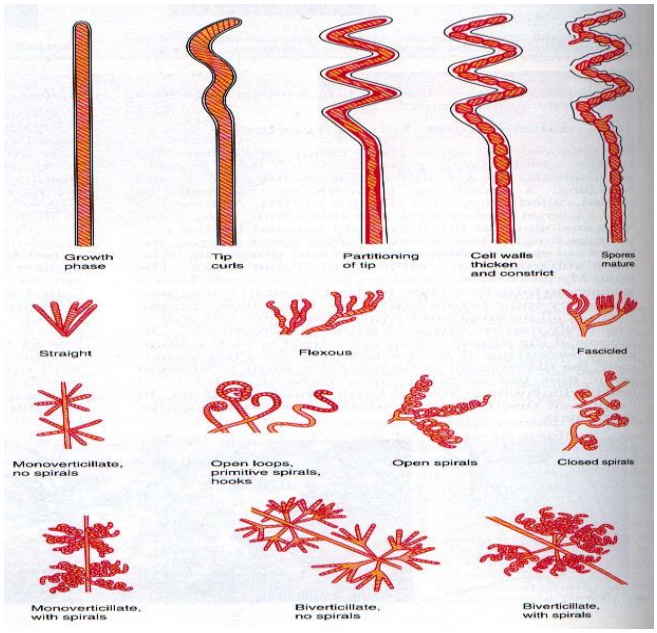
Actinobacteria were classified under four families viz. *Mycobacteriaeae*, *Actinomycetaceae*, *Streptomycetaceae* and *Actinoplanaceae.* According to Current systematic classification, actinobacteria are placed in the Order - Actinomycetales with 13 Suborders viz. 1.Actinomycineae, 2.Micrococciniae, 3.Catenulisporiniae 4.Corynebacterinae, 5.Micromonosporineae, 6.Propionibacterineae, 7. Actinopolysporineae
8. Pseudonocardinae, 9.Streptomycineae, 10.Streptosporanginae, 11.Frankineae, 12.Kineosporiineae and 13.Glycomycineae .

Morphology of Actinomycetes:

Morphologically actinomycetes resemble fungi because of their elongated cells that branch into filaments or hyphae. According to the difference of morphology and function, the mycelia can be divided into substrate mycelium and aerial mycelium



Colony on agar surface showing the substrate mycelium and aerial mycelium with chains of conidiospores



Spore chain morphology of actinomycetes

Spore chain morphology is important for the classification of actinomycetes. On the basis of spore chain morphology the strains can be placed into groups. Three major types of spore chain morphology are Rectiflexibiles, Retinaculiaperti and spirales, observed for species belonging to the genus *Streptomyces*. Other spore chain morphologies are Monoverticillus, Monoverticillus-spira, Biverticillus and Biverticillus-Spira etc. Characteristics of the spore bearing hyphae and spore chains can be determined by light microscopy using cover slip culture and slide culture

techniques .Actinomycetes are also observed by the phase-contrast microscopy for study of spore surface ornamentation.

Distribution of Actinomycetes

Actinobacteria are distributed in natural habitats like Soil, fresh water, marine, organic matter, endophytes in plants, humans and animals.

Aquatic actinomycetes: Occur in marine and fresh water environment, exhibit unique physiological and structural characteristics that enable them to survive in extremes of conditions pressure, salinity and temperature with the potential production novel bioactive compounds like Rifamycin (Micromonospora), Salinosporamide (Salinispora) Marinomycins (Marinophilus sp.) Marinopyrroles (Streptomysus sp.), Abyssomicin C (Verrucosispora) etc.

Endophytic actinomycetes ooccur as endophytes in plants/animals and give beneficial or harmful effects. They produces a variety of bioactive metabolites like antibiotics, plant growth promoters, plant growth inhibitors, hydrolytic cell wall degrading enzymes, etc.

Rhizospheric actinomycetes profusely colonize the root system of plants; protect plant roots from pathogen invasion by antibiosis, parasitism and production of extra cellular enzymes and also siderophores

Rare Actinomycetes are non-Streptomyces group that are difficult to isolates under normal conditions include Actinomadura, Actinoplanes, Amycolatopsis, Dactylosporangium, Kibdelosporangium, Ketosatospora, Microbispora, Planomonospora, Planobispora, Salinispora, Streptosporangium, and Verrucosispora.

Actinomycetes- as human pathogens.

Streptomycosis /Mycetoma - S*treptomyces* species usually cause the disease entity known as Mycetoma or streptomycosis (fungus tumor). These infections are usually subcutaneous, but they can penetrate deeper and invade the bone.

Actinomycosis: The clinically, histopathologically, and prognostically characteristic inflammatory syndrome caused by species such as *A. israelii* and *A. gerencseriae*

Nocardiopsis - Nocardia *steroides* causes tissue infections in humans. *Dermatophilus congolensis* causes dermatophilosis, a severe dermatitis of cattle, sheep, horses, and occasionally humans

Nocardiosis-*Nocardia asteroids is the cause of* pulmonary nocardiosis while *N. brasiliensis* cause sub-cutaneous lesions**.** Nocardiosis primarily presents as a pulmonary disease or brain abscess.

Potential of actinomycetes in Agriculture

Actinomycetes act as source of agro active compounds, as PGPR and biocontrol tools. They remain as the most fruitful source of microorganisms for all types of bioactive metabolites, including agro active type. Over one thousand secondary metabolites were discovered from actinomycetes of which about 60% of the new insecticides and herbicides originate from

Streptomyces itself . For e.g. Kasugamycin is a bactericidal and fungicidal metabolite derived from *S. kasugaensis* .It is an inhibitor of protein biosynthesis in microorganisms but not in mammals. Hokko Chemical Industries developed a production process to market the systemically active kasugamycin for control of rice blast (*Pyricularia oryzae)* and Pseudomonas diseases. Validamycin A was found to be a pro drug which is converted within the fungal cell to validoxylamine A, an extremely strong inhibitor of trehalase. Mildiomycin from *Streptoverticillium rimofaciens* is strongly active against powdery mildews on various crops, acting as an inhibitor of the fungal protein biosynthesis.Polyoxin B and D are natural fungicide isolated from *S. cacaoi* var. asoensis. It interferes with the fungal cell wall synthesis by inhibiting chitin synthase. Polyoxin B is used against a number of fungal pathogens in fruits, vegetables and ornamentals. Polyoxin D is used to control sheath blight of rice caused by *R. solani* and is being marketed by several companies

Actinomycetes as promising biocontrol-PGPR

Streptomyces griseus is used as seed treatment of barley, oat, wheat and carrot, to increase their growth. The isolate was originally selected for the biological control of *R.solani. S.pulche* , *S. canescens* and *S. citreofluorescens* were used in the control of bacterial, Fusarium and Verticillium wilts, early blight, and bacterial canker of tomato as well as for growth promotion as seed coating. *S.olivaceoviridis* and *S. rochei* were found effective in increasing the shoot length and shoot fresh mass of wheat plants . Arzent™ is a commercial biocontrol product is a mixture of four separate, compatible strains of *S. hygroscopicus* being used for growth promotion in radish growth .

The plants that form symbiotic relationships with actinomycete *Frankia* are called actinorrhizal plants. About 15% of the world's nitrogen fixed naturally is from symbiotic relationships between various species of the *Frankia* family of actinobacteria and their host plants. Actinomycetes have the aability to colonize plant surface, antibiosis, synthesis of extracellular proteins, and degradation of phytotoxins, siderophore production, competition and pparasitism. *S.griseoviridis* strain K61 is used in root dipping or growth nutrient treatment of cut flowers, potted plants, greenhouse cucumbers, and various other vegetables. *S.griseoviridis* is used in the biological control of Fusarium wilt of carnation, damping-off of Brassica and the root rot of cucumber. Similarly *S. griseoplanus* SAI-25, *S. bacillaris* CAI-155 and *S. albolongus* BCA-698 have entomopathogenic activity against lepidopterans.

Few commercial formulations actinomycete agrocompounds available in the market

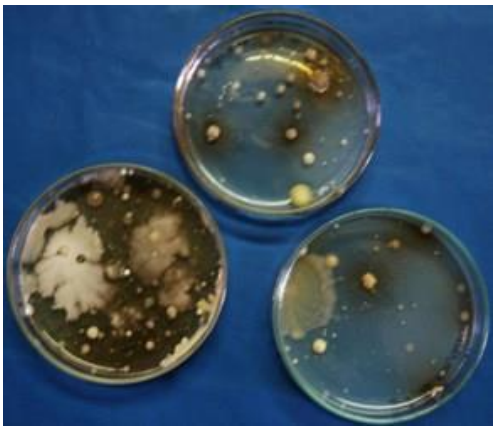
Mycostop is a commercial biofungicide formulation developed from dried spores and mycelium of *Streptomyces griseoviridis* Strain K61 for the control of seed rot, root and stem rot and wilt caused by *Fusarium*, *Alternaria* and *Phomopsis* of container grown ornamentals, vegetables and tree and forest seedlings. **Kasugamycin** is a bactericidal and fungicidal formulation from *S. kasugaensis.* -for control of rice blast and Pseudomonas diseases. **Spinosad**

is a commercial organic insecticide prepared from a soil actinomycete called *Saccharopolyspora Spinosad* active against all lepidopterans insects. **Fervenulin** is a nematicide isolated from nematicidal actinomycetes, *Streptomyces roseoverticillatus* that inhibited egg hatch and increased juvenile mortality of *Meloidogyne incognita in vitro*.

Actinogen Limited is a Western Australasian company dedicated to the discovery and isolation actinomycetes. The company has isolated two streptomycetes isolates that produce bioactive compounds which show significant antibiotic activity against "Superbug" Methicillin Resistant Staphylococcus Aureus (MRSA) known as Golden Staph and Vancomycin Resistant Enterococci (VRE) bacteria. They also discovered a series of actinomycetes that can produce Shikimic acid which is a main component in the production of Tami flu, the oral antiviral treatment. The production of Tami flu is complex and one of the most expensive components is Shikimic acid. Currently, Shikimic acid is mainly produced from the star anise fruit by fermentation process using an Escherichia-coli bacterium. The star anise tree only grows under certain climate conditions (humid, hot weather and high altitude) and takes 6 years to mature. The Shikimic acid is then extracted from the pods of the star anise. Thirty kilograms of star anise is required to produce approximately one kilogram of Shikimic acid. Actinogen also designed to discover new bioactive anti cancer molecules from soil in Western Australasia. They used to screen liquid cultures of the actinomycetes for the presence of bioactive molecules that are either cytotoxic or cytostatic for a series of cell lines derived from various human and animal cancers. The Company is also having low cost production technology for producing cellulase(s) from actinomycetes in an aerobic environment at room temperature. Cellulase(s) are an important step in the production of second generation bio-ethanol.

Isolation of actinobacteria

The soil actinobacteria can be isolated by conventional serial dilution methods. Different media were used by many workers for isolation of actinobacteria. This include Gauze's agar medium; modified Benedict's medium Czapek's agar medium, egg albumin medium, glucose asparagine medium, glycerol asparaginate agar II, chitin medium arginine glycerol salt medium; powdered chitin agar medium , Kuster's agar and starch casein agar medium . Novel genera of actinomycetes can be isolated by considering several factors during the isolation, such as the selection of natural habitats for sample collection, chemical and physical pretreatment of the samples, use of specific selective media, modification of culture conditions and genus-specific methodologies for screening of isolates



Isolation from soil

Pure culture

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GENOMIC DNA EXTRACTION

T E Sheeja

The isolation of pure intact and high quality DNA is very crucial for any molecular studies. Basically any DNA isolation shall comprise of the basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring removal of the contaminants like proteins, polysaccharides, lipids, phenols and other secondary metabolites. The DNA isolation from certain plants is usually complicated due to excessive contamination by secondary metabolites. Hence, the DNA isolation methods need to be optimized to each species of plant and in some cases even to each tissue because of the presence of these metabolites. The search for a more efficient means of extracting DNA of both higher quality and yield has lead to the development of a variety of protocols; however the basic principle of DNA extraction remains the same.

The main objective of a DNA isolation protocol is that the DNA must be purified from cellular material in a manner that prevents degradation Because of this, even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses. As mentioned earlier DNA extraction from plant can vary depending on the tissue used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer containing CTAB (cetyl trimethyl ammonium bromide) or SDS (sodium dodecyl sulphate). In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. Many factors can cause shearing of DNA during extraction. Degradation of DNA due to endonucleases is one such problem encountered in the isolation and purification of high molecular weight DNA, which interferes with the enzymatic reactions. Similarly presence of polysaccharides also inhibits enzyme activity. These polysaccharides have been found to inhibit *Taq* polymerase activity and restriction enzyme activity. The presence of polysaccharides is characterized by formation of a highly viscous solution. The oxidized form of polyphenols covalently binds to DNA giving a brown colour and reduces maintenance time, making it useless for molecular studies.

The most commonly used basic plant DNA extraction protocols are those of Dellaporta et al. 1983 and Saghai Maroof et al. 1984. In addition to these DNA isolation kits based on either

anion exchange chromatography or silica gel membranes are available commercially. However, in plants with high levels of polysaccharides, phenols and mucilage kits may not work and alternate methods need to be devised for extraction. Many protocols use phenol to separate cellular molecules and debris from the DNA, which is toxic, hazardous, expensive and require special containment facilities to maximize personnel and environment safety. Several attempts have been made to eliminate the use of hazardous chemicals, expensive kits and equipment and to reduce labour intensive steps for high throughput DNA extraction. However, the methods have limited shelf life, low purity, low recovery and poor amplification. Mostly DNA extraction protocols recommend fresh tissues; however, on the practical side many times there is a need to devise protocols from dried and recalcitrant tissues. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light. The efficiency of the isolation protocol is assessed through its amenability to downstream applications like PCR, restriction digestion etc.

Basic Procedure

The basic steps in a DNA extraction protocol is given below:

- Breaking the cells open, commonly referred to as cell disruption or cell lysis, to expose the DNA within. This is commonly achieved by chemical and physical methods-blending, grinding or sonicating the sample.
- Removing membrane lipids by adding a detergent or surfactants which also serves in cell lysis.
- Removing proteins by adding a protease (optional but often done).
- Removing RNA by adding an RNase (almost always done).
- DNA purification from detergents, proteins, salts and reagents used during cell lysis step.

The most commonly used procedures are:

- Alcohol precipitation using ice-cold ethanol or isopropanol. Insolubility of DNA in alcohols facilitate in aggregation and precipitation as pellet upon centrifugation. The process is improved by increasing of ionic strength, usually by adding sodium acetate.
- Phenol is a protein denaturant and Phenol–chloroform extraction facilitates removal of proteins. After centrifugation of the sample, denaturated proteins stay in organic phase while nucleic acid remains in the aqueous phase. On extraction and mixing of this aqueous phase with the chloroform phenol residues are removed from solution.
- Alcohol precipitation of nucleic acids after diluting with a monovalent salt. The nucleic acid precipitates spontaneously and can be pelleted by centrifugation. The salts and alcohol are removed by washing with 70% alcohol. The most commonly used salts include sodium acetate, sodium chloride and potassium chloride. Ethanol or isopropanol are the standard alcohols used for nucleic acid precipitation.
- Minicolumn purification that relies on the fact that the nucleic acid may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt content of the buffer.

- The nucleic acid pellet can be resuspended in either sterile distilled water or TE (10 mM Tris: 1mM of EDTA)
- The DNA is purified by incubating the nucleic acid solution in RNase A (10 mg/ml) at 37^0C and reprecipitation following phenol: chloroform extraction to remove the RNase

However, in certain cases specialized techniques are needed for isolation of DNA from samples like archaeological samples containing partially degraded DNA, soil samples with high humic acid and blood. Extrachromosomal DNA is generally easy to isolate, especially plasmids may be easily isolated by cell lysis followed by precipitation of proteins, which traps chromosomal DNA in insoluble fraction and after centrifugation, plasmid DNA can be purified from soluble fraction.

Reagents and equipments: The DNA isolation requires the following reagents and minor equipments:
CTAB buffer
Microfuge tubes
Mortar and Pestle
Liquid Nitrogen
Microfuge
Absolute Ethanol (ice cold)
70 % Ethanol (ice cold)
7.5 M Ammonium Acetate
55^o C water bath
Chloroform: Iso Amyl Alcohol (24:1)
Water (sterile)
Agarose
6x Loading Buffer
1x TBE solution
Agarose gel electrophoresis system
Ethidium Bromide solution
CTAB buffer 100ml
2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide) or SDS
10.0 ml 1 M Tris pH 8.0
4.0 ml 0.5 M EDTA pH 8.0 (Ethylenediaminetetra Acetic acid Di-sodium salt)
28.0 ml 5 M NaCl
40.0 ml H2O
1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000)
Adjust all to pH 5.0 with HCL and make up to 100 ml with H2O.
1 M Tris pH 8.0
Dissolve 121.1 g of Tris base in 800 ml of H_2O.

Adjust pH to 8.0 by adding 42 ml of concentrated HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 1 L with H_2O. Sterilize using an autoclave.

5x TBE buffer

54 g Tris base

27.5 g boric acid

20 ml of 0.5M EDTA (pH 8.0)

Make up to 1L with water.

To make a 0.5X working solution, do a 1:10 dilution of the concentrated stock.

1% Agarose gel

1 g Agarose dissolved in 100 ml TBE

Procedure

Modified Doyle and Doyle 1990 protocol for isolation of DNA from Turmeric leaves

- Preheat 20 ml extraction buffer with 100µl of 0.5% β-mercaptoethanol
- Grind 5 gm of leaf tissue in liquid nitrogen using mortar and pestle. Add a pinch of PVP (polyvinyl pyrrolidone) while grinding.
- Add the preheated extraction buffer into the grinded tissue and mix.
- Transfer the mixture into a centrifuge tube and incubate the tubes at 65°C for 1 hour.
- After incubation add equal volume (20 ml) of Chloroform:isoamyl alcohol and invert the tubes gently.
- Centrifuge at 3000 rpm for 20 min
- Transfer the aqueous phase to a fresh tube and add 0.6 volume of isopropanol and keep at -20°C for 1 hour or overnight.
- After incubation centrifuge the tubes at 3000 rpm for 6 mins at RT.
- Air dry the pellet and add 500µl TE buffer and dissolve the pellet
- Add RNase A to a final concentration of 10µg/ml and keep at 37°C for 30 mins.
- Add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mix gently.
- Centrifuge at 14000 rpm for 10 mins
- Transfer the aqueous phase to a new tube and add equal volume of chloroform : isoamyl alcohol (24:1)
- Centrifuge at 14000 rpm for 10 mins
- Transfer the aqueous phase to a new tube and add 2 volume of ice cold ethanol, mix and keep overnight at -20°C
- Centrifuge at 14000 rpm for 10 mins
- Pour off the supernatant and wash the pellet with 70% ethanol.
- Air dry the pellet and dissolve in TE buffer/ sterile nuclease free water according to the pellet size.

Materials

Extraction buffer

Prepare 20mM EDTA and 100mM tris HCL and mix. Adjust the pH to 8.

Add 1.4 M NaCl and 2% w/v CTAB and keep at 60°C and mix intermittently.

TE buffer

Prepare 10mM Tris HCl and 1mM EDTA. Mix and adjust the pH to 8.

-Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness. DNA quality confirmation

–Prepare a 1 % solution of agarose by melting 1 g of agarose in 100 mL of 0.5x TBE buffer in a microwave for approximately 2 min. Allow to cool for a couple of minutes then add 2.5 µl of ethidium bromide, stir to mix.

–Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface.

–Load the following into separate well so10 µL 1kb ladder o5 µL sample + 5 µL water + 2 µL 6x Loading Buffer

–Run the gel for 30 min at 100 V

–Expose the gel to UV light and photograph (demonstration)

–Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation

Detecting DNA

Quantification and visualization of DNA

Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm is used as a measure of DNA purity. DNA absorbs UV light at 260 and 280 nanometres, and aromatic proteins absorb UV light at 280 nm; a pure sample of DNA has a ratio of 1.8 at 260/280 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8. DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an agarose gel, staining with ethidium bromide or a different stain and comparing the intensity of the DNA with a DNA marker of known concentration. DNA purity is assessed in most labs now using a NanoDrop Spectrophotometer that measures the A260/A280 and A260/A230 ratios. A260/A230 ratios greater than 2 and A260/A280 ratios greater than 1.7 indicate high purity DNA and lower ratios indicate contamination.

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INTRODUCTION TO PLANT ENDOPHYTES AND ITS SIGNIFICANCE

Mohammed Faisal Peeran

Endophytes are the microorganisms, which colonize symptomless the living plant tissue without causing any immediate, overt, negative effect on the plant (Hirch and Broun 1992). Anton de Bary first introduced the term "epiphytes" for fungi that live on the surface of their host and "endophytes" for those living inside the plant tissue (De Bary 1866). Later, this term was expanded as fungi and bacteria, including actinomycetes, which spend the whole or part of their life cycle colonizing inter- or intra- cellularly, inside the healthy living tissues of the host, typically causing no apparent symptom of disease.

The word endophyte came from two Greek words, "endon" means within and "phyton" means plant. Endophytes were mentioned for first time by Bary in 19th century (Azevedo 1998) and have been defined in many ways. Wilson (1995) defined endophytes as the fungi that live internally and remain asymptomatic for at least part of their lifecycle. He also described the symptomless nature of endophyte occupation in plant tissue, symbiotic and mutualistic relationship between the endophytes and their host. But they can also be aggressive saprophytes or opportunistic pathogens.

Endophytes are plant-associated prokaryotes that form association with their host plants by colonizing the internal tissues, which has made them valuable for agriculture as a tool in improving crop performance.

Mode of endophytic infection

Foley (1962) described the path of infection of endophytic *Fusarium moniliforme* on maize. This fungus was present in the nodes earlier than in internodes and confined to the basal part of the stalk until the time of flowering. During flowering, a rapid spread of mycelium was observed throughout the plant. Maize plant may become infected by endophytic *F. moniliforme* through three different routes, namely air and/or rain splash, insect damage and seed transmission.

Advantages of endophytism

Endophytism offers several advantages to the host plants. They are (i) greater access to the nutrients, (ii) protection from desiccation, and (iii) protection from the surface feeding insects, parasitic fungi, etc. Episymbiotic fungi modify the host tissue in order to acquire their nutrients. Epidermal cells in close association to stromata are hypertrophied and lack the waxy cuticle that normally prevents the escape of water and nutrients from the leaf. Because the cuticle is absent on the leaf, the episymbiont may extract the nutrient from the leaf across the modified epidermal layer. In case of endosymbiosis, mycelium is distributed among the internal cells of the plant tissues.

1. Secondary metabolites synthesized by endophytes

Many fungal endophytes produce secondary metabolites and some of these compounds are antibiotics having antifungal, antibacterial and insecticidal properties, which strongly inhibit the growth of other microorganisms, including plant pathogens.

Antibiotics

Igarashi et al. (2000) isolated 398 actinomycetes strains from the leaves, stems and roots of cultivated or wild plants. About10-20% of the nbutanol extracts of their fermentation broths showed antagonistic activity against phytopathogenic fungi and bacteria. One of the extracts from *Streptomyces* sp. collected from *Allium fistulosum* had a potential to suppress the infection of *Alternaria brassicicola* on Chinese cabbage seedlings.

2. Enzymes

When endophyte colonizes on the plant surface, they produce enzymes such as β-1,3-glucanases, chitinases and cellulases to hydrolyze the plant cell wall. In addition, these enzymes also have a function to suppress the plant pathogen activities directly and have the capability of degrading the cell wall of fungi and Oomycetes.

3. Plant growth promoters

Endophyte may promote plant growth by secreting different hormonal substances. Porter et al. (1979) reported that endosymbionts produced auxin, which enhanced the vegetative growth of the endophyte infected plants. They also reported the mechanism of increased drought tolerance in the plants due to higher ability of meristem to recover after drought condition.

4. Mechanism of endophyte mediated plant disease resistance

Induction of plant resistance

Over the past two decades, many researchers have focused on plant resistance responses to pathogens and parasites of various scales. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two forms of induced resistances. SAR, induced by the pathogen infection, is mediated by salicylic acid and associated with the accumulation of pathogenesis-related (PR) proteins. ISR, induced by some non-pathogenic rhizobacteria, is mediated by jasmonic acid or ethylene and is not associated with the accumulation of PR proteins. These PR proteins comprise a variety of enzymes, some of which may act directly to lyse the invading cells, including chitinases and β-1,3-glucanases reinforce cell wall boundaries to resist infections, or induce the localized cell death. Fungal endophytes induced ISR may also associate with the expression of pathogenesis related genes.

F. solani, isolated from the root tissues of tomato elicited induced systemic resistance against the tomato foliar pathogen, *Septoria lycopersici* and triggered PR genes, PR5 and PR7 expression in the roots

5. Indirect effect on enhanced plant resistance

Plants develop several mechanisms against unfavorable environment such as drought, cold, salt stress or pathogens. Morphological and biochemical changes, including cellular necrosis, hypersensitive response and phytoalexin production respond to the various stresses rapidly. During the long term evolution, two types of innate resistance, non-specific (general) resistance and specific resistance are formed to resist the pathogens infestation (Kiraly et al. 2007). Since fungal endophytes may evolve from the plant pathogenic fungi, plant defense could be triggered by fungal endophytes such as pathogens. Actually, the defense of plant associated with endophytes is increased through resistance enhancement and secondary metabolites production.

Endophytes are naturally occurring biocontrol agents with potential beneficial uses in the plant. Of the nearly 3,00,000 plant species, that exist in the earth, each individual is the host to one of more endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology. The use of endophytic microorganism for the plant disease control is relatively new and unexplored area of research. Little is known about the interaction between the endophytes, host plant and pathogen. Understanding of this interaction is essential for the development of proper biocontrol strategy. Although scientific approaches on the diversity of endophytes have just recently got momentum, information on endophytes' behavior, their mutualistic interaction with crops remain scanty and more studies are needed to explore their full potentiality in plant protection sector.

ROLE OF MICROBIAL TOXINS, ENZYMES AND GROWTH REGULATORS IN PATHOGENESIS

C N Biju

Insect pests and diseases interfere with the natural growth and cause enormous damage to the crops. The interference and damage often leads to failure of plants to grow normally subsequently leading to destruction of parts or total plants. Plant disease is an abnormality in the structure and/or function of the host plant cells and/or tissue as a result of a continuous irritation caused by infectious pathogenic agents or other factors. Plant pathology is the science that studies the causes of plant diseases, the mechanisms by which diseases develop in individual plants and in plant populations and the ways and means by which plant diseases can be managed economically and sustainably. Plants have internal mechanisms of defense that protect them against diseases but, when these defense mechanisms fail, they become diseased. Pathogenic is having the characteristics of a pathogen and pathogenicity is the capability of a pathogen to cause a disease. There are several chemical weapons secreted by pathogens *viz*., enzymes, toxins and growth regulators which makes them capable to cause plant diseases.

TOXINS

It was claimed by Gaumann (1954) that, microorganisms are pathogenic only if they are toxigenic. In plant pathology, a toxin is generally defined as a non-enzymic substance which injures plant cells or disrupts their metabolism. Pathogenic fungi and bacteria often damage their host plants by producing toxins, which cause various symptoms including necrosis, chlorosis, wilting and eventually death of the plants.

In general, toxins have following effects on host plants:

- Alters cell permeability
- Disruption of normal metabolic activity
- Loss of salts from protoplasm increases
- Adversely affects respiratory process
- Uncoupling of oxidative phosphorylation
- Inhibition of host enzyme
- Affect cellular transport system
- Interfere with growth regulatory system

On the basis of pathogenesis, toxins are classified into three broad categories:

(1) Pathotoxins (Wheeler and Luke, 1963)

These toxins play an important causal role in disease and are produced by the pathogen or interaction between host and pathogen.

Properties:

- When applied on a susceptible host in low concentrations it should produce all or nearly all symptoms of the disease.

- Toxin and the pathogen should have same host range; same resistance/susceptibility spectrum.
- Pathogenicity of the pathogen should be correlated with its capacity to produce the toxin.
  E.g. Tab-toxin (*Pseudomonas tabaci*), HMT toxin (*Dreschlera maydis* race T)

(2) Vivotoxins (Diamond and Waggoner, 1953)

These toxins are produced in the infected plant /host by the pathogen and/or its host that function in the production of the disease.

Properties:

- Reproducible separation of the toxin from the diseased host.
- Purification and chemical characterization.
- Induction of at least a part of disease syndrome when applied on healthy plants.
- These toxins are generally non-specific in action.
  E.g. Fusaric acid (*Fusarium* spp.), Pyricularin (*Pyricularia oryzae*)

(3) Phytotoxins

- Phytotoxins are phytotoxic substances produced by living organisms and their role in disease induction is merely suspected rather than established.
  E.g. Alternaric acid (*Alternaria* spp.), Cochliobolin (*Dreschlera*/*Cochliobolus*)

On the basis of specificity to host, **Scheffer, 1983** classified toxins into host selective/specific toxin and non-host selective/non-specific toxins.

Host-selective toxins (HSTs) are toxic only to the host plants of the pathogen that produces the toxin. In contrast, non-specific toxins can affect a broad spectrum of plants regardless of whether they are a host or non-host of the pathogen. Yoder (1980) classified toxins of plant pathogens as a pathogenicity factor or a virulence factor by considering the possible involvement of toxins in pathogenesis: pathogenicity is the ability to cause disease (a qualitative term), whereas virulence refers to the extent or severity of the disease caused (a quantitative term).

Host Selective Toxins (HSTs)

Most HSTs are considered to be pathogenicity factors, which the pathogen producing them requires to invade tissue and induce disease. All isolates of the pathogen that produce an HST are pathogenic to a specific host and all the isolates that fail to produce HSTs lose pathogenicity to the host plants. Plants that are susceptible to the pathogen are sensitive to the toxin. Such correlations between HST production and pathogenicity in pathogens and between toxin sensitivity and disease susceptibility in plants provide persuasive evidence that HSTs can be responsible for host-selective infection and disease development.

The HSTs known to date are limited to fewer than 20 pathogenic fungi and most are low molecular weight secondary metabolites. It can be stated that, all the symptoms of a given disease result from the direct action of a toxic product of the pathogen in that disease. Tenets of

this theory can be tested by the following criteria: (a) the toxin, applied at concentrations which could be reasonably expected in or around the diseased plant, produces in a susceptible host all the symptoms characteristic of the disease, (b) the pathogen and the toxin exhibit similar host specificity, (c) the ability of the pathogen to produce the toxin varies directly with its ability to cause disease and (d) a single toxin is involved. The first toxin isolated that fulfilled the above criteria was that produced by *Cochliobolus* (*Helminthosporium*) *victoriae*.

Helminthosporium leaf blight of oats appeared in 1945 with the introduction and widespread use of the oat variety 'Victoria' and its derivatives which contained the *Vb* gene for resistance to the crown rust disease. In the Victoria lines, the pathogen infects basal portions of plants and eventually the fungus elaborates a toxin that is translocated to the leaves, causing severe leaf blight and death of the plant. All other oat varieties and other plant species are either immune or sensitivity to the toxin was proportional to their susceptibility to the pathogen. In addition to the toxin producing external symptoms identical to that induced by the pathogen, internal biochemical and histochemical induced by the toxin are identical to that induced by the pathogen. Chemically, the toxin is a complex chlorinated cyclic pentapeptide. Ultrastructural studies demonstrated the primary target of the toxin is the plasma membrane. T-toxin is another example of a host-specific toxin. The toxin is produced in common corn disease 'Southern Corn Leaf Blight' incited by the pathogen, *Cochliobolus heterostrophus* formerly known as *Helminthosporium maidis*. T-toxin is produced by race T of the pathogen that first appeared in the United States in 1968. By 1970, severe losses occurred throughout the corn belt that carried the Texas male-sterile (Tms) cytoplasm. Corn varieties with normal cytoplasm were found to be resistant to the pathogen and also to the toxin. The toxin, chemically, is a mixture of linear, long polyketols with 35 to 45 carbon atoms. The toxin specifically affects mitochondria of susceptible cells where ATP synthesis is inhibited. The Hc-Toxin, another example of a host-specific toxin produced by *Cochliobolus* (*Helminthosporium*) *carbonum* which incites a leaf spot disease in corn. The toxin is specific to only certain maize (corn) lines.

Non-host specific (non-selective) toxins

The exact roles of nonspecific toxins in pathogenesis are largely unknown, but some are thought to contribute to features of virulence, such as symptom development and *in planta* pathogen propagation. The following are the important and characterized non-host specific toxins:

Tabtoxin [*Pseudomonas syringae* pv. *tabaci*]

- Wide host range including beans, soybean, oat, maize.
- Cause necrotic spots on leaves surrounded by yellow halo.
- Dipeptide composed of amino acid threonine and tabtoxinine.
- The toxin as such is not toxic but in the cell it gets hydrolyzed and release amino acid, tabtoxinine which is toxic.
- Inhibits/inactivates the enzyme glutamine synthetase.

- Uncouples phosphorylation and photorespiration.
- Destroy thylakoid membrane thus causing chlorosis and necrosis.

Tentoxin [*Alternaria alternata*]

- Causes leaf spots and chlorosis.
- Cyclic tetrapeptide.
- Binds and inactivate the protein (chloroplast-coupling factor) involved in energy transfer into chloroplast.
- Also inhibits phosphorylation of ADP to ATP, leading to disruption of chlorophyll synthesis.

Pyricularin [*Pyricularia oryzae*]

- Exist as α-picolinic acid and pyricularin.
- Toxic to conidial germination, however, the fungus produces a pyricualrin binding protein (copper oxidase).
- Affect respiration and growth at low concentration.

Fusarial toxins

- Marticin: Produced by *Fusarium oxysporum* f sp. *pisi* – pea wilt; have nepthazarin that are red pigmented compounds.
- Fusric acid: Vivotoxin, produced by many species: *Fusarium oxysporum* f. sp. *batatis (*sweet potato); *cubense* (banana); *lini* (Linseed); *lycopersici (*Tomato); *vasinfectum* (Cotton)
- Lycomarasmin*: Fusarium oxysporum* f. sp. *lycopersici.*

ENZYMES

Enzymes like cutinases, cellulases, pectinases and lignases are often secreted by pathogenic organisms. Fungi, nematodes and bacteria are all known to produce one or more of the above enzymes in specific pathogen-host combinations. Pathogenic organisms either continually secrete enzymes or upon contact with the host plant. Enzymes are of two types:

(1) **Constitutive**: Present all time in a cell.

(2) **Inducible** : Produced only when it is needed by the cells in response to internal or external stimuli.

Plant pathogens employ an array of enzymes to breach the physical barriers offered by the plants. The cuticle is comprised of a complex wax, cutin, which impregnates the cellulose wall. The cell wall is comprised of cellulose, which makes up the structural framework of the wall, along with other matrix molecules hemicellulose, glycoproteins, pectin and lignin. The commonly secreted enzymes by the pathogenic organisms include: cutinases, pectinases, cellulases, hemicellulases, ligninases, lipases and proteinases.

Cutinases: It degrades the cutin on the cuticle layer pre-softening the tissue for mechanical penetration or as a first step in tissue degradation. Evidence indicates that, cutinases are

continually produced, albeit in low concentrations, with degradation products often inducing even higher levels of cutinase secretion.

Pectin degrading enzymes: Pectic substances comprise the middle lamella and also form an amorphous gel between the cellulose microfibrils in the primary cell wall. Pectin degrading enzymes often termed pectinases or pectolytic enzymes include pectin methyl esterases (PME), polygalacturonases (PG) and pectin lyases or transeliminases. Pectin methyl esterases remove small groups such as methyl groups (CH_3) often altering solubility and thus affecting the rate of chain splitting by polygalacaturonase and pectin lyase. Polygalacturonases split chains by adding a molecule of water, while pectin lyases split chains by removing a water molecule from the linkage. Pectin degrading enzymes are involved in a wide range of plant diseases particularly in the soft rot diseases.

Cellulases: Cellulose is the major framework molecule of the plant cell wall existing as microfibrils with matrix molecules (glycoproteins, hemicelluloses, pectins, lignins) filling the spaces between the microfibrils and cellulose chains. Cellulolytic enzymes play a role in softening and disintegration of cell walls. The cellulolytic enzymes participate indirectly in disease development by releasing soluble sugars that may be used as nutrients by pathogens and also may be involved in the release of materials in the vascular system interfering with transport or translocation of water.

Hemicellulases: Hemicelluloses are complex polysaccharide polymers that link the ends of pectic compounds to cellulose microfibrils. Since hemicelluloses are such a diverse group of polymers such as xyloglucans, glucomannans, glactomannans, arabinoglucans, etc., several hemicellulases have been identified in many plant pathogenic fungi.

Ligninases: Lignin is a phenylpropanoid which is found in the middle lamella and secondary cell wall of plants. Moreover, lignin confers the tough, woody nature to woody tissues. Most lignin degradation is by basidiomycetes known as white-rot fungi. These fungi produce ligninases that enable the fungi to utilize lignin.

GROWTH REGULATORS

Growth regulators regulate cell division, cell elongation and cell differentiation. Plant growth is controlled by a group of naturally occurring compounds with hormonal action that is often referred to as plant growth regulators/plant growth hormones. The major groups of plant growth regulators are the auxins, gibberellins, cytokinins and ethylene. Plant growth regulators act is very small concentrations, with slight changes in the normal level resulting in profound changes in the growth pattern of the plant. Pathogens often cause an imbalance in the hormonal system by causing the infected plant to produce more or less hormone, or in some cases the pathogen itself elaborates hormone thus changing the hormone level. Some of the commonly observed

symptoms related to effects on plant growth regulation are: stunting, overgrowths, galling, root branching, adventitious root formation, defoliation, rosetting, leaf epinasty, etc.

Auxin: Auxins are the major growth regulator in plants and are defined as growth regulators whose major mode of action is cell elongation. They resemble indole-3-acetic acid in their activity. The naturally occurring auxin is indole acetic acid (IAA). Increased levels of auxin are observed in plants infected with fungi, bacteria, viruses, molicutes and nematodes. Examples of important diseases directly related to altered auxin levels include; corn smut (*Ustilago maydis*), clubroot of crucifers (*Plasmodiophora brassicae*), Southern bacterial wilt (*Ralstonia solanacearum*) and crown gall (*Agrobacterium tumefaciens*).

Gibberellin: In Japan, an aberration in growth pattern became known as Bakanae (`foolish seedling disease') disease of rice. In 1926, Kurosawa, a Japanese scientist discovered that the disease was caused by, *Gibberella fujikuroi*. Later, the Japanese scientists determined that the pathogen produced hyperelongation of rice stems by secreting a chemical, which was given the name gibberellin. Gibberellins are normal constituents of green plants and also produced by several other microorganisms. The best known gibberellin is gibberellic acid. Spraying of diseased plants with gibberellin overcomes some of the symptoms (stunting) caused by several pathogens indicating that gibberellin involves in disease development.

Cytokinin: Cytokinins are necessary for cell growth and differentiation.

- Inhibit breakdown of proteins and nucleic acids, thereby causing the inhibition of senescence.
- Capacity to direct the flow of amino acids and other nutrients through the plant toward the point of high cytokinin concentration.
- Cytokinins occur in very small concentrations in green plants, in seeds and in the sap stream.
- Several cytokinins, e.g., zeatin and isopentenyl adenosine (IPA), have been isolated from plants.
- Cytokinin activity increases in clubroot galls, in crown galls, in smut and rust galls, in rust-infected bean leaves, bacterial galls and witche’s broom diseases.

Ethylene: Ethylene production in infected tissues can be dramatically induced. This induction is largely dependent on activation of the ethylene biosynthetic pathway in plant tissues. Genes encoding several key enzymes involved in the ethylene biosynthesis are highly activated at the transcriptional level. Ethylene has been considered as a signal in plant for wounding and senesces responses. Recent studies show that ethylene together with another signal component jasmonic acid may play an essential role in plant defense responses in several pathosystems.

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PLANT RESISTANCE TO INSECTS

M Balaji Rajkumar

HOST PLANT RESISTANCE (HPR)

Insects are the largest class (57%) in the animal kingdom and number of known species of insects is much more than all the other species together. Apart from their smaller size, insects have acquired much structural, developmental and behaviouristic perfection not found in any other group of organism. The insects are highly diverse group found as pests of crops, animals, public health, natural enemies and beneficial insects. As for as agricultural pests are concern, the production loss due to insect pests was estimated between 5-30 per cent. The yield loss major in
Evolution of plants and insects are believed to be together and they coexisted

Definitions

"*Relative amount of heritable qualities that influence the ultimate degree of damage done by the insect*".(Painter, R.H., 1951). In practical agriculture, resistance represents the ability of a certain variety to produce a larger crop of good quality than do ordinary varieties at the same level of insect population.

Kogan (1982) defined resistance to insect as, "*Inheritable property that enables a plant to inhibit the growth of insect populations or to recover from injury caused by populations that were not inhibited to grow. Inhibition of population growth generally derives from the biochemical and morphological characteristics of a plant which affect the behaviour or the metabolism of insects so as to reduce the relative degree of the damage these insects can potentially cause"*.

CHARACTERIZATION OF RESISTANCE

Resistance is characterised based on following parameters *viz*., Intensity, Ecological, Evolutionary, Trophic level, Crop stage, Screening conditions, Genetic nature (Number of gene/Major minor gene/biotype reaction/population concept) and range of resistance.

INTENSITY OF RESISTANCE

Painter (1951) used following scale to define the degree of resistance based on intensity. The following terms are relevant to express resistance vis-a-vis screening of varieties under field condition.

Immunity – An immune variety is one which a specific insect never injure or consume under any known condition (Cultivars immune to the attack of specific insects which are otherwise known to attack cultivar of the same species.)

High resistance – A plant possess qualities resulting in small damage by a specific insect under a given set of condition

Low resistance – A low level of resistance indicates the possession of qualities which cause a variety to show lesser damage or infestation by an insect than the average.

Susceptibility – A susceptible variety is one which shows average or more than average damage caused by an insect

High susceptibility – A variety shows high susceptibility when much more than average damage is done by the insect under consideration

An intermediate level of resistance sometime referred as **moderate resistance.**

ECOLOGICAL RESISTANCE

It is also known as *Pseudo resistance* (Painter 1951)**.** Sometimes the plant variety may be classified as resistant due to transitory characters during the unfavourable environmental conditions for insect pests and the plant is potentially susceptible. There are no heritable characters involved. It may be classified into three categories.

Host evasion - Host plants may pass through the most susceptible stage quickly or at a time when insects are less. Some plants evade injury by early maturing.

Induced Resistance – This term may be used when, there is an increase in resistance temporarily as a result of some changed conditions of plants or environment such as change in the amount of water or nutrient status of soil.

Host Escape – It refers to the absence of infestation or injury to host plant because of transitory circumstances such as incomplete infestation. This uninfected plant is cannot be taken as resistance, as it is one among the susceptible population, and their progenies needs to be tested for true expression of resistance. Host evasion pertains to whole population of the host, But host escape related to one or few individuals in the presence of insect pests.

EVOLUTIONARY CONCEPT

Resistance to insect pests is evolved either due to long host plant and insect association at the gene centres or due to pleiotropic effect of genes which are present as a result of a selective forces unrelated the insect. Based on these factors, it can be divided into types.

Sympatric resistance - It may be defined as those heritable qualities possessed by an organism which influence the ultimate degree of damage done by the insect pests having prior continuous co evolutionary history with that species of organism. This resistance evolves at the original home of the plants and insects. It is a natural selection for resistance in plants. It is a result of gene-for-gene nature of coevolution of plants and herbivores.

Allopatric resistance - It may be defined as those heritable qualities possessed by an organism which influence the ultimate degree of damage done by the insect pests having no prior continuous

co evolutionary history with that species of organism. The resistance evolved in the host plant in the absence of insects and it is unrelated to insects. It may not be the result of coevolution, but rather due to fortuitous, pleiotropic effect of genes as a result of selective forces unrelated to the insect.

TROPHIC LEVELS

In an ecosystem, interaction among the organism lead to effective defence or attack at each trophic level. The interactions may be positive & negative effects or sometime no effect.

Intrinsic resistance – The plant produces defence on its own through physical means or through production of toxins/chemicals or even both. It happens between first and second trophic levels.
Extrinsic resistance – The natural enemies of the pests benefit the host plants by reducing the pest population. It occurs in third Trophic level.

CROP STAGE

Depending on the different crop growth stages, the resistance may be divided in to two types viz., Seedling resistance and adult plant resistance. The seedling resistance measured at the seedling stage of the crop, it is also known as Juvenile resistance. Adult plant resistance is referred to the plant susceptible at its seedling stage and shows resistance when matured or adult plant. Based on screening conditions the resistance may be characterised to green house resistance and field resistance.

GENETIC RESISTANCE

Based on number and type of genes

Base on number of genes, the resistance may be due to monogenic oligogenic, polygenic control. The major gene resistance may be due to single or few genes and it is easy to incorporate or break. The minor gene resistance is the cumulative effect of several genes and it is also known as adult resistance or mature resistance or field resistance.

Based on biotype reaction

The resistance against biotypes is classified into vertical and horizontal resistance. The vertical resistance referred to the host plant showing resistance against specific biotypes. It is also known as specific resistance. The horizontal resistance referred to the host plant resistant against all the known biotypes. It is non-specific, quantitative and controlled by number of minor genes.

Based on population/Line concept

Resistance exhibited by lines which are phenotypically and genotypically similar is called as pureline resistance. The number of lines are phenotypical similar and genotypically dissimilar are multiline resistance. The multiline resistance are generally involving vertical resistance.

MISCELLANEOUS

Other terms used in host plant resistance are Cross and multiple resistances. The variety with resistance incorporated against a primary pest, confers resistance to another insect. Multiple resistances refers to the resistance incorporated in a variety against different environmental stresses like insects, diseases, nematodes, heat, drought, cold, etc.

MECHANISMS OF RESISTANCE

There are three important mechanisms of resistance *viz*., Antixenosis (Non preference), Antibiosis and tolerance.

Antixenosis

Non preference or antixenosis refers to the response of insect to the characteristics of the host plant which make ti unattractive for feeding, oviposition or shelter. It may be due to the presence of morphological characters or chemical factor in the host plants. Antixenosis denotes the plant is considered by insect pests as undesirable or a bad host. e.g. Trichomes in cotton - resistant to whitefly, Waxy bloom on crucifer leaves - deter feeding by DBM, Plant shape and colour also play a role in non preference. It is considered as much important in case of insect vectors or key pests.

Antibiosis

Antibiosis refers to adverse effect of the host plant on the survival, development and reproduction of the insects and their progeny. All the adverse effects of permanent or temporary nature following ingestion of a plant by an insect are attributed to antibiosis. The insect feeding on resistant plant may show antibiotic symptom vary from mild to acute. The common symptoms observed are, larval death in first few instars, abnormal growth rates, disr5ubption in conversion of ingested food, failure to pupate, failure of adults to emerge from pupae, abnormal adults, inability to concentrate food reserved followed by failure to hibernate, decreased fecundity, reduction in fertility, restlessness, and abnormal behaviour. It may appear due to presence of toxic substances, absence or insufficient essential nutrients, nutrient imbalances, presence of anti-metabolites and enzymes adversely affecting digestion and utilisation of nutrients.

Chemical factors in Antibiosis - Examples

Chemicals present in plants and the pest

1. DIMBOA (Dihydroxy methyl benzoxazin) Against European corn borer, *Ostrinia nubilalis*
2. Gossypol (Polyphenol) *Helicoverpa armigera* (American bollworm)
3. Sinigrin Aphids, *Myzus persicae*
4. Cucurbitacin Cucurbit fruit flies
5. Salicylic acid Rice stem borer

Physical factors in antibiosis - Thick cuticle, glandular hairs, silica deposits, tight leaf sheath, etc.

Tolerance

Tolerance refers to the ability of host plant to withstand an insect population sufficient to damage the susceptible plants. It is generally attributable to plant vigour, re-growth of damaged tissue, produce additional branches, utilisation of non vital parts by insects and compensation by growth of neighbouring plants. However, tolerance has no adverse effect on the insect pest.

The ability of tolerant varieties to support insect infestation for longer period without loss in yield or quality and enables them to frequently escape insect damage through compensation by plants than the susceptible varieties. Tolerant variety does not induce selection pressure in insect populations. Thus it can be useful to prevent the development of insect biotypes.

Antixenosis	Antibiosis
Undesirable host	Unsuitable host
Avoidance by insect	Adverse effect on insects after feeding on the host plant

Eg: *Eruca sativa*, is not a preferred host of mustard aphid *Lipaphis erysimi* (Kaltenbach). The growth and development was slower on this host as compared to *Brassica* sp. The mechanism appeared to be antibiosis, however the poor development was due to reduced feed uptake because of presence of allelochemicals in *E.sativa* indicating antixenosis.

Use of tolerance in IPM

- Tolerant varieties have high Economic threshold level and there by reduces the requirement or number of insecticide sprays.
- Does not exert any selection pressure on pests, otherwise it would lead to the development of Biotypes (A biotype is a new population capable of feeding/damaging and surviving on plants previously reported as resistant to other population of same species.

Chemical resistance in plants

In the coevolutionary struggle, plant produces diverse chemicals/deterrents through adopting diverse metabolic pathways and insects overcoming them by diverse detoxifying mechanisms.Plants adopt chemical resistance strategy against herbivores through storing less toxic precursors, which are transformed in to toxin when needed. Natural defence systems are therefore complex forming part of an intricate ecological system. Being biologically active compounds secondary metabolites aid in competitive interaction against other plants, and act as feeding stimulants and deterrents. Such as secondary compounds as terpenoids alkaloids coumarins cyanogenic glycosides, glucosinolates sequestered in different sites such as epidermal vacuoles or in cell walls or oil glands or on leaf surface waxes are important elements in plant defence against insects. Besides morphological barriers. Common toxic elements like glycoalkaloids in potato, tomatine, rutin and chlorogeneic acid in tomato and gossypol in cotton are toxic to insects. Increased phenolic and tannin production and decreased proteins and nutritional suitability are commonly evident as a response to insect attack. Within an interval of several hours after feeding several plants release protease inhibitors, cucurbitacins in squash and tepenes in sweet potato. Allelochemicals therefore tend to occur at effective concentrations in a plant or are synthesised de novo or translocate when needed (induced). Each species of plants has its own unique bouquet of secondary compounds

HPR in IPM

a. Compatibility with chemical control

HPR enhances efficacy of insecticides by increasing the mortality of leaf hoppers and plant hoppers in resistant varieties compared to susceptible variety. Insecticide concentration required to control insects on resistant variety may be lesser than the susceptible

b. Compatibility with biological control

Resistant varieties increases the efficiency of biocontrol agents (Predator or parasitoid) ratio by interrupting the activity or make the restless leads to easy exposure to natural enemies. Predatory activity of mired bug *Cyrtorhinus lividipennis* on BPH was more on a resistant rice variety IR 36 than susceptible variety IR 8. Insects feeding on resistant varieties are more susceptible to virus disease (NPV).

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The term entomopathogenic refers to those microorganisms that are capable of attacking insects using them as hosts to develop part of their life cycle those that reduce pest insect populations to levels that do not cause economic damage. They are also defined as facultative or obligate parasites of insects, with a high capacity for sporulation and survival.

Bacillus thuringiensis (Berliner)

Bacillus thuringiensis (*Bt*) are facultative anaerobic, gram-positive, spore-forming bacteria belongs to Bacillaceae with entomopathogenic properties. *Bt* produce insecticidal proteins during the sporulation phase as parasporal crystals (protein inclusions adjacent to the endospore). High efficacy and high specificity of *B. thuringiensis* favors its success in agricultural environment and it is also a best alternative for chemical insecticides in the context of saving environment.

CRYSTAL PROTEIN

The crystalline inclusions are predominantly comprised of one or more proteins Crystal (Cry) and Cytolitic (Cyt) toxins, also called delta-endotoxins. Cry proteins are parasporal inclusion (Cry) proteins from *B. thuringiensis* that exhibit experimentally verifiable toxic effect to a target organism or have significant sequence similarity to a known Cry protein. Similarly, Cyt proteins are parasporal inclusion proteins from *Bt* that exhibits haemolytic (Cyt) activity or has obvious sequence similarity to a known Cyt protein. Techniques commonly used to characterize *Bt* strains or the ICP (Insecticidal crystal proteins) includes cell wall fatty acid analysis, monoclonal antibodies, oligonucleotide DNA probes, plasmid profiles, polymerase chain reaction (PCR) analysis, DNA fingerprinting and SDS-PAGE profiles.

APPLICATIONS OF CRY TOXINS

Three major applications of *Bt* toxins have been achieved: (i) in the control of defoliator pests in forestry, (ii) in the control of mosquitoes that are vectors of human diseases, and (iii) in the development of transgenic insect-resistant plants. Two major forms of *Bt*, one is formulations and another one is transgenic plants expressing crystal proteins

Bt formulations

Commercial *Bt* products are microbial pest control agents containing specific insecticidal crystalline proteins and most often living spores as well as formulating agents. They are processed fermentation products. The *Bt* formulation came into commercial arena during 1940's and now occupies 90 per cent of global microbial insecticides market. It proves the best alternative of chemical insecticides in the context of environmental safety. Spore counts do not accurately reflect the insecticidal activity of a *Bt* strain or *Bt* product because the number and

amount of ICPs produced per bacterial cell can vary. The potency (ITU/mg) of each *Bt* product is bioassayed using an international standard that uses a specific test insect

_Bt_ transgenic plants

The insect-resistant transgenic plants were among the first products of plant biotechnology to reach the marketplace. Recent commercial releases of genetically engineered crops have included transgenic corn, cotton and potato, which express *Bacillus thuringiensis* toxins.

In 1995, *Bt*-potato (NewLeaf™, Monsanto, St Louis, MO, USA) became the first *Bt*-crop to be commercialized. These potatoes were engineered to express the Cry3A protein, for protection against Colorado potato beetles. Insecticide use was reported to be reduced by up to 40% for *Bt*-plants in 1997. In 1996, *Bt*-cotton (Bollgard™, Monsanto) was released to protect against tobacco budworm, and to a lesser extent, cotton bollworm and pink bollworm. The transgenic *Bt*-cotton expresses the Cry1Ac protein. In 1997, *Bt*-cotton was reported to give an average yield increase of 14%, with a reduction in insecticide use of 300 000 gallons.

Several companies have developed *Bt*-maize and commercialized its use since 1996 by licensing it to seed companies, YieldGard™, NatureGard™ produce the Cry1Ab protein. In field experiments, *Bt*-maize gives 99% control of first generation European corn borer larvae.

MODE OF ACTION OF _Cry_ TOXIN

The mode of action of *Cry* toxins is a multi-step process, Ingestion, solubilisation, activation, binding and pore formation .

INGESTION

Enough toxins have to be ingested by the target insect by means of spores (formulations) or a crystal protein (transgenic plants). The toxin has to reach midgut of the insect to start infection. Then the crystal protein gets solubilised in the midgut environment.

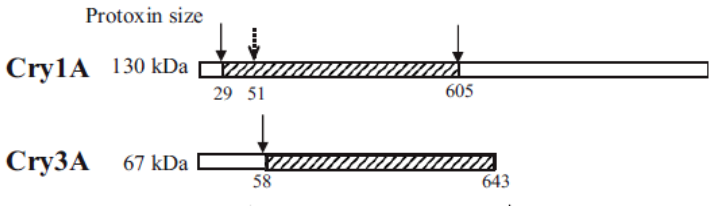
SOLUBILIZATION

The stabilization of crystal protein is maintained by the formation of disulphide bonds between the Cysteine residues. This disulphide bonds should be broken down in the midgut in a highly reductive environment with high gut pH. All crystal protein needs a specific gut pH to dissolve and release full length of protoxin. Some of the crystal protein and their gut pH requirement are given below.

***Bt* Subspecies**	**Protein**	**Soluble pH**
Bt kurstaki	Cry 1 Cry 2	9.5 12
Bt israelensis	Cry 4 A, Cry 4 B, Cyt A	9.5
	Cry 4 D	12
	Cry 1 Ac	<3.5 and >9.5

ACTIVATION

After the release of full length of protoxin, it should be activated into a toxic peptide or Trypsin resistant fragment. For example in a lepidopteran specific Cry 1 A proteins, the 130 to 140 KDa larger protoxins is activated by gut proteases like serine proteases, trypsin and chymotrypsin. These enzymes cleaved the 29 amino acids from N-terminus, 500 amino acids from C- terminus of the protoxin i.e., 130 KDa cleaved to 65-67 KDa (Fig)**.** This remaining small toxin is known as Trypsin or proteases resistant fragment. The peritrophic membrane (PM) act as a molecular sieve and it can be allow up to molecular mass of 100 KDa. The activated toxin can easily diffuse across the



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TOXIN BINDING AND MEMBRANE INTERACTION

- Toxin specificity binds to receptors present in brush border membrane of midgut epithelium.
- For Cry 1 A toxins, at least four binding proteins have been described in Lepidoptera.
    1. Cadherin receptor (CADR)
    2. Glycosylphosphatidylinositol (GPI) anchored aminopeptidases (APN)
    3. GPI anchored alkaline phosphatases (ALP)
    4. Glycolipids

ION PORE FORMATION

- ❖ In Lepidoptera the epithelial apical membrane of midgut actively transports K^+ ions there by creating relatively large membrane potential.
- ❖ This K^+ transport is sustained by electrogenic V-type *ATPase* pump that actively pumps H^+ ions to the gut lumen K^+/H^+ exchange occurs through an electroneutral antiport (Dow, 1986).
- ❖ The amino acid uptake depends on existence of the transpithelial electrical potential differences. Disruption of amino acid uptake by symport mechanism located apical border of columnar epithelial cell allows K^+ and amino acid into cytoplasm.
- ❖ Activated *Bt* toxin apparently insert in midgut membrane and increase K^+ conductance of Columnar epithelium and disruption of electrical K^+ and pH gradients. Then the toxin inhibits (dose dependent) the Na^+, K^+ gradient dependent Phenylalanine uptake in brush border membrane vesicle (BBMV) of *M. sexta* larva.
- ❖ There are three hypothesis proposed for ion pore formation are,
    i. *Bt* toxin directly inhibits plasma membrane *ATPase*
    ii. Selectively increase permeability of ions
    iii. Toxin form a nonspecific pore

The putative membrane spanning amphipathic helices located in the N terminal hydrophobhic toxic domain might be involved in pore formation. Form a non selective pore radius 0.6 – 1.0 nm is larger than the crystal radius of 0.13, 0.10, 0.14nm for K, Na, NH_4+ respectively. Both Cry and Cyt toxins may ultimately act by similar mechanism but different cell membrane components are required for the initial stages of interaction between these toxins and cell membrane. Cry toxin bind to a midgut glycoprotein receptor while unsaturated phospholipids act as a binding sites for the Cyt toxins. Ultimately toxin molecule insert into the plasma membrane to forms a pore is permeable to small ions and molecules. The pore will enlarge from osmotic swelling and eventually cell lysis will occur.

DEVELOPEMENT OF RESISTANCE

A major threat to the use of *Bt* is the appearance of insect resistance, which has been documented in the field with lepidopteran insects. The continuous exposure to cry toxin create selection pressure among the insect population leads to development of resistance. The development of pest resistance in two ways, one is lab or real world and another is in field condition. Resistance is typically observed as an increase in population LC_{50} or as enhanced growth and survival at a discriminating concentration, compared with susceptible colony.

Laboratory estimates has been done to achieve early warning of reduced larval susceptibility. Here defining the resistance is important in the case of *Bt*. Field resistance is defined as a genetically mediated increase in the ability of a target pest to feed and complete development on one or more commercial line(s) of *Bt* crops under field condition.

MECHANISMS OF RESISTANCE

The resistance mechanism located at each of the various steps involved in the mode of action. If any alterations or changes in those steps of mode of action will confer resistance to the target insect. Altered proteolytic processing and binding site modifications are the common mechanisms of resistance.

ALTERED PROTEOLYTIC ACTIVITY

Reduced activation of toxin

The activation of protoxin is reduced by lowering the activity of proteases or absence of proteases. For example, reduced conversion of protoxin to active toxin in *Plutella xylostella.* Then slower activation of protoxin to toxin and faster degradation of Cry 1 Ab protein was also observed in laboratory. Eg. *H. virescens.*

BINDING SITE MODIFICATION

Reduced binding

The receptors found in brush border membrane vesicles. In case of *Plodia interpunctella* is observed that, there has been 50 fold reduction in binding affinity with toxin. Resistance

development in the field by population of *Plutella, Pectinophora gossypiella* due to reduced binding of toxin and *CADP* alleles are associate with resistance.

Low levels of receptor

In *Spodoptera litura,* the inhibition of APN & production by dsRNA interference showed that an insect with low APN levels confers resistance to Cry 1 C toxin. *Heliothis virescens* – Resistance of YHD 2 (mutant) larva to Cry 1 Ac toxin correlated with reduced levels of 65 Kda ALP.

Slightly altered binding site

There are 4 different receptor binding sites has been identified in *P. xylostella* that, Site 1 is a binding site for Cry 1Aa, likewise Site 2 for Cry 1Ab, Cry 1Ac, Cry 1F, Cry 1J, Site 3 for Cry 1B and Site 4 for Cry 1C. If any alteration in single site confers resistance and the sites recognized by more than one toxin will confer cross resistance. In *Heliothis virescens* the epitope binds loop-3 of Cry 1 Ac domain II & that a single nucleotide change in this region results in loss of binding.

Increased cell repair or cell replacement

In case of Cry 1Ac ingested as a sub lethal dose by 4th instar of *H.virescens* it can be able to repair or replace damaged midgut cells more efficiently. The other mechanisms also observed are increased detoxification, increased non specific binding of toxin. Cadherin gene responsible for 40-80% of Cry1Ac resistance.

SPECIES RESISTANCE TO *Bt* FORMULATIONS - EXAMPLES

Species	**Type**	**Environment**	**Toxin**	**Mechanism**
*Plodia interpunctella (*Mc Gaughey, 1985)	Dipel	Storage bin/ lab	*Cry* 1Ab	Reduced binding
Plutelll xylostella (Tabshnik, 1990)	*Bt Kurstaki Bt aizwai*	Field	*Cry* 1 Ab	Reduced binding
		Field	*Cry* 1 Aa Cry 1 Ac	Reduced binding
			Cry 1 Ca	Altered binding site
Heliothis virescens	Dipel	Lab	*Cry* 1 Ab	Increased detoxification and altered binding site
			Cry 1 Ac *Cry* 1 Ca	Increased cell repair Non specific binding

FIELD RESISTANCE AGAINST *BT* TRANSGENICS

Field resistance occurs in an isolated population of different locations

SPECIES	**ENVIRONMENT**	**TOXIN**
Helicoverpa zea	Field – USA (Mississipi) – *Bt* cotton	Cry 1 Ac

Spodoptera frugiperda	Field- Puerto Rico - *Bt* corn	Cry 1 F
Busseola fusca	Field – South Africa – *Bt* corn	Cry 1 Ab
Pectinophora gossypiella	Field – Gujarat, India – *Bt* cotton Reported by Monsanto India.	Cry 1 Ac

FITNESS COST (FC)

The fitness cost can be defined as, a trade off in which alleles conferring higher fitness in one environment (eg. Presence of *Bt*) reduce fitness in an alternative environment (eg. Absence of *Bt*). In *H. armigera the* resistance population had decreased fitness and took longer time to complete development in untreated diet (non Bt) than susceptible one. FC can incorporate into model to predict evolution of resistance. If FC increases the delay of resistance increases. If pest resistance to one *Cry* protein is may be more susceptible to other *Cry* protein.

Non-toxic plants in resistance management

This strategy provides non-toxin bearing plants to maintain susceptible insects within the field in seed mixtures or external to the transgenic crop in refuges. Seed mixtures once seemed attractive because they required little effort on the part of growers to implement resistance management. They were also expected to favor random mating between susceptible and resistant insects. Subsequent studies have shown, however, that for some pests, interplant movement by larvae would render this strategy less effective. Larvae may actively avoid feeding on *Bt* plants or larvae developing on non-*Bt* plants may move to toxic plants and die, thus reducing the effective size of the refuge.

In field tests using the diamondback moth and transgenic broccoli, our group found that unsprayed refuges external to the *Bt* crop were more effective for conserving susceptible alleles and reducing the overall number of resistant insects on transgenic plants compared to mixed or sprayed refuges. However, care must be taken to ensure the insects in the refuge are managed from both an economic and IRM standpoint. Managing insects in the refuge, while conserving *Bt* susceptible alleles, continues to be a challenge. Evaluating the potential effectiveness of IRM strategies is made especially difficult by the limited ability of researchers to run empirical tests.
Most resistant strains are unable to survive on *Bt* hosts, and field tests using resistant insects raise concerns about creating a wider resistance problem. One exception to this rule is the use of *Bt* broccoli and resistant diamondback moth strains in regions where the insect does not overwinter. This system has been used extensively by our laboratory to evaluate IRM concepts and strategies, although it is limited by the inability to conduct landscape-sized, long-term trials. For the most part, the development of current IRM strategies has relied largely on computer simulations. When *Bt* crops were first commercialized in 1996, most experts agreed that the best strategy available at the time was a combination of high expression and a refuge to conserve susceptible alleles.

The cry toxins are highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable. Therefore, *Bt* is a viable alternative for the control of insect pests in agriculture and of important human disease vectors. The identification of receptor and binding epitopes in the toxins will help in the development of novel toxins and strategies to deal resistance. Needs to develop resistance management strategies appropriate for Indian conditions specific to crop and location before commercialization of any *Bt*-transgenic crops to delay resistance development. Monitoring the development of resistance and studies on the susceptibility of target pests before and after commercialization for longer period. Transgenic plants are one of the components of IPM in managing insect pests. So we have to incorporate all other feasible agronomic practices to support the plants against pests. Especially at the later stage when the toxin expression will be less. Indiscriminate use of *Bt* transgenics with single gene Cry 1 Ac will leads to rapid development of resistance. Transgenic plants with new novel toxins should be developed to combat resistance development correlates with possible time taken for resistance development by using prediction models. Treating *Bt* crops as a silver bullet for pest management will almost certainly hasten the evolution of resistance, incorporation of transgenic crops with traditional, integrated approaches to pest management should help ensure their long-term sustainability and maximize their environmental and human health benefits.

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MOLECULAR TECHNIQUES FOR DETECTION OF PLANT VIRUSES

A I Bhat

1. Serological (Immunological) methods

Serological methods are successful in the detection of viruses and a few bacteria. The method is not generally used for the detection of fungi due to cross reactions seen among different species. The reaction between antibodies and antigen paved the way for the detection of plant pathogens especially viruses and these techniques are referred to as serological techniques. The primary requirement of serological method is the production of pathogen specific antiserum. Antisera are produced by immunizing animals with a highly purified virus or viral coat protein. The coat protein of a virus that is cloned and expressed in *Escherichia coli* can also be used as source of antigen. Although various animal species may be used to raise the antiserum, rabbits are usually preferred animal for most applications. Purified viral antigens at a concentration of 0.5-1.0 mg are usually administered in complete Freund's adjuvant, followed by subsequent immunization in complete adjuvant. Good-quality antisera of adequate titer can be obtained by injecting animals with as little as 50-100 pg of nucleoprotein with some viruses. The antigen is usually introduced intramuscularly or intravenously into the animal. The production of high-titered antiserum depends on the amount of virus injected and viral antigenicity. The number of injections will also affect the specificity of antiserum (Bhat and Maheshwari, 2017). A variety of serological methods have been developed for detection of pathogens. The traditional methods involved direct observation of specific precipitates of virus and antibody, either in liquid media or in agar gels. Over about the past 30 years these methods have been progressively superseded by the use of enzyme-linked immunosorbent assay (ELISA), immunosorbent electron microscopy and dot immunobinding assay. Some of the important methods currently used in the serological detection methods include, Enzyme linked immunosorbent assay (ELISA), Dot Immunobinding Assay (DIBA) or Dot-ELISA Assay, Tissue blot immunoassay (TIBA), Electro-blot immunoassay (EBIA) and Lateral flow technique

2. Polymerase Chain Reaction (PCR) based methods

Polymerase chain reaction (PCR) is the most sensitive method (10^2-10^5 times more than ELISA) available presently for detection of pathogens. It is important when pathogens occur at low concentration. It also has the potential to detect more than one pathogen in one reaction and diagnosis is amenable to automation. PCR technique involves the specific amplification of the target DNA fragment enzymatically under *in vitro* conditions. The method involves the hybridization of synthetic complementary oligonucleotide primers to the target sequence and synthesis of multiple copies of cDNA of the sequence between primers using heat stable DNA polymerase. Each amplification cycle consists of melting the double stranded template DNA at high temperature, annealing (hybridization) of the primers with complementary sequences in the template DNA at low temperature, extension of the primers with DNA polymerase (DNA synthesis). During each cycle, the sequence between the primers is doubled. A normal PCR

involves 30-50 such amplification cycles (Mullis *et al*., 1986; Saiki *et al*., 1988). As PCR is based on DNA, it is not directly applicable to most plant viruses that have RNA genomes. However, a complementary DNA (cDNA) can be made to the desired region of the RNA using a primer and reverse transcriptase, and this used as the initial template. This procedure now widely used, is termed as RT-PCR (Reverse Transcription PCR). PCR and RT-PCR has proved to be a very powerful tool for pathogen detection and diagnosis. Several variants of PCR named as IC-PCR, IC-RT-PCR, ITS-PCR, ARDA-PCR, nested PCR, multiplex PCR and mRT-PCR are available.

2.1. PCR for detection of pathogens

In addition to fungal, nematode, bacterial and phytoplasmal pathogens, plant viruses containing single or double stranded DNA as their genomes in the genera such as *Badnavirus, Begomovirus, Caulimovirus, Curtovirus, Mastrivirus, Nanovirus,* can be detected by PCR using DNA isolated from these pathogens or infected plants as template. ITS-PCR, RAPD or SCAR are the commonly used PCR methods for the detection of fungal pathogens while PCR based on rDNA is commonly used in the detection of bacterial, nematode and phytoplasma. The important steps in the PCR: (i) extraction of total DNA from infected plant or from pathogen; (ii) synthesis of two virus specific primers; (iii) set up the PCR reaction in a vial by adding extracted nucleic acid, primers, nucleotides, magnesium chloride and *Taq* polymerase. Use nucleic acid extracted from a known infected and healthy plants to serve as positive and negative controls respectively; (iii) keep the vials in the PCR machine and start the run as per the pre-decided program and (iv) identify positive reactions by running contents of PCR on the agarose gel. The presence of bands at the expected position indicate that sample under test is positive (Henson and French, 1993).

2.2 Reverse transcription (RT) PCR for detection of RNA viruses

RT-PCR is a method to amplify complementary DNA (cDNA) copies of RNA. In this, the first step is the enzymatic conversion of RNA to a single stranded cDNA template. An oligo deoxynucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase (Reverse transcriptase) to create cDNA copy that can be amplified by PCR. Either RNA isolated from purified viral preparations or total RNA isolated from infected plant or dsRNA isolated from infected plant can be used as template to initiate cDNA synthesis. The primer for first strand cDNA synthesis can be specifically designed to hybridize to a particular target gene or it can be an oligo (dT) (for viruses containing Poly (A) tail at their 3' ends). Amplification of the desired portion of cDNA can be achieved in PCRs primed by sense and antisense oligonucleotide primers corresponding to specific sequences in particular cDNAs. For maximum specificity, the antisense primer should be located upstream of the oligonucleotide used to prime cDNA synthesis. Positive and negative controls should always be included when setting up RT-PCRs (Hadidi *et al.*, 1995).

3. Real-time PCR

The process of quantifying target DNA has been simplified considerably with the advent of real time PCR. This method avoids the usual need for post reaction processing as the amplified

products are detected by an in built fluorimeter as they accumulate. This is done by using fluorescent probes or non specific DNA binding dyes that are specific to the target DNA. The principle underlying real time PCR is that the larger the amount of target DNA present in the sample being tested, the quicker the reaction process and enters the exponential phase of amplification. The quantity of PCR amplicon produced at each cycle is measured using the fluorescent dyes or probes. The cycle threshold (Ct) is calculated for each sample tested. This is the cycle number at which a statistically significant increase in fluorescence is detected. The Ct increases with the decreasing amount of target DNA. A calibration curve relating to Ct to know amounts of target DNA is constructed and used to quantify the amount of initial target DNA in an unknown sample. In real time PCR, TaqMan oligonucleotide probes which contain a fluorescent reported dye with a quencher dye in close proximity to reduce the fluorescent signal is used. During the PCR process, the fluoregenic probe binds to its target sequence, within the DNA fragment being amplified. Other types of fluorescent probes and primers are also used in real time PCR. It is possible to several targets simultaneously by using probes with different fluorescent reporter dyes. Such assay can be used to quantify different organisms. Real time PCR assay has been used to detect several plant pathogens including virus, bacteria, fungi and nematodes infecting different crops (Roberts *et al*., 2000; Boonham *et al.,* 2002; Lievens *et al*., 2006; Fanelli *et al*., 2007; Yan *et al.* , 2008; Bhat and Siljo, 2014).

4.0 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a single tube technique for the amplification of DNA. It may be combined with a reverse transcription step to allow the detection of RNA. LAMP is a novel approach to nucleic acid amplification which uses a single temperature incubation thereby obviating the need for expensive thermal cyclers. Detection of amplification product can be by photometry for turbidity caused by increasing quantity of magnesium pyrophosphate in solution or with addition of SYBR green, a color change can be seen without equipment (Notomi *et al*., 2000; Mori *et al*., 2001; Nagamine *et al*., 2002). Also in-tube detection of DNA amplification is possible using manganese loaded calcein which starts fluorescing upon complexation of manganese by pyrophosphate during *in vitro* DNA synthesis. LAMP has the potential to be used as a simple screening assay in the field. Due to its simplicity, ruggedness, and low cost, LAMP could provide major advantages compared to PCR without compromising on the sensitivity. In LAMP, the target sequence is amplified at a constant temperature of 65 °C using either two or three sets of primers and a polymerase with high strand displacement activity. Due to the specific nature of the action of these primers, the amount of DNA produced in LAMP is considerably higher than PCR based amplification. Hence, LAMP can also be quantitative. Real-time detection is also possible by running LAMP reaction on a real time LAMP machine (example- Genie II from Optigene, UK) by measuring fluorescence emitted by SYBR green or other dyes on real time basis. LAMP has been successfully used for the detection several fungal, bacterial and viral pathogens of plants (Notomi *et al*., 2000; Mori *et al*., 2001; Nagamine *et al*., 2002; Fukuta *et al*., 2003; 2004; Nie, 2005; Tomlinson *et al.*, 2010; Bhat *et al*., 2013; Siljo and Bhat, 2014) .

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POLYMERASE CHAIN REACTION FOR DETECTION OF PLANT VIRUSES

A I Bhat and Anju Mohandas

Polymerase Chain Reaction (PCR) is a technique used to amplify a segment of DNA that lies between two regions of known sequences. It was invented by Kary Mullis during 1983. It is a method by which any number of copies (several millions) of any specific piece of DNA can be made within a few hours in the laboratory.

When any cell divides, enzymes called polymerases make a copy of all the DNA in the genome. The first step in this process is to "unzip" the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template. To copy DNA, polymerase requires two other components: four nucleotide bases and primer. DNA polymerases cannot copy a chain of DNA without a short sequence of nucleotides to "prime" the process, or get it started. So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer. Once the primer is made, the polymerase can take over making the rest of the new chain.

PCR mimics the above process in a small vial in the laboratory. A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, large quantities of the primers (primers must be duplicates of nucleotide sequences on either side of the piece of DNA of interest and sequence of nucleotides of a primer must be already known) and DNA polymerase. The automation of PCR process was possible because of the use of polymerase named *Taq* polymerase. *Taq* is a nickname for *Thermus aquaticus*, a bacterium that lives in hot springs. Unlike other polymerases, the polymerase extracted from *Taq* is stable in the fluctuating temperatures.

1. Steps in PCR

There are three basic steps in PCR. The first step separates the two DNA chains in the double helix called as melting of DNA (Denaturation). This is done by heating the vial containing PCR components to 90–95^{o}C for about 30 seconds. The second step is hybridization or annealing of the primers to their complementary bases on the now single stranded DNA (Primer extension). This is achieved by cooling the vial to 45–65^{o}C (depending on the melting temperature of the primers) for about 30 seconds. The final step is to make a complete copy of the template called as extension. During this the temperature in the vial is raised to 72^{o}C as *Taq* polymerase works best at this temperature. The *Taq* polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. This completes one cycle of PCR, during which each piece of DNA in the vial gets duplicated. Each newly synthesized DNA piece can now act as a new template for the subsequent cycles, so after 30 cycles, several millions copies of a single piece of DNA can be produced. Instrument called as thermocycler (PCR Machine) is used to automatically control the required temperature for each of the three steps.

2. Components of PCR

In order to perform PCR, the following components are essential:

2. 1. Oligonucleotide primer pair

In order to synthesize primers, sequence of at least a portion of the DNA molecule that one wishes to amplify should be known. Primers consist short oligonucleotides (containing about two dozen nucleotides) that are precisely complementary to the sequence at the 3' end of each strand of the DNA to be amplified. Primers are synthesized on an automated DNA synthesizer. Careful design of primers is required to obtain the desired products in high yield.

2.2 Thermostable DNA polymerase

Taq DNA polymerase is most commonly used enzyme, although, many other thermostable enzymes are now available.

2.3. Deoxynucleoside triphosphates (dNTPs)

Standard PCRs contain equimolar amounts of dATP, dTTP, dCTP and dGTP. Concentrations of 200-250 µM of each dNTP are recommended per each reaction.

2.4 Divalent cations:

All thermostable DNA polymerases require free divalent cat ions such as Mg+2 for activity. The optimal concentration of Mg+2 must be determined for each combinations of primers and template. Concentration would vary from 1.5 mM to 4.5 mM depending on the primers and template.

2.5 Buffer

Tris-Cl, adjusted to a pH between 8.3 to 8.8 at room temperature is included in standard PCRs at a concentration of 10 mM.

2.6 Template DNA

Template DNA can be in single- or double stranded form. Closed circular DNA templates are amplified slightly less efficiently than linear DNAs.

3. Protocol for PCR amplification of DNA viruses

The important steps in the PCR for plant viruses containing either single- or double stranded DNA as their genomes include: (i) extraction of total DNA from virus infected plant (ii) synthesis of two virus specific primers; (iii) set up the PCR reaction in a vial by adding extracted nucleic acid, primers, nucleotides, magnesium chloride and *Taq* polymerase. Use nucleic acid extracted from a known infected and healthy plants to serve as positive and negative controls respectively; (iii) keep the vials in the PCR machine and start the run as per the pre-decided program and (iv) identify positive reactions by running contents of PCR on the agarose gel. The presence of bands at the expected position indicate that sample under test is positive (see Figure).

3.1 Requirements

3.1.1 Materials

Thermal cycler, Microcentrifuge, Micropipette and tips, Eppendorf tubes, Thin walled PCR tubes

Ice flaking machine and ice, Agarose , Microwave, Gel apparatus with power pack, Transilluminator

3.1.2 Reagents

Template DNA, 10x PCR buffer, $MgCl_2$, dNTP mix, Primers, *Taq* DNA polymerase, TAE buffer Ethidium bromide, Gel loading dye

3.2 Protocol

3.2.1. In a sterile 0.2 ml thin walled PCR tube, add the following

10x PCR buffer	5.0 µl
10 mM dNTP mix	1.0 µl
100 ng/µl Forward Primer	0.5µl
100 ng/µl ReversePrimer	0.5µl
Taq DNA polymerase (3Units/µl)	0.5 µl
Sterile water	32.5 -37.5 µl
Template DNA	5-10 µl
Total volume	50µl

Also, set up similar reaction using template DNA from a known template (positive control) and another reaction without any template (negative control).

3.2.2. Amplify the nucleic acid using the denaturation, annealing and extension times, and temperatures required for each of the steps. Times and temperatures needed to be set up depend on the primer annealing temperature and the length of fragment being amplified. Extension should be carried out for 1 min for every 1000 bp of length of fragment being amplified. Denaturing and extension are usually carried out at 94^{o}C and 72^{o}C respectively. Temperature for annealing of primers should be either Tm or Tm-2^{o}C of the primers.

3.2.3. Run about 10-15 µl of the reaction mixture in 1% agarose gel for 1 h. through electrophoresis. Include DNA markers of appropriate size.

3.2.4. Visualize the DNA by placing the gel on a transilluminator.

3.4 Anticipated Results

A successful amplification reaction should yield a readily visible DNA fragment of expected size. The gel containing samples of positive controls and template DNA under test should contain a prominent band of DNA of appropriate molecular weight. This band should be absent from the lanes containing samples of negative controls.

ISOLATION OF TOTAL RNA FROM PLANTS

D Prasath, R Karthika and M Snigdha

Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments including northern analysis, nuclease protection assays, RT-PCR, RNA mapping, in vitro translation and cDNA library construction. To be successful, however, the RNA isolation procedure should include some important steps both before and after the actual RNA purification.

When working with RNA, care must be taken to create a ribonuclease-free environment.
Research into optimizing RNA analysis has identified two points in the RNA isolation process that can be improved; treatment and handling of tissue or cells prior to RNA isolation and storage of the isolated RNA. Since most of the actual RNA isolation procedure takes place in the presence of strong denaturants (e.g. GITC, LiCl, SDS, phenol) that render RNases inactive. It is typically prior to, and after the isolation, when RNA integrity is at risk.

Reagents required

- Liquid nitrogen
- Extraction buffer
- Acid Phenol :Chloroform
- Plant RNA isolation aid (ambion)
- β-mercaptoethanol
- RNA Later
- Cold isopropanol
- Cold absolute alcohol
- 75% ethanol
- RNA Storage solution
- 0.1% DEPC treated water

Equipments

- Pre cooled Mortar and pestle
- Refrigerated centrifuge
- Oak ridge tubes
- Gel electrophoresis unit
- Power pack
- Vortex mixer
- Deep freezer
- Ice flaker
- Eppendorf tubes
- Spectrophotometer

Total RNA isolation from the leaf or rhizome tissues of ginger (Deepa et al. 2014)

Procedure

- 100mg of sample tissues were ground in 2ml of pre-warmed extraction buffer using mortar and pestle and transferred to an epperdorf tube and mixed.
- The homogenate was centrifuged at 13,000 rpm for 15 min and the supernatant was transferred to a fresh tube.
- Added equal volume of Acid phenol: Chloroform, mixed gently but thoroughly. Centrifuged at the tubes at 13,000 rpm for 10 minutes at 4°C.
- 700µL of the supernatant was transferred to 1.5 mL eppendorf tube and added 0.3V of Sodium acetate (5M) and 0.7V acid phenol chloroform and mixed gently. The tubes were centrifuged at 13,000 rpm for 10 minutes at 4°C.
- 700µL of supernatant was transferred to 1.5mL eppendorf tube, added 0.1V of sodium acetate (3M) and an equal volume of isopropanol and mixed gently.
- The mixture was transferred to an RNA spin column and the tubes were centrifuged at 10,000 rpm for 1 minute at 4°C and the flow through was discarded.
- The column was washed with 350µl of RW1 buffer (wash buffer). The tubes were centrifuged at 10,000 rpm for 1 minute at 4°C.
- Added 80µl of DNase buffer mix to the column and incubated for 30 min at room temperature.
- After incubation the column was washed with 300µl of RW1 buffer. The tubes were centrifuged at 10,000 rpm for 1 minute at 4°C and flow through was discarded.
- The column was washed with 500µl of RPE buffer and centrifuged at 10,000 rpm for 1 minute at 4°C. The step was repeated again.
- The column was given an empty spin at 10,000 rpm for 2 min at 4°C. The column was then transferred to clean eppendorf tube and eluted with 30µL of DEPC water and stored at 4°C.

Reagents and Preparations

- 0.01% DEPC (Diethyl pyrocarbonate): Add 1ml DEPC in 1L distilled water.
- 1M Tris: Dissolve 121.1 g of Tris base in 800 ml of DEPC treated water. Adjusted pH to 8 by adding concentrated HCl and make up to 1 L.
- 0.5 M EDTA (Ethylene Diamine Tetra Acetate): Add 186.12 g of disodium Ethylene Diamine Tetra acetate to 800 ml of DEPC treated water. Stirred vigorously on a magnetic stirrer. Adjusted the pH to 8 with NaOH and make up to 1L.
- 2- Mercaptoethanol (Sigma-Aldrich): Usually obtained as 14.4M Solution. Stored in dark bottle at 4°C.
- 3M Sodium-acetate: Added 246.09 g of Sodium-acetate trihydrate to 800 ml of DEPC treated water. Adjusted the pH to 5.2 with glacial acetic acid and made up to 1 L.

- 5M Sodium acetate: Added 246.09 g of Sodium-acetate trihydrate to 800 ml of DEPC treated water. Adjusted the pH to 5.2 with glacial acetic acid and made up to 1 L.
- RPE Buffer: Wash buffer consisting of Tris-HCl.
- DNase Buffer mix: 10 µl DNase stock was mixed with 70 µl DNA digestion buffer.
- Buffer RW1: Contains ethanol, guanidine thiocyanate.
- Extraction buffer: (100 mM Tris-HCl, 25 mM EDTA (pH 8), 2% SDS, 1% β-mercaptoethanol and 2% PVP)
    - Tris 100ml
    - EDTA 50ml
    - SDS 20g

Make up the remaining volume to 1L using DEPC treated water.

DEPC Treatment

RNases are very stable and difficult to inactivate. Solutions, plastic wares and glass wares used in the lab should be treated using 0.1 % DEPC and incubated overnight at room temperature. The treated solutions should be autoclaved for 30 minutes to remove any trace of DEPC and dried in hot air oven before used.

Agarose gel electrophoresis

Preparation of reagents

- Tris acetate EDTA (TAE) buffer (50X) : Dissolve 242 g of Tris base in DEPC treated water, added 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA (pH 8) and make up the volume to 1L. Sterilize by autoclaving.
- 10x loading dye
    - Bromophenol blue 0.25%
    - Xylene cynol 0.25%
    - Glycerol 50%
    - TAE buffer 1x
- Ethidium bromide: 1 g of ethidium bromide was added to100 mlwater.Stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved .Transferred to a dark bottle and stored at 4°C.

Protocol for electrophoresis

- 1.2% agarose gel in 1X TAE buffer was prepared with 2µl ethidium bromide.
- 3µl of RNA samples were loaded with 2µl of RNA loading dye.
- Gel electrophoresis was carried out for 30 minutes at 60-80volts.
- The RNA bands were visualized on a UV trans-illuminator and the gel picture was captured by gel sys.

RNA quality

The quality and quantity of the RNA were assessed spectrophotometrically by a standard procedure using biophotometer (Eppendorf, Hamburg, Germany). An A_{260}/A_{280} value of 1 corresponds to 40µg/mL RNA. Contamination due to phenols, carbohydrates and proteins was determined by recording A_{260}/A_{230} and A_{260}/A_{280} respectively. In order to verify RNA integrity, extracts were fractioned by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light.

cDNA synthesis

- 500ng of DNase treated RNA was reverse transcribed in a total volume of 25 µL.
- 1µl of 50mM EDTA was added and incubated at 65°C for 10 min.
- After incubation, 1µl of oligo (dT) was added and incubated at 65°C for 10 min. After incubation the mix was kept in ice immediately.
- This step is followed by the addition of reverse transcriptase and dNTPs (Table 4).
- Reaction conditions: 42°C for 1 hour, followed by heating at 70° for 10 min, storage at 4°C.

Components of RT-PCR Reaction mix

Components	Volume (µl)
RT buffer (5X)	4
Reverse transcriptase(200U/µl)	1
Ribolock-Rnase inhibitor(40U/µl)	0.5
dNTP (10mM)	2

Reaction mixture:

Components	Volume (µl)
RT buffer (5X)	4
Reverse transcriptase(200U/µl)	1
Ribolock-Rnase inhibitor(40U/µl)	0.5
dNTP (10mM)	2

The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

Control reactions

Positive and negative control reactions should be used to verify the results of the first strand cDNA synthesis steps.

Reverse transcriptase minus (RT-) negative control is important in RT-PCR or RT-qPCR reactions to assess for genomic DNA contamination of the RNA sample. The control RT- reaction contains every reagent for the reverse transcription reaction except for the RT enzyme.
No template negative control (NTC) is important to assess for reagent contamination. The NTC reaction contains every reagent for the reverse transcription reaction except for RNA template.

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REAL-TIME PCR

D Prasath, Karthika R and M Snigdha

The real-time polymerase chain reaction uses fluorescent reporter dyes to combine DNA amplification and detection steps in a single tube format. The increase in fluorescent signal recorded during the assay is proportional to the amount of DNA synthesised during each amplification cycle. Individual reactions are characterised by the cycle fraction at which fluorescence first rises above a defined background fluorescence, a parameter known as the threshold cycle (Ct) or crossing point (Cp). Consequently, the lower the Ct, the more abundant the initial target. This correlation permits accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays. The homogeneous format eliminates the need for post-amplification manipulation and significantly reduces hands-on time and the risk of contamination. Real-time PCR is often abbreviated to qPCR, although that abbreviation is not universally accepted.

Real-Time PCR Applications

Real-Time PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. With the ability to collect data in the exponential growth phase, the power of PCR has been expanded into applications such as:

- Viral Quantitation
- Quantitation of Gene Expression
- Array Verification
- Drug Therapy Efficacy
- DNA Damage measurement
- Quality Control and Assay Validation
- Pathogen detection
- Genotyping

Advantages of using Real-Time PCR:

- Traditional PCR is measured at End-Point (plateau), while Real- Time PCR collects data in the exponential growth phase
- An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated
- The cleaved probe provides a permanent record amplification of an Amplicon
- Increase dynamic range of detection
- No-post PCR processing
- Detection is capable down to a 2-fold change

Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.

Gene expression studies using qPCR

Gene of interest: HMG CoA Synthase (*Zingiber officinale*)
Reference gene: Actin

Reaction Set up

- Real-time PCR analysis is performed with Quantifast SYBR Green Mastermix kit (Qiagen, USA) on the Rotor Gene Q Real-Time PCR system (Qiagen, USA).
- Gently vortex and briefly centrifuge all solutions after thawing.
- Prepare a reaction master mix by adding the following components (except template DNA)
- Usually the total reaction volume is 25µl, prepare reaction as follows

Reagents	Con. required	Volume
SYBR Green Qiagen master Mix (2X)	2X	12.5µl
Forward primer	10 pM	1.0 µl
Reverse Primer	10 pM	1.0 µl
Template cDNA	(diluted cDNA)	5.0 µl
Nuclease free water	-	9.5 µl
	Total	25 µl

- Fold change of the transcripts was calculated relative to the control (0 hpi) using 2^{-ddCt} method (Livak and Schmittgen 2001) using *β*-actin as an internal control (Prasath et al. 2013).
- Fold differences were transformed by using a binary logarithm (log 2).
- The specificity of all amplifications was confirmed by single-peak melting curves.
  The thermal conditions are as follows:

Step	Time	Temperature	Ramp rate
PCR initial heat activation	5min	94℃	maximal/fast mode
2-step cycling: Denaturation	30s	94℃	maximal/fast mode
Combined annealing/extension	30s	60℃	maximal/fast mode

Number of cycles	35-40		

Data analysis

For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the β-actin as an internal standard.

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CLONING OF PCR PRODUCT

A I Bhat and Anju Mohandas

The validity of the amplification reaction can be assessed by cloning product followed by sequencing. There are three basic methods use for cloning a PCR product. The include: (i) TA cloning: Since the PCR product generated by *Taq* polymerase is appended with a single extraneous dA at 3' ends, the easiest way of cloning is by using plasmid tailed with dT. (ii) Blunt end cloning: The blunt ended PCR product generated by *Pwo* or *Pfu* polymerase can be cloned into a plasmid restricted with blunt end generating enzymes. (iii) Directional cohesive end cloning: In this case PCR product is first restricted with appropriate restriction enzymes followed by ligating them onto plasmid linearized by same restriction enzymes. In all the above methods PCR products are first purified to remove enzymes, unused primers, dNTPs etc. For this the PCR product is first run on low melting agarose gel, the band of interest is excised and purified. Alternatively PCR product can also be purified using commercial kits. The purified PCR product is then ligated with linearized dT or blunt end or cohesive end vector using *E.coli* T_4 DNA ligase. If directional cloning is planned, restriction of PCR product is carried out with appropriate enzymes before ligation with vector. The ligated vector is then used to transform competent *E.coli* cells and transformants are selected using appropriate markers such as antibiotic or blue / white colony morphology. The following protocol describes cloning using TA vector.

Purification of PCR product through low melting point (LMP) agarose gel

Requirements

Materials

Balance, Beakers, measuring cylinders, Microwave oven, Low melting point (LMP) agarose, Gel apparatus and power pack, Microcentrifuge tubes, microtips, pH meter, magnetic stirrer, transilluminator, waterbath**,** microcentrifuge, micropipette, razor blade, Refrigerator/cold room

Reagents

Buffer saturated phenol, chloroform, Phenol: chloroform, 3M sodium acetate pH 5.2 absolute and 70% ethanol, sterile water, TAE buffer

Protocol

1. Prepare 0.8% LMP agarose in 1x TAE buffer and cool it to room temperature. Cast the gel on a pre-cooled gel set and allow it to solidify at 4^{o}C.

2. Load PCR product with loading dye and run at 100 V for 60-90 min.

3. Examine the gel under UV transilluminator to locate the DNA band of interest. Excise the band using a clean sterile razor blade or scalpel.
4. Trim off any excess agarose and transfer to a 1.5 ml sterile eppendorf tube.
5. Incubate the tube at 70^{o}C in a heating block for 7 min.
6. Cool the solution to room temperature, add equal volume of buffered saturated phenol and vortex for 30 sec.
7. Centrifuge for 2 min at 10,000 rpm and collect aqueous phase.
8. Re-extract the aqueous phase once with phenol: chloroform and then twice with chloroform alone.
9. Precipitate the DNA by adding 0.1 volume of 3M chilled sodium acetate and 2 volumes of ethanol. Incubate the mixture at -80^{o}C for 1 h.
10. Centrifuge for 15 min at 12,000 rpm at 4^{o}C to palletize the DNA.
11. Pour off the supernatant carefully and wash the pellet with 70% cold ethanol and re-centrifuge for 5 min.
12. Pour off the supernatant carefully, air dry the pellet and resuspend the pellet in a small volume (about 10-20 µl) of sterile water and store at -20^{o}C until ready to use

Ligation of the purified PCR product into vector

Requirements
Materials
Refrigerator, Micropipette and tips, Microcentrifuge

Reagents
10x T4 DNA ligase buffer, T4 DNA ligase , Linearized TA vector, Purified PCR product

Protocol

1. Set up ligation reaction with purified PCR product in a TA plasmid vector.

10x T4 DNA ligase buffer	1.0 µl
Linearized TA vector (20 ng/µl)	1.0 µl
Purified PCR product (10 ng/µl)	7.0 µl
T4 DNA ligase (3 Weiss units/µl)	1.0 µl

   Mix the reactants by pipetting and incubate at 4^{o}C for 24 h.

2. Ligated plasmid is now ready for transformation.

Preparation of competent E.coli cells
Plasmid DNA do not enter bacteria under their own power, but require assistance traversing the outer and inner cell membranes and in reaching an intracellular site where they can be expressed and replicated. This can be achieved by using competent bacterial cells for transformation.

Bacterial cells can be made competent (to take up foreign DNA) either by chemical or physical methods. Most of the chemical methods are based on the observations of Madel and Higa (1970) who showed that bacteria treated with ice cold solutions of $CaCl_2$ and then briefly heated to 37^oC or 42^oC could be transfected with bactriophage lamda DNA. The same method was later used to transform bacteria with plasmid DNA. The physical method known as elctrophoration involve exposure of bacterial cells to electrical charge that destabilizes their membranes and thus induces the formation of transient membrane pores through which DNA molecules can pass. Although electrophoration is easy, fast and efficient, it is expensive requiring costly electrical equipment and specially designed cuvettes. In the following the commonly and most frequently used method of preparation and transformation of competent *E.coli* using $CaCl_2$ is described.

Requirements

Materials

Ice flaking machine, High speed centrifuge and centrifuge tubes, Autoclave, Glasswares
Micropipette and tips, Shaker incubator, Laminar flow, Refrigerator, Sterile petriplats
Inoculation needle, Eppendorf tubes

Reagents

E.coli bacterial strain (DH 5 α), Luria broth and Luria agar, 0.1M $MgCl_2$, 0.1M $CaCl_2$

Protocol

1. Pick a single bacterial colony from a freshly prepared plate and transfer into 100 ml broth. Incubate the culture with vigourous shaking (200 rpm) at 37^oC till the O.D reaches 0.4 at 600 nm.
2. When the required O.D is reached, transfer the bacterial cells to sterile disposable, ice cold 50 ml polypropylene tubes. Store on ice for 10 min.
3. Centrifuge the cells at 2700 g for 10 min at 4^oC.
4. Decant off the supernatant and resuspend the pellet by swirling in 30 ml of ice cold MgCl2-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$).
5. Recover the cells by centrifugation as above.
6. Decant the medium from pellets and resuspend the pellet in 2 ml of ice cold 0.1M $CaCl_2$ for each 50 ml of original culture.
7. At this point cells achieve competence. The cells can be directly used for transformation or can be dispersed into aliquots and frozen at -70^oC for later use.

Transformation of *E. coli*

Requirements

Materials

Micrcentrifuge, Eppendorf tubes, Micropipette and tips, Laminar flow, Incubator cum shaker, Ice flakes, Water bath, Luria broth and Luria agar, Spreader

Reagents

Appropriate antibiotics solution, Competent cells, Recombinant ligated plasmid
X-gal, IPTG

Protocol

1. Take 200 µl of competent cells in a sterile chilled tube. Add about 50 ng recombinant plasmid (ligation mix) in a volume of 10 µl or less. Also, keep a control for transformation, by incubating 2 ng of plasmid with 200 µl of competent cells. Have one extra tube of cells as untransformed control to check for contamination.
2. Mix gently and incubate on ice for 30- 45 min.
3. Transfer the tubes to 42^{o}C in a water bath for 90-120 sec (to give heat shock to cells).
4. Quickly transfer tubes to an ice bath and chill for 1-2 min.
5. Add 1 ml of LB medium and incubate at 37^{o}C for 1 h with shaking (at 200 rpm).
6. Plate the cells on appropriate selective medium (Luria agar containing x-gal, IPTG and appropriate antibiotics) using different volumes (200 µl, 400 µl, 500 µl). Spread uniformly with the help of spreader.
7. Incubate the plate invertedly overnight at 37^{o}C and check for the transformants next morning.

Screen light blue colonies for inserts

The *lacZ* gene has been used in prokaryotic cloning vectors as a marker for insertion of the gene of insert. Typically, when transformations are plated onmedium containing the substrate X-gal, colonies which contain vector with an insert appear white while those that contain vector without an insert are blue. In some cases, however, light blue colonies or white colonies with blue centres will appear. These clonies often contain vector with inserts.

Alpha Complementation

Alpha complementation was first reported in 1967 after researchers found that coexpression of the N- and C- terminal coding sequences of the *lacZ* gene results in wild type β-galactosidase activity. In most prokaryotic cloning vectors, the small α fragment is present on the vector with a multiple cloning site inserted into the open reading frame of the gene. The lacZ w fragment is expressed in the *E.coli* strain (encoded by lacZ M15). Insertion of a gene into the multiple cloning site disrupts the lacZ α open reading frame and results in an inactive, β-galactosidase protein. The substrate X-gal is hydrolyzed by functional β-galactosidase and produces a blue colour.

Screen the blues: Many prokaryotic cloning vectors carry the α fragment of the lacZ gene to allow blue/white screening of colonies. Sometimes small inserts, in general 1 kb or less will not disrupt the lacZ α reading frame or will cause reinitiation of translation therefore allowing expression of an intact, however less active β-galactosidase protein. This results in colonies

which contain inserts that are light blue or white with a blue centre. It is important, therefore, to screen these colonies for the insert, particularly if the PCR product you are cloning is small.

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DEVELOPMENT OF MOLECULAR MAPS AND MARKER ASSISTED SELECTION IN CROP PLANTS

M S Shivakumar

Mapping population

Population used for mapping the genes, is commonly called as mapping population and they usually obtained from controlled crosses. Selection of parents is the 1st step for production of mapping population. Parents selected to develop mapping population should have sufficient variation for trait of interest both at DNA sequence level and at phenotypic level. Higher the variation, it is easier to find the recombination. Parents should not be so diverse that they unable to cross.

Types of mapping population:

1) F2 Population

The simplest form of a mapping population is F2 population. Parent 1 (P1) and parent 2 (P2) are two parents contrasting for trait of interest crossed to get F1 population. Individual F1 plant is then selfed to produce an F2 population. F2 populations are outcome of single meiotic cycle. The segregation ratio for codominant marker is 1:2:1 (homozygous like P1:heterozygous:homozygous like P2) while segregation ratio for each dominant marker is 3:1.

Merits:

- Developed with minimum efforts
- Best for preliminary mapping
- Require less time for development

Demerits:

- Outcome of only one meiotic cycle
- Limited use for fine mapping
- Not immortal population
- Difficult to map quantitative trait

2) F2:3 Population

Selfing of F2 individuals for single generation results in F2:3 populations. Such populations suitable for specific situations like, mapping of quantitative trait, mapping of recessive genes. Demerit of F2:3 population is, like f2 population it is also not immortal.

3) Double Haploids (DH)

DH produced from chromosome doubling of pollen/egg derived haploid plants from F1. Another method of producing the haploids is wide crossing. For example haploids in barley, can be

produced by wide crossing with *Hordeum bulbosum*. During the early stages of seed development the *H. bulbosum*chromosomes are eliminated producing haploid embryo. In DH all markers segregate in 1:1 ratio.

Merits:
• Immortal/ permanent population
• Used in mapping of both qualitative and quantitative trait
• Fast production of homozygous lines

Demerits:
• High cost involved in establishing tissue culture
• Somaclonal variation arise during DH production

4) Back cross population (BC)
Backcrossing is a method of crossing of a F1 hybrid with one of its parents. The offspring produced has genetic identity similar to that of the parent. In genetic analysis usually recessive parent is used.

Merits:
• Elite genotype of recurrent parent will produce elite genotype at the end of the backcrossing programme
• The elite combination is not lost
• Less time require to develop

Demerits:
• Cant used for quantitative traits
• More restricted for recessive traits
• Require many seasons to develop new cultivar
• Not immortal

5) Recombinant Inbreed Line (RIL)
RIL population is developed using continuous selfing or sibmating progeny of individual member of F2 population until complete homozygosity is achieved. Single seed descent method is best for development, bulk or pedigree method without selection can be used. Segregation ratio of RILs is 1:1.

Merits:
• Immortal population
• Can be replicated over different locations

• Important for QTL mapping
• Product of many meiotic cycles so useful to identify tightly linked markers

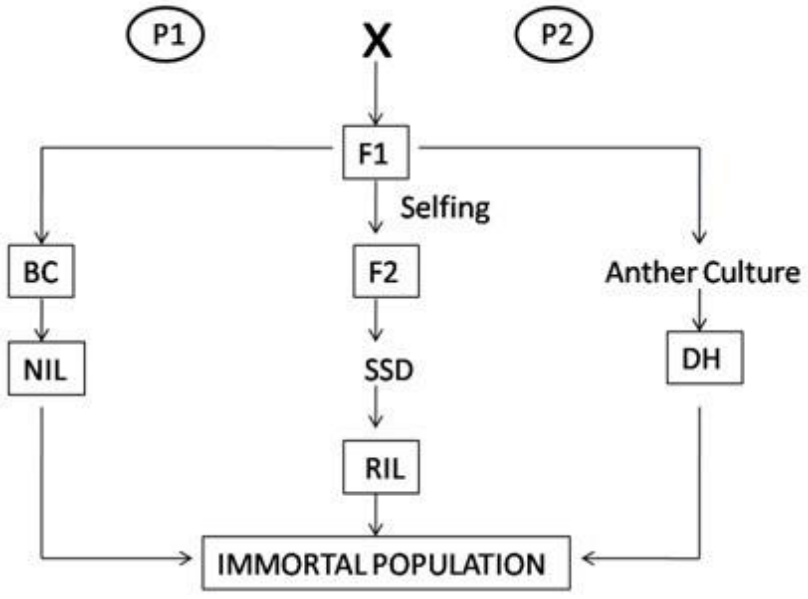
Demerits:
• Require many seasons to develop RILs
• RIL development is difficult in crops having high inbreeding depression

6) Near Isogenic Lines (NILs)
NILs are lines of genetic codes that are identical except for differences at a few specific locations or genetic loci. NILs can be developed by repeated selfing or backcrossing of F1 with recurrent parent. Irrespective of dominant or codominant marker NILs segregate in 1:1 ratio.

Merits:
• Immortal population
• Used for tagging traits
• Used in functional genomics
• Used for fine mapping

Demerits:
• Many generations require to develop NILs
• Can't used in linkage mapping
• Increased cost, time and efforts
• Linkage drag

Pictorial representation of development of mapping population



Molecular markers types and applications

- A genetic marker is a gene or known DNA sequence on a chromosome that can be used to Identify individuals or species
- There will be no need if indentified traits have these three features
- Traits were easily score
- Individuals were easily classified into few distinct phenotypic classes
- Complete corresponding between phenotypic and genotypes

Based on Hybridization Markers are classified

1) Morphological Markers :- Height, Colour, Shape etc.
2) Biochemical Markers :- Isozyme Protein Banding Pattern
3) Molecular Markers.

On the bases of chronology

1) First generation (RFLP and RAPD and there modifications)
2) Second generation (SSRs and AFLP and there modifications)
3) Third generation (ESTs and SNPs) markers.

Depending on the use of PCR

(1)PCR-based and
(2) non-PCR-based markers

Based on their molecular basis

(1) SNPs (generated by variation in DNA sequence)
(2) non-SNPs (produced by variation in sequence length, e.g., SSRs

On this basis of the location and the functional significance of markers

(1)Random, (2) gene-based, and (3) functional markers.
On the basis of the above and the throughput criteria,
(1) low-throughput hybridization-based markers,
(2) medium-throughput PCR-based markers,
(3) high-throughput sequence-based markers.

Molecular Markers variations are arise due to:

- Base pair changes.
- Rearrangements (translocation or inversion).
- Insertions or deletions.
- Variation in the number of tandem repeats.
- Reflect heritable differences in homologous DNA sequences among individuals.

Advantage over previously detected makers

- Ubiquitous nature
- Stably inherited
- Multiple alleles for each marker
- Devoid of pleiotropic effects
- Detectable in all tissues, at all ages specially in early age to save time and cost, labour
- Long shelf life of the DNA samples.

Hybridization based (non-PCR)

Technique:

RFLPs (Restriction Fragment Length Polymorphism analysis) Botstein *et al*. (1980)

These genetic markers resulting from the variation or change in the length of defined DNA fragments produced by digestion of the DNA sample with restriction endonucleases and Electrophoretic comparison of the size of defined restriction fragments derived from genomic DNA .

Steps involved in

1. Isolate high quality DNA
2. Digest with a combination of restriction enzymes
3. Fractionate digested samples by electrophoresis
4. Transfer fragments to membrane
5. Hybridize with radioactively labeled DNA probe(s); detect by autoradiography or can also use non-radioactive labeling system.

Considerations for use of RFLPs - Relatively slow process -Use of radioisotopes has limited RFLP use to certified laboratories (but non-radioactive labelling systems are now in wide use) - Co-dominant markers; often species-specific - Need high quality DNA - Need to develop polymorphic probes –expensive

PCR based techniques (RAPD, ISSR, SSR, AFLP, EST , SNP).

Random Amplified Polymorphic DNA (RAPD) are genetic markers resulting from PCR amplification of genomic DNA sequences recognized by random primers of arbitrary nucleotide sequence (Williams et al., 1990). RAPDs are dominant markers that require no prior knowledge of the DNA sequence, which makes them very suitable for investigation of species that are not well known (Williams et al. 1993). A single, random-sequence oligonucleotide primer in a low stringency PCR (35–45°C) simultaneously amplifies several discrete DNA fragments random amplified polymorphic DNA (RAPD) by Williams et al. (1990) arbitrary primed PCR (AP-PCR) by Welsh and McClelland (1990)

DNA amplification fingerprinting (DAF) by Caetano-Anollés et al., (1991) 10-mer oligonucleotide several discrete DNA products up to 3 kb are amplified (amplicons) these are considered to originate from different genetic loci visible in conventional agarose gel

electrophoresis as the presence or absence of a particular RAPD band RAPDs predominantly provide dominant markers

Application

- Construction of genetic map eg Arabidopsis
- Mapping of traits
- Fingerprinting of individuals
- Identification of somatic hybrids

AFLP (Amplified Fragment Length Polymorphisms) is a combination of PCR and RFLP Involved in Informative DNA fingerprints of amplified fragments. It is based on the selective amplification of a subset of genomic restriction fragments using PCR.
Steps involved in AFLP process
1. Digest genomic DNA with restriction enzymes
2. Ligate with adaptors (defined sequences) to both ends of the fragments 3. Carry out PCR on the adaptor-ligated mixture, using primers that target the adaptor, but that vary in the base(s) at the 3' end of the primer. AFLP technology is a DNA fingerprinting technique that combines RFLP and PCR.

Advantages of AFLP's

- Very sensitive
- Good reproducibility but technically demanding
- Relatively expensive technology
- Discriminating homozygotes from heterozygotes
- Requires band quantitation (comparison of pixel density in images from a gel scanner)

SSR (Simple Sequence repeats) variants

1. Microsatellites
2. Short tandem repeats (STRs)
3. Sequence-tagged microsatellite sites (STMS)
    - Tandemly arranged repeat units 1–6 bp long
    - Di-, tri- and tetranucleotide repeats – (CA)n, (AAT)n and (GATA)n with different length of reaptes motif formed by slipped strand mispairing
    - Widely distributed in genomes (plants• &animals (Tautz and Renz, 1984).

Construction steps
I) Microsatellite library construction
II) Identification of unique microsatellite loci
III) Identification a suitable area for primer design
IV) Obtaining a PCR product

V) Evaluation of banding pattern
VI) Assessing PCR product for polymorphisms

Advantages of SSRs:

- Hypervariability
- Reproducibility
- Codominant nature
- Locus specificity random dispersion throughout most genomes
- More variable than RFLPs or RAPDs.
- Readily analysed by PCR and easily detected on PAGE SSLPs with large size differences detected on agarose gels SSR markers can be multiplexed genotyping throughput is high and can be automated start-up costs are low for manual assay methods (once the markers have been developed) SSR assays require only very small DNA samples(ca.100 ng / individual)

The disadvantages

- Labour intensive development process
- High start-up costs for automated methods

Inter-Simple Sequence Repeats (ISSR)

- Amplification of DNA samples present between two SSRs opposite oriented direction
- SSR used as primers for IISR

Advantage: Highly polymorphic, No sequence required
Limitation: Dominant, Reproducibility, Homology same as RAPD

SNP (Single Nucleotide Polymorphisms) Pronounced as snip - an individual nucleotide base difference There are three types recognized

- Transitions (C/T or G/A)
- Transversions (C/G, A/T, C/A or T/G) e.g., AAGCCTA AAGCTTA The two alleles are C and T
- Insertions and deletions (Indels)

Some of more interesting point about sinps

- Human genome has at least 1.42 million SNPs as comprising to 100000 of which result in an RFLP 1% of the population should have SNP 90% of all human genetic variation are SNPs and occur every 100–300 bases C/T transitions constitute 2/3 or 67% of the SNPs
- Single base variants in cDNA (mRNA) or single Nucleotide base - the smallest unit of inheritance
- Typical SNP frequencies are in the range of one SNP every 100–300 bp

- SNPs may fall within coding sequences of genes – if same polypeptide then called synonymous SNP or Silent SNP its does not altered amino acid if different polypeptide then called non-synonymous i.e. altered amino acid sequence
- Non-coding regions of genes may have SNPs by Gene splicing
- Transcription factor binding the sequence of non-coding RNA
- The intergenic regions between genes at different frequencies in different chromosome regions
-

Approaches adopted for discovery of novel SNPs:

I) in vitro discovery, where new sequence data is generated

II) in silico methods that rely on the analysis of available sequence data

III) Indirect discovery, where the base sequence of the polymorphism remains unknown SNP

Sobrino et al. (2005) classified SNP genotyping assays into 4 groups (based on the molecular mechanisms)

- Allele-specific hybridization
- Primer extension
- Oligonucleotide ligation
- Invasive cleavage

The advantages of SNPs are their most abundant in numbers even located within gene would provide ideal marker and do not involve gel electrophoresis. SNP detection is more rapid because it is based on oligonucleotide hybridization analysis.

Application of Molecular markers

- Phylogenetic studies
- Trait Identification and Mapping
- DNA finger printing
- Genetic diagnostics
- Expression Profile Analysis
- Study of genome
- Gene mapping / Gene tagging
- Seed testing
- Identifying location of QTL's
- Marker Assisted Selection (MAS)
- Marker Assisted Backcrossing Breeding (MABB)

APPROACHES IN PLANT BIOFORTIFICATION - CONVENTIONAL BREEDING AND SECOND GENERATION GM CROPS

S Aarthi

Agricultural production increased tremendously after green revolution. Productivity increased with the help of high inputs and modern technologies which in turn met the quantity in global food demands. The shift from quantity to quality is emphasised to fight back malnutrition as Malnutrition is indeed a complex problem caused by policy, production, distribution and marketing in the food system which has a major impact on health and the economics of the regions affected. 3 billion people worldwide suffer micronutrients deficiency. 2.5 billion world population suffer from Zinc deficiency, 1.6 billion population suffer from Iron deficiency, 1 billion people reside in iodine deficient regions, 400 million people have vitamin A deficiency, Malnutrition accounts ~30 million death/year.

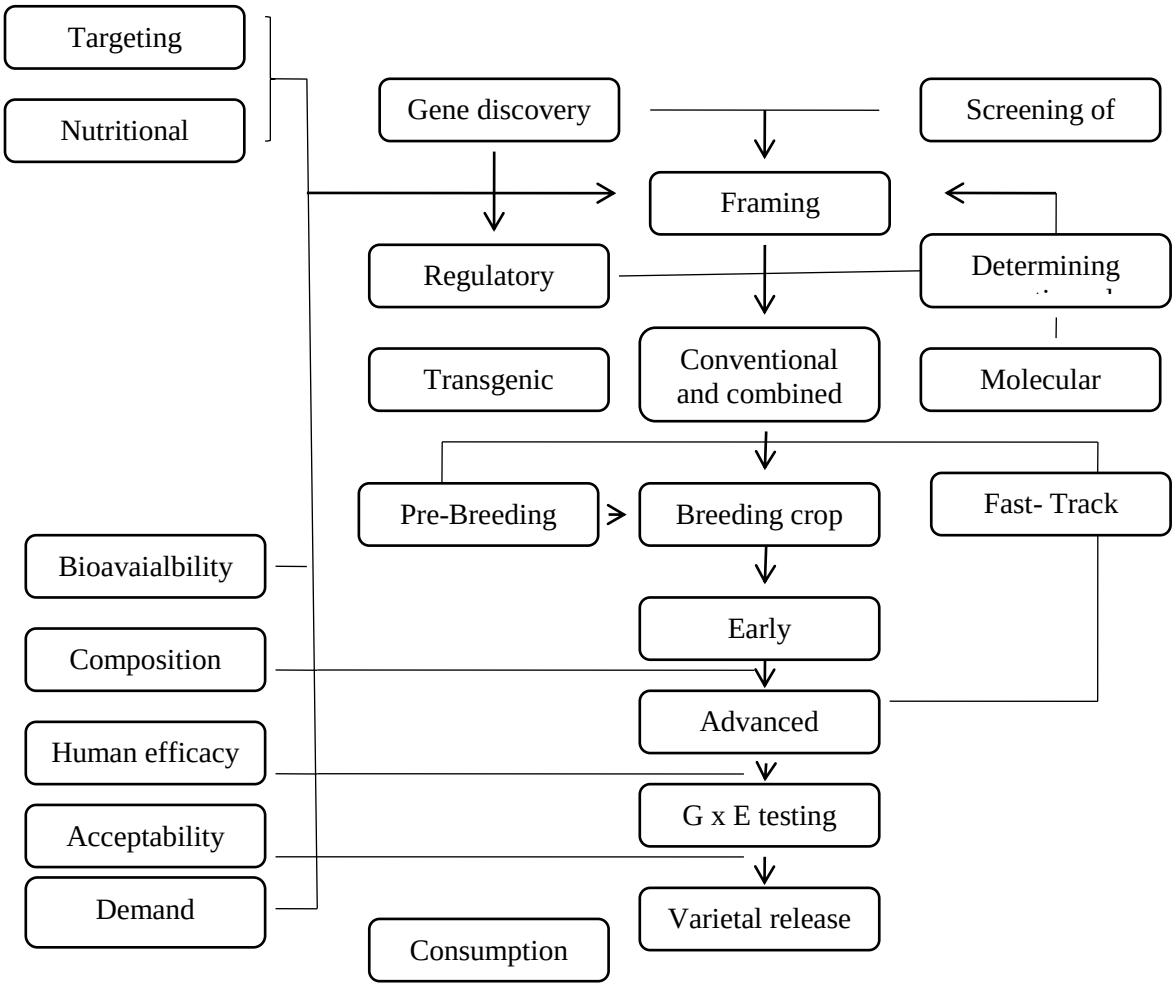
Improving the phytonutrient content of crops, referred to as biofortification, has been promoted as one potential solution. Biofortification is a process by which the nutritional quality of food crops is improved by adding nutrients or other health promoting properties through agronomic practices, conventional plant breeding, or modern biotechnology such as genetic modification. Conventional breeding programmes are highly concentrated to increase the productivity along with increased nutritive quality and genetically modified (GM) foods with an increased micronutrient level, one type of biofortification, are foods whose genetic composition is altered in a way that does not occur spontaneously in nature.

Case study on biofortification application:

Iron crops

Iron nutrition research has demonstrated the efficacy of biofortified iron bean and iron pearl millet in improving the nutritional status of target populations. In Rwanda, iron-depleted university women showed a significant increase in hemoglobin and total body iron after consuming biofortified beans for 4.5 months. The efficacy of iron pearl millet was evaluated in secondary school children from Maharashtra, India. A significant improvement in serum ferritin and total body iron was observed in iron-deficient adolescent boys and girls after consuming biofortified pearl millet flat bread twice daily for four months. The prevalence of iron deficiency was reduced significantly in the high-iron biofortified pearl millet group.

Approaches for Biofortification



Vitamin A bioavailability studies found efficient conversions from provitamin A to retinol, the form of vitamin A used by the body. Efficacy studies demonstrated that increasing provitamin A intake through consuming vitamin A-biofortified crops results in increased circulating beta-carotene, and has a moderate effect on vitamin A status, as measured by serum retinol. Consumption of orange sweet potato (OSP) can result in a significant increase in vitamin A body stores across age groups. The primary evidence for the effectiveness of biofortification comes from OSP, assessed through a randomized controlled trial. The OSP intervention reached 24,000 households in Uganda and Mozambique from 2006 to 2009 with adoption rates of OSP greater than 60% above control communities. Introduction of OSP in rural Uganda resulted in increased vitamin A intakes among children and women, and improved vitamin A status among children – a decrease in the prevalence of low serum retinol by 9 percentage points. Women who got more vitamin A from OSP also had a lower likelihood of having marginal vitamin A deficiency. Consumption of orange maize has been demonstrated to improve total body vitamin A stores as effectively as supplementation and significantly improve visual function in marginally vitamin A deficient children. To date, only a small provitamin A cassava efficacy study has been completed

in Eastern Kenya with 5–13-year-old children. This trial demonstrated small but significant improvements in vitamin A status, measured both by serum retinol and beta-carotene, in the yellow cassava versus the control group. A larger-scale efficacy trial is underway in Nigeria.

Zinc studies have demonstrated that zinc in biofortified wheat is bioavailable. Because plasma zinc concentration, the biomarker widely used to estimate zinc status, has limitations in measuring changes in dietary zinc, foundational research to identify and test more sensitive biomarkers is underway. These biomarkers will be tested in the zinc rice and wheat efficacy trial scheduled for 2017. A recent study showed that DNA strand breaks are a sensitive indicator of modest increases in zinc intake, such as the amount of additional zinc that might be delivered by a biofortified crop.
The above case studies give the glimpse of application oriented bifortification by breeding strategies.

Conventional selective breeding combined with marker-assisted selection has produced germplasm with enhanced vitamin content. Such efforts by collaborators in the HarvestPlus program exploited heterogeneity in the β-carotene contents among available varieties to produce new, high-β-carotene varieties:

- **Orange-fleshed sweet potato**—Forty-six improved varieties with β-carotene contents as great as 24,900 μg/g (compared to 30–100 μg/g in common cultivars) have been released in throughout Africa, South America, and China.[34]
- **Yellow cassava**— Screening of germplasm revealed a range of β-carotene contents of 0–19 μg/g. Three hybrids with β-carotene contents of 6–8 μg/g have been released in Nigeria.[35]
- **High-β-carotene corn (maize)**—Screening revealed a range of β-carotene contents of 0–19 μg/g in existing lines. Genetic loci associated with β-carotene level were identified, and DNA markers were developed that facilitated breeding using marker-assisted selection to take advantage of rare genetic variation in the β-carotene hydroxylase-1 (*CRTRB1*) gene, and increase expression of *PSY1*, which encodes for phytoene synthase, the rate-limiting step in the carotenoid biosynthetic pathway.[36] Hybrids were produced with β-carotene contents of 6–8 μg/g; five have been released in Zambia, Nigeria, and Ghana.
- **High-β-carotene plantain**[38]—Screening of >300 genotypes revealed a range of β-carotene contents of 1–345 μg/g (fresh weight). Five varieties with β-carotene contents of 17–106 μg/g have been identified for dissemination in Burundi and the Democratic Republic of the Congo.

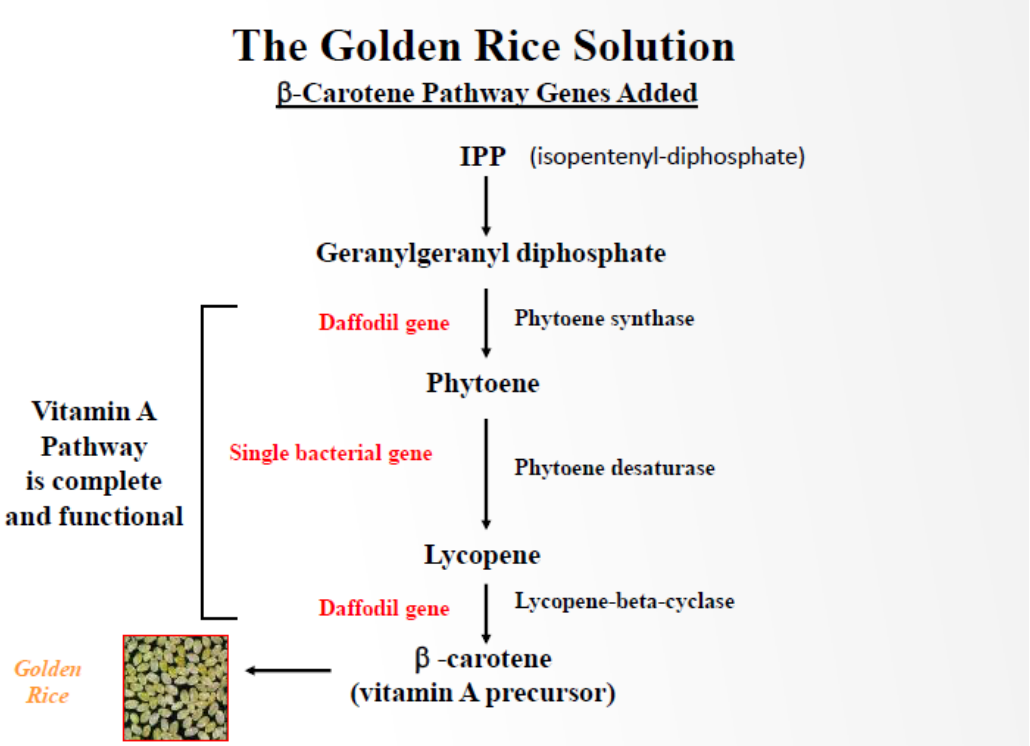
Others have used selective breeding to produce high-β-carotene carrots and tomatoes and high-anthocyanin carrots. Proof-of-concept experiments have been done to increase other vitamins species used as models in plant research: tocopherols and vitamin B_6 in *Arabidopsis thaliana*;

tocopherols in *Brassica napus*.Strain selection and optimization of culture conditions of baker's yeast (*Saccharomyces cerevisiae*) have been used to make three- to fivefold increases in the folate contents of breads.

Biotechnological approach

Plant biotechnology has been around since the advent of humankind, resulting in tremendous improvements in plant cultivation through crop domestication, breeding and selection. Genetic engineering has changed the surface of our planet by further expanding the gene pool used by plant breeders for plant improvement. Transgenic approaches in food plants have raised concerns on the merits, social implications, ecological risks and true benefits of plant biotechnology. The recently acquired ability to precisely edit plant genomes by modifying native genes without introducing new genetic material offers new opportunities to rapidly exploit natural variation, create new variation and incorporate changes with the goal to generate more productive and nutritious plants.

Genetic engineering has been shown to be useful in adding vitamin biosynthetic capacity to plant species with incomplete biosynthetic pathways. This concept was demonstrated by the pioneering work of producing "**Golden Rice**," i.e., various rice containing ~35 μg/g β-carotene in the endosperm. This was achieved by inserting into the rice genome two missing genes needed for β-carotene synthesis : *PSY* (encoding phytoene synthase), initially from daffodil (*Narcissus pseudonarcissus*) and subsequently from corn (maize); and *CRT1* (encoding β-carotene hydroxylase-1) from a soil bacterium (*Erwinia uredovora*). As the rice genome already contained the third gene required for this pathway, LYC (encoding *lycopene* cyclase), these additions completed β-carotene biosynthetic capacity.



Purple Tomatoes' – Tomatoes normally accumulate low levels of anthocyanin pigments, but two regulatory proteins from snapdragon were introduced, and tomato fruits accumulating very high levels of health-promoting anthocyanin

Biofortification by conventional breeding and advanced biotechnological tools have the potential to complement the existing micronutrient interventions, in particular by targeting the rural poor who eat only the staple food and often have less access to commercially processed food

GENE DISCOVERY THROUGH TRANSCRIPTOME ANALYSIS

K Johnson George

Study on transcriptome, the entire pool of transcripts in an organism or single cells at certain physiological or pathological stage, is indispensable in unraveling the connection and regulation between DNA and protein. During the past decade, next-generation sequencing (NGS) has revolutionized our understanding of genomics in a fast, high-throughput, cost-effective, and tractable manner. By adopting NGS, efficiency and fruitful outcomes concerning the efforts to elucidate genes responsible for producing active compounds in medicinal plants were profoundly enhanced. The unprecedentedly rapid development of such technologies provides so many choices to facilitate the task, which can cause confusion when choosing the suitable methodology for specific purposes. Here, we review the general approaches for deep transcriptome analysis and then focus on their application in discovering genes in plants.

The unique feature of transcriptome, the entire RNA molecules that are transcribed from a genome and the key bond between information embodied in genotype and phenotype, is what determines its exclusive importance in studies of elucidating functional genes. The scope of transcriptomics covers: archiving all transcripts, including mRNAs, small RNAs, and micro RNAs, in target organisms or even one single cell; tackling the structural composition of genes and their related characteristics, such as posttranscriptional modifications; measuring expression levels in different developmental stages, or to quantify impacts of environmental factors.

Deep transcriptome, also called RNA sequencing (RNA-Seq), provides a new way to address the challenges. It employs deep sequencing technologies that sequence the arrayed amplified cDNA many times, and the coverage or depth indicating the times for which a nucleotide is detected.

The Technology

The revolution in sequencing represented by short-read (otherwise known as 2nd generation or next-generation) technologies has enabled the sequencing approach to leap ahead of the microarray approach once again. In 2006 the first RNA-seq paper was published utilizing 454/Roche technology. The data generated comprised just 200,000 reads of length 110 bp for a total of 20 Mbases of data. However for sequencing approaches to be successful a lot of sequencing is needed (the more the better) and it was not until the advent of greater through-put that RNA-seq was able to compete with microarrays. The era of RNA-seq dominance began in earnest in 2008 with a trio of papers utilizing the new short-read tech-nology developed by Solexa (now Illumina). From the outset the Illumina/Solexa technology has generated gigabases of data per run (initially 1 GB per run for the Genome Analyzer when it was initially released in 2006 rising to 600 GB per run for the HiSeq 2500 as of 2012).

The major limitation of short-read RNA-seq is the difficulty in accurately reconstructing expressed full-length transcripts from the assembly of reads. This is particularly complicated in complex transcriptomes, where different but highly similar isoforms of the same gene are expressed, and for genes that have many exons and possible alternative promoters or 3' ends. Long-read technologies, such as Pacific-Biosciences (PacBio) SMRT and Oxford Nanopore, that were initially applied to genome sequencing are now being used for transcriptomics and have the potential to overcome this assembly problem. Oxford Nanopore Technologies has developed a method to do direct RNA sequencing on its MinIon nanopore sequencing device. After the sequencing adaptors are ligated to the mRNA/cDNA complex, the mRNA is sequenced. The RNA molecule is sequenced in the reverse direction — 3' to 5' — since the initial adaptors were ligated to the poly A tail.

Pacific Biosciences (PacBio) has developed a commercially successful platform for single-molecule real-time sequencing that provides very long read length. It has to be mentioned, though, that the PacBio single-molecule long-read technology is extremely helpful for the de novo generation of reference transcriptomes. The recently developed full-length transcript sequencing method by PacBio in principle circumvents the assembly step since it provides full-length sequences of single complementary DNA molecules.

To acquire a transcriptomic profile, cDNA is commonly synthesized from mRNA for sequencing. Typically, it involves the following steps: purifying mRNA molecules; producing fragmented RNA with desired length (alternatively, fragmentation is performed after cDNA is synthesized from intact RNA); synthesizing first-strand and then double-stranded (ds) cDNA; end repair and ligating oligonucleotide adapters to the ends of the ds cDNA; validation and quantification of the cDNA library for high-throughput sequencing. Poly-A containing mRNAs can be hybridized to oligo-dT-attached carriers, such as magnetic beads, and then the nonbound irrelevant material can be washed away using commercial mRNA purification kits. Finally, mRNA is eluted from the oligo-dT carrier. Different technologies/platforms have distinct sequencing abilities. Currently, the read length that can be sequenced by Illumina (San Diego, USA) and Ion PGM system (Life technologies, USA) is under 500 bases. GS FLX+ system (454 Life Sciences, USA) delivers up to 1000 bp in read length and PacBio RS II (Pacific Biosciences, USA) is capable of producing reads with length of 15 kb. The size of input RNA has to be decided before constructing a cDNA library.

Because mRNAs are longer than the read-lengths of typical high-throughput sequencing methods, transcripts are usually fragmented prior to sequencing in case of Illumina sequencing. The fragmentation method is a key aspect of sequencing library construction when shot read sequencing is employed. To fragment nucleotide sequences, physical methods like sonication, acoustic shearing, nebulization, and heat, as well as enzymatic ways including restriction endonucleases and nonspecific nuclease cocktails, can be used. Subsequently, mRNA fragments

are primed with random oligomers and reverse transcribed to produce first stand cDNA. Sheared RNA templates are replaced to generate ds cDNA. Before adapters can be ligated to cDNA for sequencing, overhangs caused by fragmentation have to be repaired. When a pair of adapters is ligated to both ends of a cDNA fragment, it therefore can be synthesized from both sides, resulting in "paired-end" reads (in case of Illumina). Finally, the size and purity of the cDNA library will be checked to ensure its reliability. Once the transcript molecules have been prepared, they can be sequenced in just one direction (single-end) or both directions (paired-end) in case of Illumina based technology.

Data interpretation

The interpretation of the NGS datasets requires sophisticated and powerful computational programs. The common output file recording information obtained from deep sequencing is in FASTQ format (eg. Illumina). The FASTQ output files may contain adapters and poor quality input data that are generated during sequencing process, and they have to be removed before any subsequent handling. RNA-Seq experiments generate a large volume of raw sequence reads, which have to be processed to yield useful information. Data analysis usually requires a combination of bioinformatics software tools that vary according to the experimental design and goals. The process can be broken down into the following four stages: quality control, alignment, quantification, and differential expression. Sequence reads are not perfect, so the accuracy of each base in the sequence needs to be estimated for downstream analyses. Raw data are examined for high quality scores for base calls, guanine-cytosine content matches the expected distribution, the over representation of particularly short sequence motifs (k-mers), and an unexpectedly high read duplication rate. In general, two major types of analyses are conducted on RNA-Seq data: assembly of reads into contiguous sequences (contigs) and mapping of reads to a reference either to obtain an account of transcript amounts or to verify/modify gene models or discover splice or sequence variants. Of these two types of analyses, the assembly of RNASeq data into contigs, in particular, de novo assembly from short reads without a guiding reference, is still problematic. In order to link sequence read abundance to expression of a particular gene, transcript sequences are aligned to a reference genome, or de novo aligned to one another if no reference is available. In principle, two different assembly strategies exist: overlap-based assemblers such as CAP3 and De Bruijn graph-based assemblers, such as Velvet/ Oases and Trinity. The list of softwares available for de nono assembly is listed below.

RNA-Seq de novo assembly softwares

Software (Manufacturer)	Strengths and weaknesses
Velvet-Oases	The original short read assembler, now largely superseded.
SOAPdenovo-trans	Early short read assembler, updated for transcript assembly
Trans-ABySS	Short reads, large genomes, MPI-parallel version available

Trinity	Short reads, large genomes, memory intensive
miraEST	Repetitive sequences, hybrid data input, wide range of sequence platforms accepted.
Newbler	Specialized for Roche 454 sequence, homo-polymer error handling.
CLC genomics workbench	Graphical user interface, hybrid data.

Gene function annotation and gene discovery

The transcriptomic techniques have been particularly useful in identifying the functions of genes and identifying those responsible for particular phenotypes. Integration of RNA-Seq datasets across different tissues has been used to improve annotation of gene functions in commercially important organisms or threatened species.

For the study of model plants, clean reads can be directly mapped back to their draft genomes to measure the expression of genes. For nonmodel plants whose genome information is usually unavailable, the inconvenient fact concerning convenient NGS is that relatively short reads have to be assembled to restore sequence information sacrificed by fragmentation for the massive parallel process. With the assembled contigs at hand, reads can be mapped back to check the expression level of transcripts. Metrics include RPKM (reads per kilobase of transcript per million mapped reads), FPKM (substitute reads with fragments), and TPM (transcripts per million) The expression of any transcript is quantified by the number of sequenced reads or fragments that are mapped back, and it is dependent on sequencing depth as well as the expression levels of other transcripts. The Differentially Expressed Genes (DEG) of the transcriptome libraries were used to discover the unigenes with significant differences in expression. Differential Expression Genes (DEGs) under certain condition have become powerful strategies for the global analysis of plant genes. For differential expression (DE) analysis, the raw reads are typically modeled using negative binomial or Poisson distribution, followed by various methods for normalization. Similarity between experimental transcripts and the identified genes deposited in public databases provides hints for their possible function. BLAST (Basic Local Alignment Search Tool) and its newer version BLAST+ are algorithms for this purpose.

Gene annotation concerns several aspects to make specific definitions about function or structure. Gene Ontology (GO) describes gene products with three independent categories: biological process, cellular component, and molecular function, which may produce multiple GO terms assigned to one query sequence. To collect GO information, gene identifiers (GI) from NCBI or accession number (AC) can be extracted from BLAST outputs and used to retrieve GO through online tools (http://www.uniprot.org/mapping/). Then, the GO information of corresponding sequences can be displayed using web gene ontology annotation plot (WEGO).

Adopting Fisher's exact test, researchers can investigate the over expressed GO terms by incorporating expression levels of contigs with associated GO terms. Alternatively, commercial software blast2go provides an algorithm to annotate sequences against public databases, including GO, from which enrichment analysis can be conducted. KEGG (Kyoto Encyclopedia of Genes and Genomes) , InterPro, and CathaCyc are also popular for gene annotation.

Finding genes involved in secondary metabolism

Plants synthesize an impressive number of different secondary metabolites involved directly in the interaction with other organisms and with environment or indirectly in the regulation of plant responses. Moreover many secondary metabolites hold beneficial effects on human health and are used as bioactive components of drugs. Plant nutraceuticals, natural plant food and use of nutritional therapies and phytotherapies have become progressively popular to improve health and to prevent and treat diseases. Improving the traits related to plant secondary metabolism (i.e. defense responses, fitness, stress tolerance, nutraceutical value, etc.) has become a main target of plant breeding and biotechnology industry.

The diffusion of new technologies have rapidly increased the release rate of sequence and expression data. The large amount of sequence information now available for plant genomes and transcriptomes, provides an opportunity to identify genes involved in secondary metabolic pathways in many plant species of agronomical interest. An effective approach to predict genes involved in the same metabolic pathway is the co-expression analysis. A set of genes involved in a biological process can be co-regulated and thus co-expressed under the control of a shared regulatory system, therefore, if a gene with unknown function is co-expressed with known genes of a particular metabolic pathway, this gene has potentially a role in the same pathway.

Among the 17 groups affiliated with KEGG, the pathway database is frequently used to study secondary metabolic profiles of medicinal plants. The identified entire, or partial, pathways were drawn with enzyme information connecting chemical intermediates and products. To do a pathway search, KEGG supports various ways, including KO (KEGG Orthology), and EC (Enzyme Commission) number. Known enzymes presented in metabolic procedures provide important clues for plants of interest due to similarity in amino acid sequences.

Measuring the expression of an organism's genes in different tissues, conditions, or time points gives information on how genes are regulated and reveals details of an organism's biology. It can also help to infer the functions of previously unannotated genes. Transcriptomic analyses may be validated using an independent technique, for example, quantitative PCR (qPCR), which is recognisable and statistically assessable. Gene expression is measured against defined standards both for the gene of interest and control genes. Whole-transcriptome sequencing is an effective approach for functional gene discovery and for insights into the expression and regulation networks of genes

Plant metabolic engineering and synthetic biology have the potential to produce environmentally clean and low in put chemicals and biofuels. The production of specialized metabolites in crops enhances their nutritional value and resistance against insects and microorganisms. For this purpose, it is necessary to understand gene regulatory networks
and the functioning of appropriate enzyme genes.

Using modern biotechnology to dissect biosynthesis pathways and manipulate the functional genes involved in biosynthesis of the active components in medicinal plants are two main development directions for the modernization of herbal medicines. Linking biosynthetic genes to secondary metabolites and *vice versa* can potentially help not only in characterization of new secondary metabolites, but also in redesigning known biosynthetic pathways of secondary metabolites to produce novel compounds. As model, a study in garden sage (*Salvia officinalis*) by Ali *et al.* (2017) can be considered. There they characterized genes that are involved in terpenoid biosynthesis in *S. officinalis* and determined their biological significance in *S. officinalis* for terpenoid production in various tissues. In this study, a transcriptome database was established for *S. officinalis* leaves using NGS technology to identify and to characterize genes that are related to the terpenoid biosynthesis pathway. The criteria used to achieve these objectives and to elucidate the complex metabolic pathways and genes for the understanding of terpenoid production in *S. officinalis* included the following: (i) transcriptome analysis of leaves using Illumina HiSeq 2000 sequencing; (ii) Gas Chromagraphy coupled Mass Spectrometry (GC-MS) analysis for three fresh plant parts (old leaves, young leaves, and stems); (iii) characterization of five terpene genes in transgenic *N. tabacum*; (iv) qRT-PCR of highly expressed genes that are involved in the biosynthesis of terpenoids; (v) and the combination of data from the transcriptome, qRT-PCR, and metabolome with GC-MS for revealing the functions of metabolic genes that are involved in the biosynthesis of valuable terpenoids.

Wide-scale use of any identified compounds requires a reliable and scalable source of material that is both ethically and commercially viable. This requirement is often not met by the natural plant source due to difficulties with cultivation, endangered wild populations and the inherent chemical complexity found in most medicinal plant species. While chemical synthesis can potentially provide a solution to this problem, the large number of stereocenters present in natural products makes this route challenging technically and economically. Another approach to solving this problem is via the engineering of microbial organisms to produce specific natural
products using biosynthetic pathways reconstructed from the original or even a combination of plant species. An example is by using Saccharomyces cerevisiae as a host to make important plant-derived pharmaceuticals and nutraceuticals are already available, and include: artemisinic acid, a precursor of the anti-malarial drug artimisinin, naturally produced by the plant *Artemisia annua.*

Transcriptomics has revolutionised our understanding of how genomes are expressed. Over the last three decades, new technologies have redefined what is possible to investigate, and integration with other omics technologies is giving an increasingly integrated view of the complexities of cellular life. As more and more RNA-seq data become publically available, there is a great need to develop new algorithms to formulate both the global and local characteristics of co-expression networks, especially those dynamic changes associated with biological processes. Efforts are on at this institute for identification and functional analysis of genes involved in disease resistance, drought tolerance and biosynthesis of secondary metabolites in crops like black pepper and small cardamom.

NEXT GENERATION SEQUENCING TECHNOLOGIES

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NGS technologies have demonstrated the capacity to sequence DNA at unprecedented speed, thereby enabling previously unimaginable scientific achievements and novel biological applications. But, the massive data produced by NGS also presents a significant challenge for data storage, analyses, and management solutions. Advanced bioinformatics tools are essential for the successful application of NGS technology. As evidenced throughout this review, NGS technologies will have a striking impact on genomic research and the entire biological field. With its ability to tackle the unsolved challenges unconquered by previous genomic technologies, NGS is likely to unravel the complexity of the human genome in terms of genetic variations, some of which may be confined to susceptible loci for some common human conditions. This chapter reviews basic concepts, general applications, and the potential impact of next- generation sequencing (NGS) technologies on genomics, with particular reference to currently available and possible future platforms and bioinformatics.

Since the time DNA was discovered as the code to all biological life on earth, man has sought to unravel its mysteries. If the genetic code could be sequenced or "read", the origins of life itself may be revealed. Although this thought might not be entirely true, the efforts to date made have certainly revolutionized the biological field. The "original" sequencing methodology, known as Sanger chemistry, uses specifically labeled nucleotides to read through a DNA template during DNA synthesis. This sequencing technology requires a specific primer to start the read at a specific location along the DNA template, and record the different labels for each nucleotide within the sequence. After a series of technical innovations, the Sanger method has reached the capacity to read through 1000–1200 basepair (bp); however, it still cannot surpass 2 kilo basepair (Kbp) beyond the specific sequencing primer.

In order to sequence longer sections of DNA, a new approach called shotgun sequencing was developed during Human Genome Project (HGP). In this approach, genomic DNA is enzymatically or mechanically broken down into smaller fragments and cloned into sequencing vectors in which cloned DNA fragments can be sequenced individually. The complete sequence of a long DNA fragment can be eventually generated by these methods by alignment and reassembly of sequence fragments based on partial sequence overlaps. Shotgun sequencing was a significant advantage from HGP, and made sequencing the entire human genome possible. The core philosophy of massive parallel sequencing used in next- generation sequencing (NGS) is adapted from shotgun sequencing.

New NGS technologies read the DNA templates randomly along the entire genome. This is accomplished by breaking the entire genome into small pieces, then ligating those small pieces of DNA to designated adapters for random read during DNA synthesis (sequencing- by-

synthesis). Therefore, NGS technology is often called massively parallel sequencing. The read length (the actual number of continuous sequenced bases) for NGS is much shorter than that attained by Sanger sequencing. At present, NGS only provides 50–500 continuous basepair reads, which is why sequencing results are defined as short reads. These short reads are a major limitation in current technology; however, developing NGS technologies, such as single-molecule sequencing, may surpass Sanger methodologies and have the potential to read several continuous kilo basepairs (Kbps) (Table 1). Since next-generation technologies currently produce short reads, coverage is a very important issue. Coverage is defined as the number of short reads that overlap each other within a specific genomic region. For example, a 30-fold coverage for CYP2D6 gene means that every nucleotide within this gene region is represented in at least 30 distinct and overlapping short reads. Sufficient coverage is critical for accurate assembly of the genomic sequence.

In addition to the need for adequate coverage, short reads create many sequences that cannot be interpreted or "mapped" to any reference DNA or be accurately assembled. This is simply because some of the short reads are too short and may match with many different regions of the genome and are not unique to any specific region of the sequence. Short-read sequences that can be assembled and matched with a reference sequence are generally called "mappable reads". NGS is a rapidly evolving technology that is changing on an almost daily basis. The purpose of this review is to highlight these advances and bring the reader up to date on the latest technological achievements in DNA sequencing technologies, particularly as related to genomics. Following completion of the HGP, a new approach, genome-wide association study (GWAS), was widely applied to genomics. Although several early GWAS studies reported potentially promising results, the majority of GWAS studies were disappointing because of inadequate sample size, limitation of arrays for certain genetic variations, and/or heterogeneity in phenotype. These obstacles may be overcome by new genomic technology, i.e., next-generation sequencing (NGS), also known as massively parallel sequencing or multiplex cyclic sequencing. Since many genetic variants which contribute to many human conditions are still unknown, unbiased whole-genome sequencing will help to identify these genetic variants, including single nucleotide variants (SNVs) or single nucleotide polymorphisms (SNPs), small insertions and deletions (indels, 1–1000 bp), and structural and genomic variants (>1000 bp).

Previously, DNA sequencing was performed almost exclusively by the Sanger method, which has excellent accuracy and reasonable read length but very low throughput. Sanger sequencing was used to obtain the first consensus sequence of the human genome in 2001 and the first individual human diploid sequence. Shortly thereafter, the second complete individual genome was sequenced using next-generation technology, which marked the first human genome sequenced with new NGS technology. Since then, several additional diploid human genomes have been sequenced with NGS utilizing a variety of related techniques to rapidly sequence genomes with varying degrees of coverage. A common strategy for NGS is to use DNA

synthesis or ligation process to read through many different DNA templates in parallel. Therefore, NGS reads DNA templates in a highly parallel manner to generate massive amounts of sequencing data but, as mentioned above, the read length for each DNA template is relatively short (35–500 bp) compared to traditional Sanger sequencing (1000–1200 bp).

Several NGS methods recently developed allow larger-scale DNA sequencing. The number of large short-read sequences from NGS is increasing at exponential rates. Currently, five NGS platforms are commercially available, including the Roche GS-FLX 454 Genome Sequencer (originally 454 sequencing), the Illumina Genome Analyzer (originally Solexa technology), the ABI SOLiD analyzer, Polonator G.007 and the Helicos HeliScope platforms. These NGS instruments generate different base read lengths, different error rates, and different error profiles relative to Sanger sequencing data and to each other. NGS technologies have increased the speed and throughput capacities of DNA sequencing and, as a result, dramatically reduced overall sequencing costs.

History of DNA Sequencing

First- and second-generation sequencing technologies have led the way in revolutionizing the field of genomics and beyond, motivating an astonishing number of scientific advances, including enabling a more complete understanding of whole genome sequences and the information encoded therein, a more complete characterization of the methylome and transcriptome and a better understanding of interactions between proteins and DNA. Nevertheless, there are sequencing applications and aspects of genome biology that are presently beyond the reach of current sequencing technologies, leaving fertile ground for additional innovation in this space. We also describes a new generation of single-molecule sequencing technologies (third generation sequencing) that is emerging to fill this space, with the potential for dramatically longer read lengths, shorter time to result and lower overall cost.

First-generation sequencing

First-generation sequencing was originally developed by Sanger in 1975 (the chain-termination method) and in parallel by Maxam and Gilbert in 1977 (a chemical sequencing method). From these first-generation methods, Sanger sequencing ultimately prevailed given it was less technically complex and more amenable to being scaled up. For Sanger sequencing practiced today, during sample preparation, different-sized fragments of DNA are generated each starting from the same location. Each fragment ends with a particular base that is labeled with one of four fluorescent dyes corresponding to that particular base. Then all of the fragments are distributed in the order of their length via capillary electrophoresis. Information regarding the last base is used to determine the original sequence. This method results in a read length that is _800 bases on average, but may be extended to above 1000 bases. While fully automated implementations of this approach were the mainstay for the original sequencing of the human genome, their chief limitation was the small amounts of DNA that could be processed per unit time, referred to as

throughput, as well as high cost, resulting in it taking roughly 10 years and three billion dollars to sequence the first human genome .

Second-generation sequencing

Commercial SGS tools emerged in 2005 in response to the low throughput and high cost of first-generation methods. To address this problem, SGS tools achieve much higher throughput by sequencing a large number of DNA molecules in parallel. With most SGS technologies, tens of thousands of identical strands are anchored to a given location to be read in a process consisting of successive washing and scanning operations. The 'wash-and-scan' sequencing process involves sequentially flooding in reagents, such as labeled nucleotides, incorporating nucleotides into the DNA strands, stopping the incorporation reaction, washing out the excess reagent, scanning to identify the incorporated bases and finally treating the newly incorporated bases to prepare the DNA templates for the next 'wash-and-scan' cycle. This cycle is repeated until the reaction is no longer viable. The array of DNA anchor locations can have a very high density of DNA fragments, leading to extremely high overall throughput and a resultant low cost per identified base when such instruments are run at high capacity. For example, Illumina's HiSeq 2000 instrument can generate upwards of 300 or more gigabases of sequence data in a single run. The time-to-result for these SGS methods is generally long (typically taking many days), due to the large number of scanning and washing cycles required. Furthermore, because step yields for the addition of each base are, 100%, a population of molecules becomes more asynchronous as each base is added. This loss of synchronicity (called dephasing) causes an increase in noise and sequencing errors as the read extends, effectively limiting the read length produced by the most widely used SGS systems to significantly less than the average read lengths achieved by Sanger sequencing. Further, in order to generate this large number of DNA molecules, PCR amplification is required. The amplification process can introduce errors in the template sequence as well as amplification bias. The effects of these pathologies are that neither the sequences nor the frequencies with which they appear are always faithfully preserved. In addition, the process of amplification increases the complexity and time associated with sample preparation. Finally, the massively high throughput achieved by SGS technologies per run generates mountains of highly informative data that challenge data storage and informatics operations, especially in light of the shorter reads (compared with Sanger sequencing) that make alignment and assembly processes challenging. First-generation sequencing and SGS technologies have led the way in revolutionizing the field of genomics and beyond, motivating an astonishing number of scientific advances. Nevertheless there are sequencing applications and aspects of genome biology that are presently beyond the reach of current sequencing technologies, leaving fertile ground for additional innovation in this space.

Next-generation sequencing platforms

Among the five commercially available platforms, the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, and the Applied Biosystems (ABI) SOLiD Analyzer are currently dominating

the market. The other two platforms, the Polonator G.007 and the Helicos HeliScope, have just recently been introduced and are not widely used. Additional platforms from other manufacturers are likely to become available within the next few years and bring NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript new and exciting technologies, faster sequencing speed, and a more affordable price. Methodologies used by each of the current available NGS systems are discussed below.

(i) Roche GS-FLX 454 Genome Sequencer

The Roche GS-FLX 454 Genome Sequencer was the first commercial platform introduced in 2004 as the 454 Sequencer. The second complete genome of an individual was sequenced with this platform). The 454 Genome Sequencer uses sequencing-by-synthesis technology known as pyrosequencing. The key procedure in this approach is emulsion PCR in which single-stranded DNA binding beads are encapsulated by vigorous vortexing into aqueous micelles containing PCR reactants surrounded by oil for emulsion PCR amplification. During the pyrosequencing process, light emitted from phosphate molecules during nucleotide incorporation is recorded as the polymerase synthesizes the DNA strand. Initially, the 454 Sequencer had a read length of 100 bp but now can produce an average read length of 400 bp. The maximum ~600 bp capacity of 454 systems approaches the halfway of current Sanger sequencing capacities (~1200 bp). At 600 bp, the 454 Sequencer has the longest short reads among all the NGS platforms; and generates ~400–600 Mb of sequence reads per run; critical for some applications such as RNA isoform identification in RNA-seq and de novo assembly of microbes in metagenomics. Raw base accuracy reported by Roche is very good (over 99%); however, the reported relatively error-prone raw data sequence, especially associated with insertion-deletions, is a major concern. Low yield of sequence reads could translate into a much higher cost if additional coverage is needed to define a genetic mutation.

(ii) Illumina/Solexa Genome Analyzer

The Illumina/Solexa Genome Analyzer was the second platform to reach market, and currently is the most widely used system. The Illumina platform uses sequencing-by- synthesis approach in which all four nucleotides are added simultaneously into oligo-primed cluster fragments in flow-cell channels along with DNA polymerase. Bridge amplification extends cluster strands with all four fluorescently labeled nucleotides for sequencing. The Genome Analyzer is widely recognized as the most adaptable and easiest to use sequencing platform. Superior data quality and proper read lengths have made it the system of choice for many genome sequencing projects. To date, the majority of published NGS papers have described methods using the short sequence data produced with the Genome Analyzer. At present, the new Illumina HiSeq 2000 Genome Analyzer is capable of producing single reads of 2 × 100 basepairs (pair-end reads), and generates about 200 giga basepair (Gbp) of short sequences per run. The raw base accuracy is greater than 99.5%.

(iii) ABI SOLiD platform

The ABI SOLiD platform uses a unique sequencing-byligation approach in which it uses an emulsion PCR approach with small magnetic beads to amplify the DNA fragments for parallel sequencing. During SOLiD sequencing, DNA ligation is carried out to link specific fluorescent labeled 8-mer oligonucleotides for "dinucleotide-encoding", whose 4^{th} and 5th bases are encoded by specific fluorescence. Each fluorescent marker on a 8-mer identifies a two-base combination, which can be further distinguished with a universal primer offsetting scheme. The primer offsetting scheme allows a universal primer that is offset by one base from the adapter-fragment position to hybridize to DNA templates in five cycle sets permitting the entire fragment to be sequenced and each base position sequenced twice during each cycle. Each ligation step is followed by fluorescence detection and another round of ligation. SOLiD4 analyzer has a read length of up to 50 bp and can produce 80–100 Gbp of mappable sequences per run. The latest model, 5500×l solid system (previously known as SOLiD4hq) can generate over 2.4 billion reads per run with a raw base accuracy of 99.94% due to its 2-base encoding mechanism. This instrument is unique in that it can process two slides at a time; one slide is receiving reagents while the other is being imaged. The SOLiD4 platform probably provides the best data quality as a result of its sequencing-by-ligation approach but the DNA library preparation procedures prior to sequencing can be tedious and time consuming. The newly marketed EZ-Bead system may provide some resolution to this problem.

(iv) Danaher/Dover/Azco Polonator G.007

The Danaher/Dover/Azco Polonator G.007 is a new platform on the market with emphasis on competitive pricing. The Polonator platform employs a sequencing-by-ligation approach using a randomly arrayed, bead-based, emulsion PCR to amplify DNA fragments for parallel sequencing. The short-read length is 26 bp, and 8–10 Gbp of sequence reads are generated per run, with 92% of the reads mappable. The random bead-based array will likely be replaced with their patented rolonies technology (rolling circle colonies) on an ordered array to increase accuracy and improve read length.

(v) Helicos HeliScope

The Helicos HeliScope platform is the first single molecular sequencing technology available that uses a highly sensitive fluorescence detection system to directly detect each nucleotide as it is synthesized. The distinct characteristic of this technology is its ability to sequence single DNA molecules without amplification, defined as Single-Molecule Real Time (SMRT) DNA sequencing. The short-read length ranges from 30 bp to 35 bp at present time, with a raw base accuracy greater than 99%, and 20–28 Gbp of potential sequence reads per run in the near future. The advantage of single-molecule DNA sequencing technology is its potential to read extremely long sequences and fast sequencing speed, which could translate into a dramatic reduction in overall sequencing cost. As such, advanced single DNA molecule sequencing technology has been defined as the next-NGS technology. However, the basic philosophy of massive parallel

sequencing is still the same and the term next- generation sequencing (NGS) will only be used in this review. More detailed technical description of these platforms is available elsewhere.

Transitioning from SGS to TGS

The genomics community has been enormously enabled by first- and second-generation sequencing (SGS) technologies in comprehensively characterizing DNA sequence variation, de novo sequencing of a number of species, sequencing of microbiomes, detecting methylated regions of the genome, quantitating transcript abundances, characterizing different isoforms of genes present in a given sample and identifying the degree to which mRNA transcripts are being actively translated. One of the hallmark features of the SGS technologies is their massive throughput at a modest cost, with hundreds of gigabases of sequencing now possible in a single run for several thousand dollars. Despite the recent and rapid acceptance of SGS technologies, a new generation of single-molecule sequencing (SMS) technologies is emerging. Unlike major SGS sequencing by synthesis (SBS) technologies that rely on PCR to grow clusters of a given DNA template, attaching the clusters of DNA templates to a solid surface that is then imaged as the clusters are sequenced by synthesis in a phased approach, the new generation of SBS technologies interrogate single molecules of DNA, such that no synchronization is required (a limitation of SGS), thereby overcoming issues related to the biases introduced by PCR amplification and dephasing. More importantly, this new generation of sequencing technologies has the potential to exploit more fully the high catalytic rates and high processivity of DNA polymerase or avoid any biology or chemistry altogether to radically increase read length (from tens of bases to tens of thousands of bases per read) and time to result (from days to hours or minutes). The promises then of this new, third generation of sequencing technologies in offering advantages over current sequencing technologies are (i) higher throughput; (ii) faster turnaround time (e.g. sequencing metazoan genomes at high fold coverage in minutes); (iii) longer read lengths to enhance de novo assembly and enable direct detection of haplotypes and even whole chromosome phasing; (iv) higher consensus accuracy to enable rare variant detection; (v) small amounts of starting material (theoretically only a single molecule may be required for sequencing); and (vi) low cost, where sequencing the human genome at high fold coverage for less than $100 is now a reasonable goal for the community.

There may not yet be consensus on what constitutes a third generation, or next–next-generation sequencing instrument, given advances are being made on rapid time scales that do not easily fit into generational time scales. However, SMS without the need to halt between read steps (whether enzymatic or otherwise), where reads from SMS instruments represent sequencing of a single molecule of DNA. SMS technologies that do not purposefully pause sequencing reaction after each base incorporation represent the most thoroughly explored TGS approaches in hopes of increasing sequencing rates, throughput and read lengths, lowering the complexity of sample preparation and ultimately decreasing cost. However, as a result of using these criteria to define

TGS, a number of exciting technologies do not fit neatly into this definition, but are nevertheless exciting in terms of how they complement current SGS technologies.

Table 1. Comparison of First generation, second generation and third generation sequencing techniques.

	First generation	Second generation[a]	Third generation[a]
Fundamental technology	Size-separation of specifically end-labeled DNA fragments, produced by SBS or degradation	Wash-and-scan SBS	SBS, by degradation, or direct physical inspection of the DNA molecule
Resolution	Averaged across many copies of the DNA molecule being sequenced	Averaged across many copies of the DNA molecule being sequenced	Single-molecule resolution
Current raw read accuracy	High	High	Moderate
Current read length	Moderate (800–1000 bp)	Short, generally much shorter than Sanger sequencing	Long, 1000 bp and longer in commercial systems
Current throughput	Low	High	Moderate
Current cost	High cost per base Low cost per run	Low cost per base High cost per run	Low-to-moderate cost per base Low cost per run
RNA-sequencing method	cDNA sequencing	cDNA sequencing	Direct RNA sequencing and cDNA sequencing
Time from start of sequencing reaction to result	Hours	Days	Hours
Sample preparation	Moderately complex, PCR amplification not required	Complex, PCR amplification required	Ranges from complex to very simple depending on technology
Data analysis	Routine	Complex because of large data volumes and because short reads complicate assembly and alignment algorithms	Complex because of large data volumes and because technologies yield new types of information and new signal processing challenges
Primary results	Base calls with quality values	Base calls with quality values	Base calls with quality values, potentially other base information such as kinetics

Next- Next-generation sequencing platforms (Third Generation Sequencing)

SMS technologies can roughly be binned into three different categories: (i) SBS technologies in which single molecules of DNA polymerase are observed as they synthesize a single molecule of DNA; (ii) nanopore-sequencing technologies in which single molecules of DNA are threaded through a nanopore or positioned in the vicinity of a nanopore, and individual bases are detected as they pass through the nanopore; (iii) direct imaging of individual DNA molecules using advanced microscopy techniques etc. Each of these technologies provides novel approaches to sequencing DNA and has advantages and disadvantages with respect to specific applications. These technologies are at varying stages of development, making the writing of a review on TGS difficult given there is still much to prove regarding the utility of many of the TGS technologies. However, if the full potential of these technologies is realized, in several years time, whole genome sequencing will likely be fast enough and inexpensive enough to resequence genomes as needed for any application. Here we discuss many of the emerging TGS technologies that have the potential to make such stunning advances possible.

Since single DNA molecule sequencing technology can read through DNA templates in real time without amplification, it provides accurate sequencing data with potentially long-reads and efforts have focused recently in this new direction. Several unique single- molecule DNA

sequencing technologies are currently under development; however, little information has been made publically available.

(i) SMS sequencing by synthesis

Single-molecule real-time sequencing: The single-molecule real-time (SMRT) sequencing approach developed by Pacific Biosciences is the first TGS approach to directly observe a single molecule of DNA polymerase as it synthesizes a strand of DNA, directly leveraging the speed and processivity of this enzyme to address many of the shortcomings of SGS. Given that a single DNA polymerase molecule is of the order of 10 nm in diameter, two important obstacles needed to be overcome to enable direct observation of DNA synthesis as it occurs in real time are: (i) confining the enzyme to an observation volume that was small enough to achieve the signal-to-noise ratio needed to accurately call bases as they were incorporated into the template of interest; and (ii) labeling the nucleotides to be incorporated in the synthesis process such that the dye–nucleotide linker is cleaved after completion of the incorporation process so that a natural strand of DNA remains for continued synthesis and so that multiple dyes are not held in the confinement volume at a time (something that would destroy the signal-to-noise ratio). The problem of observing a DNA polymerase working in real time, detecting the incorporation of a single nucleotide taken from a large pool of potential nucleotides during DNA synthesis, was solved using zero-mode waveguide (ZMW) technology.

(ii) Nano-technologies for single-molecule sequencing

Thousands of nano-tunnels on a chip can be used to monitor the movement of a polymerase molecule on a single DNA strand during replication to perform single-molecule DNAsequencing-by-synthesis. Nano-technologies have long been considered a cutting-edge technology for single-molecule DNA sequencing and several nanopore sequencing concepts and technologies are currently under development. One concept is based on the observation that when a DNA strand is pulled through a nanopore by an electrical current, each nucleotide base (A, T, C, G) creates a unique pattern in the electrical current. This unique nanopore electrical current fingerprint can be used for nanopore sequencing.

(iii) Fluorescence-based single-molecule sequencing

Pacific BioSciences is developing a single-molecule real time (SMRT) DNA sequencing technology. This approach performs single-molecule sequencing by identifying nucleotides which are phospholinked with distinctive colors. During the synthesis process, fluorescence emitted as the phosphate chain is cleaved and the nucleotide is incorporated by a polymerase into a single DNA strand.

(iv) Electronic detection for single-molecule sequencing

Reveo is developing a technology to stretch out DNA molecules on conductive surfaces for electronic base detection. A stretched and immobilized strand of DNA will be read through by

multiple nano-knife edge probes. Each nano-knife edge probe specifically recognizes only one nucleotide for single-molecule sequencing. Intelligent Biosystems is also developing a platform using the electronic detection approach which will allow for high speed and high sensitivity single-molecule analysis with decreased background noise.

(v) Electron microscopy for single-molecule sequencing

Electron microscopy (EM) was the first proposed and attempted approach to sequence DNA molecules before the Sanger sequencing was established and this concept has recently been reevaluated with the emergence of new technologies. Since scanning tunneling microscopy (STM) can reach atomic resolution, STM for single-molecule sequencing is being explored. LightSpeed Genomics is developing a microparticle approach by capturing sequence data with optical detection technology and new sequencing chemistry from a large field of view to reduce the time consuming sample and detector rearrangement. Halcyon Molecular is developing a DNA sequencing technology by atom-by-atom identification and EM analysis. The key advantage of this technology is very long read lengths. ZS Genetics is also developing EM-based technologies for single-molecule DNA sequencing.

Other approaches for single-molecule sequencing

Ion Torrent developed an entirely new approach to sequencing based on the well- characterized biochemistry that when a nucleotide is incorporated into a strand of DNA by a polymerase, a hydrogen ion is released as a byproduct. They have developed an ion sensor that can detect hydrogen ions and directly convert the chemical information to digital sequence information. In essence, their NGS platform can be defined as the world's smallest solid-state pH meter.

Focusing on resequencing specific sections of the human genome combined with genome- region enrichment, Genizon BioSciences is developing a sequencing-by-hybridization technology based on known reference sequences. Avantome (acquired by Illumina) is also exploring the single-molecule sequencing technologies.

Road to the personal genome project

Since the initiation of 1000 genome project the cost of sequencing an individual genome has been rapidly decreasing and will likely reach $1000 per person within a short period of time, making personalized medicine become a possible reality. In genomics, the personal genome era made available by NGS technologies will mark a significant milestone in entire genomic research field in the foreseeable future. It is not clear which NGS technology will eventually dominate the genomic research field, but it is almost certain that further reductions in cost, rapid increases in sequencing speed with improved accuracy, and the advantages conferred by these new technologies will assure that NGS will become an essential molecular tool affecting all aspects of the biological sciences. Detailed information of the NGS technologies and platform discussed above is summarized in Table 2.

Table 2. Platforms and detailed information on NGS technologies

Technology	Amplification	Read length	Throughput	Sequence by synthesis
Currently available				
Roche/GS-FLX Titanium	Emulsion PCR	400–600 bp	500 Mbp/run	Pyrosequencing
Illumina/HiSeq 2000, HiScan	Bridge PCR (Cluster PCR)	2 × 100 bp	200 Gbp/run	Reversible terminators
ABI/SOLiD 5500xl	Emulsion PCR	50–100 bp	>100 Gbp/run	Sequencing-by-ligation (octamers)
Polonator/G.007	Emulsion PCR	26 bp	8–10 Gbp/run	Sequencing-by-ligation (monomers)
Helicos/Heliscope	No	35 (25–55) bp	21–37 Gbp/run	True single-molecule sequencing (tSMS)
In development				
Pacific BioSciences/RS	No	1000 bp	N/A	Single-molecule real time (SMRT)
Visigen Biotechnologies	No	>100 Kbp	N/A	Base-specific FRET
U.S. Genomics	No	N/A	N/A	Single-molecule mapping
Genovoxx	No	N/A	N/A	Single-molecule sequencing by synthesis
Oxford Nanopore Technologies	No	35 bp	N/A	Nanopores/exonuclease-coupled
NABsys	No	N/A	N/A	Nanopores
Electronic BioSciences	No	N/A	N/A	Nanopores
BioNanomatrix/nanoAnalyzer	No	400 Kbp	N/A	Nanochannel arrays
GE Global Research	No	N/A	N/A	Closed Complex/nanoparticle
IBM	No	N/A	N/A	Nanopores
LingVitae	No	N/A	N/A	Nanopores
Complete Genomics	No	70 bp	N/A	DNA nanoball arrays
base4innovation	No	N/A	N/A	Nanostructure arrays
CrackerBio	No	N/A	N/A	Nanowells
Reveo	No	N/A	N/A	Nano-knife edge
Intelligent BioSystems	No	N/A	N/A	Electronics
LightSpeed Genomics	No	N/A	N/A	Direct-read Sequencing by EM
Halcyon Molecular	No	N/A	N/A	Direct-read Sequencing by EM
ZS Genetics	No	N/A	N/A	Direct-read Sequencing by TEM
Ion Torrent/PostLight	No	N/A	N/A	Semiconductor-based pH sequencing
Genizon BioSciences/CGA	No	N/A	N/A	Sequencing-by-hybridization

Current strategies for the NGS project

To ensure the correct identification of genetic variants, short-read coverage must be sufficient to ensure the complete and accurate sequence assembly. Currently, at least 30× coverage is recommended in whole-genome scans for rare genetic variants in human genomes, which is a burden on computer resources and cost management. Although the cost of whole-genome sequencing has dropped substantially, the cost remains a major obstacle; whole-genome sequencing of a single individual currently costs approximately $100,000.00. By targeting specific regions of interest, selective DNA enrichment techniques improve the overall cost and efficiency of NGS; however, targeted enrichment must maintain uniform coverage, high reproducibility, and no allele bias for any genomic region. Targeted sequencing generally focuses on all protein-coding subsequences (the functional exome), which only requires ~5% as

much sequencing compared to that required for the entire human genome. This strategy currently reduces the overall cost to around $10,000 or less for the sequencing of a single individual. An important consideration to the cost of such experiments is the depth of sequence coverage required to achieve a desired sensitivity and specificity of at least 25-fold nominal sequence coverage. The most common techniques for targeted sequence enrichment are either microarray-based or solution hybrid- based. Several targeted selection technologies have been marketed and successfully applied in different NGS projects with variable success and may become the tools of choice to lower the burden of time and cost. For example, using targeted selection strategy, the mutations in DHODH from four individuals from three unrelated families with Miller syndrome have been successfully identified, illustrating that selective DNA enrichment techniques will dramatically reduce overall cost and accelerate discovery of genetic variants that cause rare and yet to be discovered genetic disorders. Other genetic loci for rare diseases have also been successfully identified through exome sequencing, further validating this strategy. Commercially available products for targeted sequence-enrichment include Agilent's SureSelect and NimbleGen's SeqCap/EZ Exome (both array- and solution-based technologies), RainDance and Illumina's TruSeq (solution-based technology), Febit's HybSelect and LC Sciences (microarray-based strategy), Qiagen and Fluidigm (PCR-based method) (Table 3).

Table 3. Targeted sequence- enrichment technologies for NGS.

Technology	Approach	Platform	Website
Agilent/SureSelect	Array- and solution-based	Illumina/Roche/ABI	http://www.chem.agilent.com/
RainDance	Microdroplet-based	Illumina/Roche/ABI	http://www.raindancetechnologies.com/
NimbleGen/SeqCap/EZ Exome	Array- and solution-based	Illumina/Roche/ABI	http://www.nimblegen.com/products/seqcap/index.html
Febit/HybSelect	Microarray-based	Illumina/Roche/ABI	http://www.febit.com/microarray-sequencing/index.cfm
Fluidigm	PCR-based	Illumina/Roche/ABI	http://www.fluidigm.com/targeted-resequencing.html
Mycroarray/Myselect	Solution-based	Illumina/Roche/ABI	http://www.mycroarray.com/products/myselect.html
LC Sciences	Microarray-based	Illumina/Roche/ABI	http://www.lcsciences.com/applications/genomics/
Qiagen/SeqTarget	Long-range PCR-based	Illumina/Roche/ABI	http://www.qiagen.com/products/seqtargetsystem.aspx
Illumina/TruSeq	Solution-based	Illumina/Roche/ABI	http://www.illumina.com/applications.ilmn

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GENOME EDITING USING CRISPR/CAS (CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS -ASSOCIATED PROTEINS) SYSTEM

A I Bhat

Clustered regularly-interspaced short palindromic repeats (CRISPR) is widely found in bacterial and archaeal genomes as a defense mechanism against invading viruses and plasmids. The CRISPR locus consists of segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a bacterial virus or plasmid. CRISPR spacers recognize and cut these exogenous genetic elements in a manner analogous to RNA interference in eukaryotic organisms. CRISPRs are found in approximately 40% of sequenced bacteria genomes and 90% of sequenced archaea. The CRISPR interference technique has enormous potential application, including altering the germline of humans, animals and other organisms, and plants. By delivering the Cas9 protein and appropriate guide RNAs into a cell, the organism's genome can be cut at any desired location. CRISPRs have been used in concert with specific endonuclease enzymes for genome editing and gene regulation in species throughout the tree of life.

Discovery

Clustered repeats were first described in 1987 for the bacterium *Escherichia coli* by Yoshizumi Ishino. Later observed by Francisco Mojica in 1993 and named them as short regularly spaced repeats (SRSR). SRSR was renamed as CRISPR in 2002. In 2005, three independent research groups showed that some CRISPR spacers are derived from phage DNA and plasmids. Source of the spacers was a sign that the CRISPR/cas system could have a role in adaptive immunity in bacteria. Koonin and colleagues proposed that spacers serve as a template for RNA molecules, analogous to a system called RNA interference used by eukaryotic cells. CRISPR was first shown to work as a genome engineering/editing tool in human cell culture in 2012. It has since been used in a wide range of organisms including baker's yeast (*S. cerevisiae*), zebrafish (*D. rerio*), fruit flies (*D. melanogaster*), nematodes (*C. elegans*), plants, mice, monkeys and human embryos. CRISPR has been modified to make programmable transcription factors that allow scientists to target and activate or silence specific genes.

CRISPR associated (Cas) genes

A set of genes was found to be associated with CRISPR repeats, and was named the *cas*, or *CRISPR-associated*, genes (Fig. 1). The *cas* genes encode putative nuclease or helicase proteins, which are enzymes that can cut or unwind DNA. Jennifer Doudna and Emmanuelle Charpentier found that bacteria respond to an invading phage by transcribing spacers and palindromic DNA into a long RNA molecule. The cell then uses transacting CRISPR RNA (tracrRNA) and Cas9 to cut this long RNA molecule into pieces called CRISPR RNAs (crRNAs). Cas9 is a nuclease, an enzyme specialized for cutting DNA. It has two active cutting sites (HNH and RuvC), one for

each strand of the DNA's double helix. The team demonstrated that they could disable one or both sites while preserving Cas9's ability to home in on its target DNA. In 2012, a group including both Doudna and Charpentier combined tracrRNA and spacer RNA into a "single-guide RNA" molecule that, mixed with Cas9, could find and cut the correct DNA targets. Their study proposed that such synthetic guide RNAs could be used for gene editing.

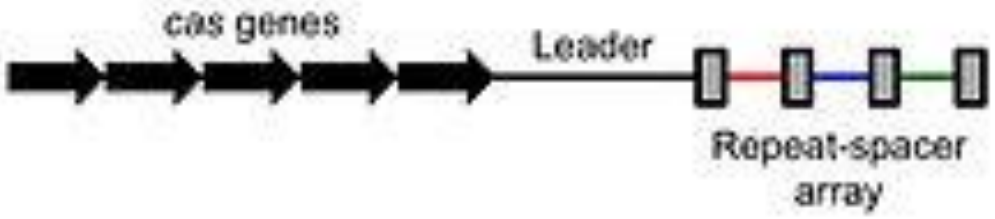

Fig. 1 Diagram of a CRISPR locus. The three major components of a CRISPR locus are shown: *cas* genes, a leader sequence, and a repeat-spacer array

Common terminologies used in CRISPR/Cas9 system

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas9	CRISPR associated protein 9
Protospacer	Complementary nucleotide sequence available on the target genome (DNA)
PAM	Protospacer Adjacent Motif: NGG available at the 3‘ end of the protospacer
crRNA	CRISPR RNA
sgRNA	Single guide RNA: sgRNA containing an approximately 20 base sequence specific to the target DNA 5′ of a non-variable scaffold sequence
Spacer	The specific sequence available on sgRNA which binds to the protospacer
HDR	Homology directed DNA repair
NHEJ	Non-homologous end-joining
DSBs	Double-strand DNA breaks

Functions of various components of CRISPR/cas system

Component	**Function**
crRNA	Contains the RNA used by Cas9 to guide it to the correct section of host DNA along with a region that binds to tracrRNA (generally in a hairpin loop form) forming an active complex with Cas9
tracrRNA	Binds to crRNA and forms an active complex with Cas9
sgRNA	Single guide RNAs are a combined RNA consisting of a tracrRNA and at least one crRNA

Cas9	Protein that in its active form is able to modify DNA utilizing crRNA as its guide. Many variants exist with differing functions (i.e. single strand nicking, double strand break, DNA binding) due to Cas9's DNA site recognition function that is independent of its two DNA cleaving domains (one for each strand).
Repair template	DNA that guides the cellular repair process allowing insertion of a specific DNA sequence

Repeats and spacers

CRISPR repeats range in size from 24 to 48 base pairs. They usually show some dyad symmetry, implying the formation of a secondary structure such as a hairpin, but are not truly palindromic. Repeats are separated by spacers of similar length. Some CRISPR spacer sequences exactly match sequences from plasmids and phages, although some spacers match the prokaryote's genome (self-targeting spacers). New spacers can be added rapidly as part of the immune response to phage infection.

Cas genes and CRISPR subtypes

Cas genes are often associated with CRISPR repeat-spacer arrays. Comparative genomics identified multiple cas genes; an initial analysis of 200 bacterial and archaeal genomes suggested as many as 45 cas gene families. Only cas1 and cas2 genes are present in all 45 families. Three types of CRISPR/Cas system have been described (i) Type I, which cleaves and degrades target DNA (ii) Type II cleaves target DNA without causing degradation and (iii) Type III, which cleaves DNA or RNA. Most work on engineering DNA editing has entailed use of the type II system. The type II CRISPR locus contains a cluster of four genes, Cas9, Cas1, Cas2 and Csn1 as well as two non-coding RNA elements, tracr RNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, 30 bp each). Each spacer is typically derived from foreign genetic material (protospacer) and drives the specificity of CRISPR- mediated nucleic acid cleavage.

Mechanism of CRISPR/Cas

The type II CRISPR/Cas system comprised of CRISPR RNA (crRNA), RNA-guided Cas9 nuclease, and a partially complementary *trans*-acting crRNA (tracrRNA), is most widely used for genome editing due to its simplicity. In this effector system, short sequences (also known as protospacers) from invading genes are copied into the host genome to form the spacer-repeat CRISPR locus that is transcribed into pre-crRNA. After hybridization with tracrRNAs, these pre-crRNAs are cleaved by endogenous RNase III and mature to guide the Cas9 to the complementary genomic target sequence. The resulting crRNA-tracrRNA-Cas9 complex requires a protospacer-adjacent motif (PAM) site at the 3′ end of the target 20-bp sequence matching the protospacer to cleave and introduce DSBs (Fig. 2). The widely-used *Streptococcus pyogenes* Cas9 (SpCas9) recognizes a 5′-NGG-3′ PAM (N is any nucleotide) sequence, whereas Cas9 proteins from other species bind to different PAM sequences. Further simplification of this

system by fusing the crRNA and tracrRNA into a chimeric RNA, called single-guide RNA (sgRNA or gRNA), has resulted in the widespread employment of CRISPR/Cas9 system for genome editing.

Once these have been assembled into a plasmid and transfected into cells the Cas9 protein with help of the crRNA finds the correct sequence in the host cell's DNA and – depending on the Cas9 variant – creates a single or double strand break in the DNA (Fig. 3). Properly spaced single strand breaks in the host DNA can trigger homology directed repair, which is less error prone than non-homologous end joining that typically follows a double strand break. The non-homologous end joining (NHEJ) pathway generates insertions and deletions during double-stranded break (DSB) repair. However, in the presence of a DNA template with homology to the sequences flanking the DSB location, homology-directed repair (HDR) can seal the DSB in an error free manner (Fig. 3). In most cells both of these repair pathways are active, however the HDR pathway is generally less efficient that the NHEJ. The efficiency of HDR is determined by the concentration of donor DNA present at the time of repair, the length of the homology arms of the donor DNA, the cell cycle, and the activity of the endogenous repair systems. Donor arms are at least 500 bp in length. Inserts between the homology arms are frequently in the 1–2 kb range longer inserts are possible, but the efficiency of recombination decreases as the insert size increases

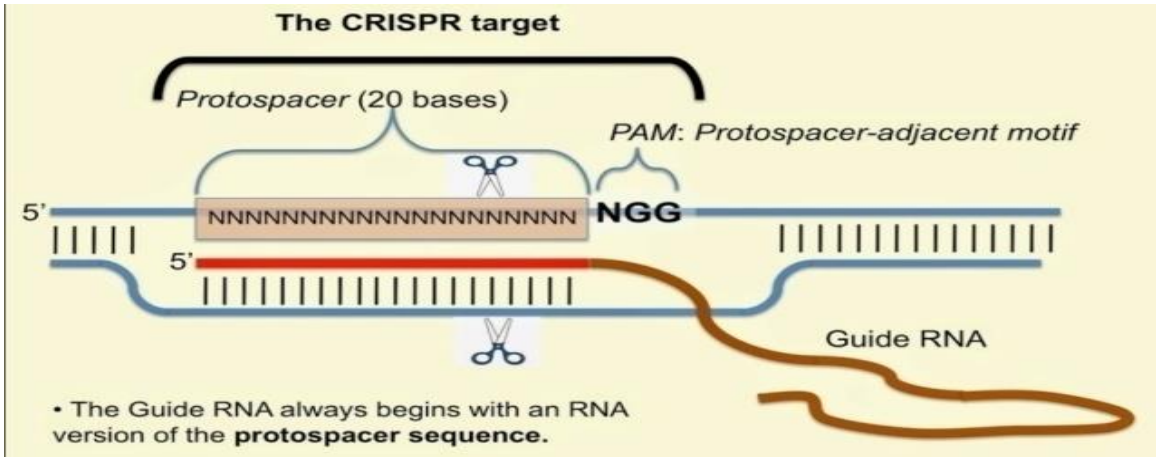

Fig. 2 Binding of crRNA to the target DNA. Note the PAM region at 3´ end of the target (protospacer) region

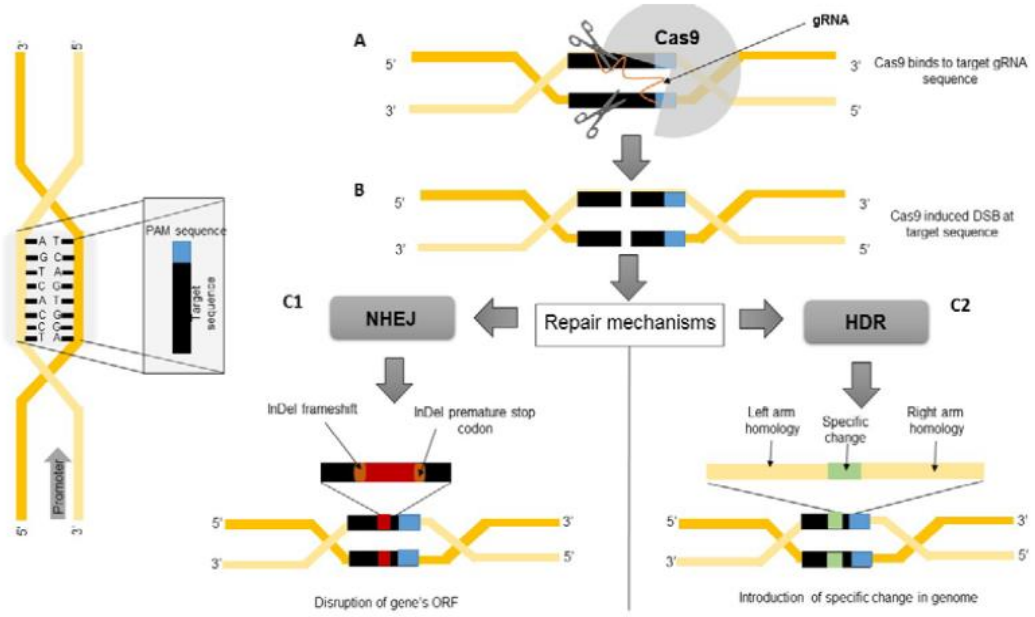

Fig. 3 Gene editing using CRISPR/Cas9 based cleavage and subsequent repair of the target DNA region

Application in plants

A schematic diagram showing various steps involved in genome editing in plants using CRISPR/Cas system is provided in Fig. 4. CRISPR/cas system generates stable and heritable mutants which can easily segregate from cas9/sgRNA construct that results in the development of transgene-free plants. It is a new plant breeding technique that is faster than the traditional method. Plants produced through CRISPR/cas are not GM crops; they are genetically edited (GE) crops hence probably socially acceptable. It can be used to get pest resistance, enhanced nutrional value, climate resilient crops, increased productivity etc. Multiplex genome editing using CRISPR/Cas 9 based binary vector set and a gRNA module vector set shown possible in plants. Plant viruses (*Cabbage leaf curl virus, Tobacco rattle virus*) can be used as vectors to deliver cas 9/sgRNA to plants. Recombinant viruses carrying cas 9/sgRNA can systemically spread throughout the plant system editing the genome in all cells of the plant. CRISPR can also be used to get virus resistance in plants by editing plant genome (not viral genome).

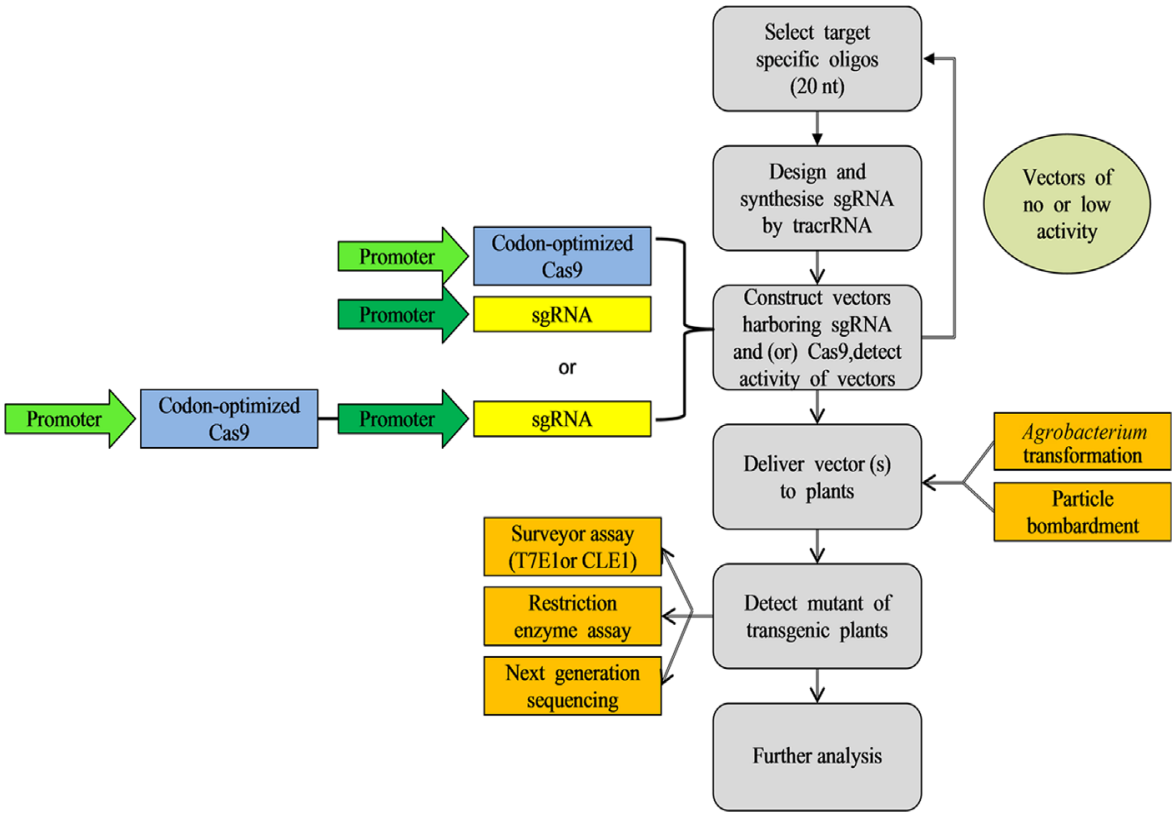

Fig. 4 Schematic diagram showing various steps involved in genome editing in plants using CRISPR/Cas system

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INTRODUCTION TO BIOINFORMATICS

I P Vijesh Kumarand Blessy M. Baby

Bioinformatics is all about searching biological databases, comparing sequences, analyzing protein structures and using computer as a means for asking various biological and biomedical questions. It actually saves months of work in the lab at the minute cost of few hours work with the computer. Before the advent of bioinformatics there were only two means of performing biological experiments: *in vivo* which is within a living organism or *in vitro* which is in external or artificial environment. Taking cue from this we can say that bioinformatics is *in silico* biology as the computers are mainly manufactured by silicon chips.

Bioinformatics is at the forefront of the most recent developments in biology, system biology, decoding of the human genome, new legal and forensic techniques, new biotechnologies, as well as personalized medicine in the near future.

History of sequence analysis

The first sequence of a protein to be deciphered was that of insulin by Alfred Sanger. It was a beginning for sequencing and sequence analysis. In pre-computer era sequences were analyzed and assembled by writing them on paper and then pasting them on wall and then looking for an optimal alignment.

BIOLOGICAL DATABASE

Sequence databases are great tools because they offer a unique window on the past. They make it possible to answer today's biological questions by enabling us to analyze sequences that may have been determined as many as 25 years ago, when the whole technology emerged. By doing this, they connect past and present molecular biology. The first databases were in fact created as some sort of sequence museum, where sequences could be preserved for all eternity in pristine form, just as they were determined, interpreted, and published by their original authors. This historical perspective pretty much remains in GenBank, the leading nucleotide sequence repository maintained as a consortium between the U.S. National Center for Biotechnology Information (NCBI), the European Molecular Biology Laboratory (EMBL), and the DNA Data Bank of Japan (DDBJ).

Databases in general can be classified in to primary, secondary and composite databases. A primary database contains information of the sequence or structure alone. Examples of these include Swiss-Prot & PIR for protein sequences, GenBank & DDBJ for Genome sequences and the Protein Databank for protein structures. All published genome sequences are available over the internet, as it is a requirement of every scientific journal that any published DNA or RNA or protein sequence must be deposited in a public database. The main resources for storing and distributing sequence data are three large databases: the NCBI database (www.ncbi.nlm.nih.gov/), the European Molecular Biology Laboratory (EMBL) database (www.ebi.ac.uk/embl/), and the

DNA Database of Japan (DDBJ) database (www.ddbj.nig.ac.jp/). They are known as primary databases. These databases collect all publicly available DNA, RNA and protein sequence data and make it available for free. They exchange data nightly, so contain essentially the same data.

Fig. 1: Various classification of biological database

A secondary database contains derived information from the primary database, like the conserved sequence, signature sequence and active site residues of the protein families. A secondary structure database contains entries of the PDB in an organized way or information on conserved secondary structure motifs of a particular protein. Some of the secondary databases created and hosted by various researchers at their individual laboratories include SCOP, CATH, PROSITE, eMOTIF

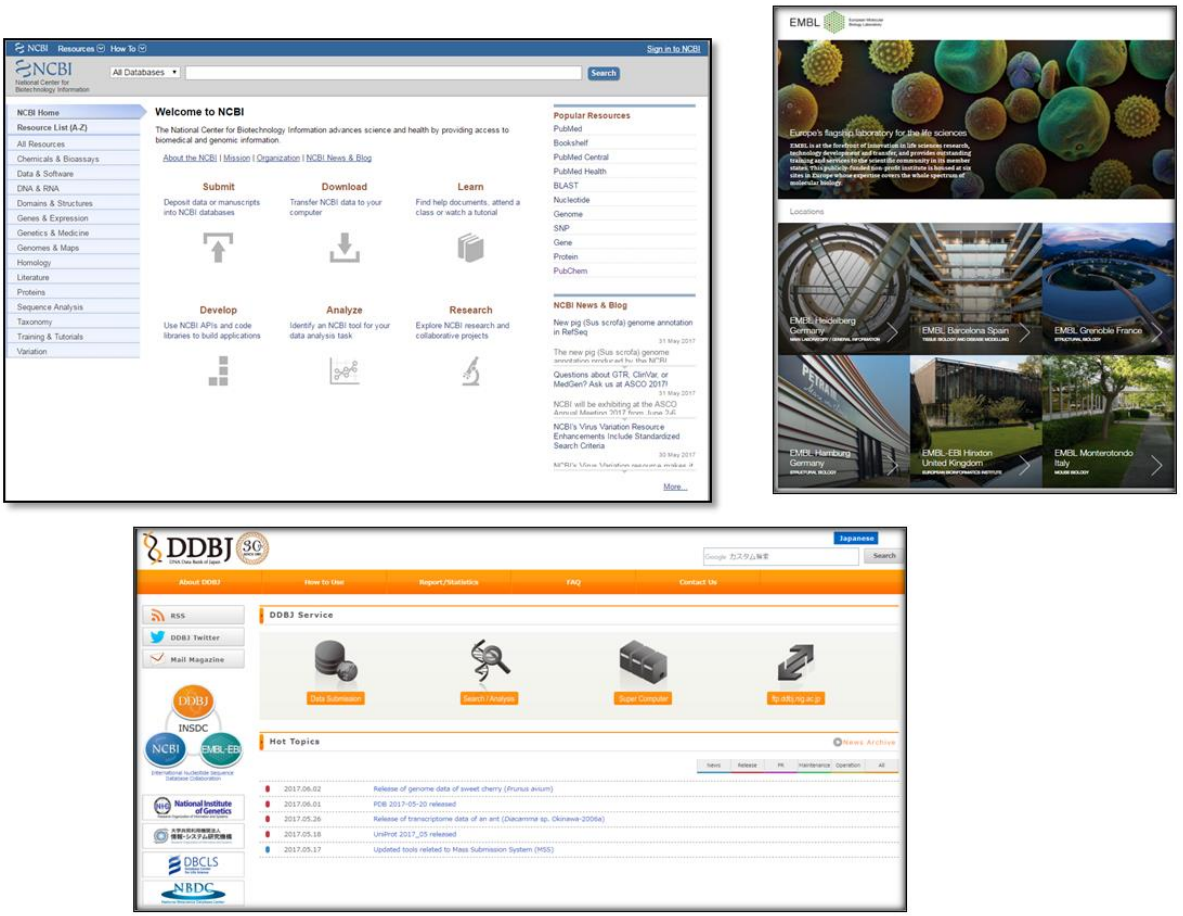

Fig. 2: Home page of the three major nucleotide database: NCBI, EMBL and DDBJ

Other than these, there are several other type of databases:

- Secondary Databases
    - RefSeq
    - OMIM
    - HapMap
- Gene Expression Databases (mostly Microarray data)
    - ArrayExpress
    - Gene Expression Omnibus
- Genome Databases
    - Ensembl Genomes
    - Wormbase
    - TAIR
- Phenotype Databases
    - PhenCode
    - PhenomicDB
- RNA Databases
    - Rfam

    - miRBase
- Protein Sequence Databases
    - UniProt
    - Protein Information Resource
    - Swiss-Prot
    - PROSITE
    - Pfam
    - PRINTS

SEQUENCE ANALYSIS

Sequence analysis is the process of subjecting a DNA, RNA or peptide sequence to any of a wide range of analytical methods to understand its features, function, structure, or evolution. Methodologies used include sequence alignment, searches against biological databases, and others. Sequence alignment is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences.

Global and local alignments

Global alignments, which attempt to align every residue in every sequence, are most useful when the sequences in the query set are similar and of roughly equal size. (This does not mean global alignments cannot start and/or end in gaps.) A general global alignment technique is the Needleman-Wunsch algorithm, which is based on dynamic programming. Local alignments are more useful for dissimilar sequences that are suspected to contain regions of similarity or similar sequence motifs within their larger sequence context. The Smith-Waterman algorithm is a general local alignment method also based on dynamic programming.

Local vs. Global

- Global alignment – finds the best alignment across the whole two sequences.

```
ADLGAVFALCDRYFQ
||||      ||||  |
ADLGRTQN-CDRYYQ
```

- Local alignment – finds regions of similarity in parts of the sequences.

```
ADLG    CDRYFQ
||||    ||||  |
ADLG    CDRYYQ
```

PAIRWISE ALIGNMENT

Pairwise sequence alignment methods are used to find the best-matching piecewise (local) or global alignments of two query sequences. Pairwise alignments can only be used between two sequences at a time, but they are efficient to calculate and are often used for methods that do not require extreme precision (such as searching a database for sequences with high similarity to a query). The three primary methods of producing pairwise alignments are dot-matrix methods, dynamic programming, and word methods

The technique of dynamic programming can be applied to produce global alignments via the Needleman-Wunsch algorithm, and local alignments via the Smith-Waterman algorithm Word methods, also known as k-tuple methods, are heuristic methods that are not guaranteed to find an optimal alignment solution, but are significantly more efficient than dynamic programming. Word methods are best known for their implementation in the database search tools FASTA and the BLAST family.

BLAST is the most common tool used for sequence analysis. There are different types of BLAST programs available. Blastp and blastx for protein. Blastn, tblastn, tblastx for DNA. Sometimes BLAST cannot give us all the members of a very large protein family, starting with the one sequence that we have. When running BLAST, the most closely related sequences are obtained. For finding the other distant cousins PSI-BLAST is used.

Some of the blast and PSI-BLAST servers around the world

Table	BLAST and PSI-BLAST Servers around the World	
Country/ Continent	*Program*	*URL*
USA	BLAST/PSI-BLAST	www.ncbi.nlm.nih.org/BLAST
Europe	BLAST	www.expasy.ch/tools/blast/
Europe	BLAST	www.ch.embnet.org/software/bBLAST.html
Europe	BLAST	www.ebi.ac.uk/blast
Japan	BLAST/PSI-BLAST	www.ddbj.nig.ac.jp/search/blast-e.html

Fig. 5: Commonly used BLAST programs and their host countries and website

MULTIPLE SEQUENCE ALIGNMENT Multiple sequence alignment is an extension of pairwise alignment to incorporate more than two sequences at a time. Multiple alignment methods try to align all of the sequences in a given query set. Multiple alignments are often used in identifying conserved sequence regions across a group of sequences hypothesized to be evolutionarily related. Such conserved sequence motifs can be used in conjunction with structural and mechanistic information to locate the catalytic active sites of enzymes. Alignments are also used to aid in establishing evolutionary relationships by constructing phylogenetic trees.

Main Criteria for Building a Multiple Sequence Alignment

Structural similarity-Amino acids that play the same role in each structure are in the same column. Structure-superposition programs are the only ones that use this criterion.

Evolutionary similarity-Amino acids or nucleotides related to the same amino acid (or nucleotide) in the common ancestor of all the sequences are put in the same column. No automatic program explicitly uses this criterion, but they all try to deliver an alignment that respects it.

Functional similarity-Amino acids or nucleotides with the same function are in the same column. No automatic program explicitly uses this criterion, but if the information is available, you can force some programs to respect it — or you can edit your alignment manually.

Sequence similarity-Amino acids in the same column are those that yield an alignment with maximum similarity. Most programs use sequence similarity because it is the easiest criterion. When the sequences are closely related, their structural, evolutionary and functional similarities are equivalent to sequence similarity.

Main Applications of Multiple Sequence Alignments

APPLICATIONS	PROCEDURE
Extrapolation	A good multiple alignment can help convince you that an uncharacterized sequence is really a member of a protein family. Alignments that include Swiss-Prot sequences are the most informative.
Phylogenetic analysis	If you carefully choose the sequences you include in your multiple alignment, you can reconstruct the history of these proteins.
Pattern identification	By discovering very conserved positions, you can identify a region that is characteristic of a function (in proteins or in nucleic-acid sequences).
Domain identification	It is possible to turn a multiple sequence alignment into a profile that describes a protein family or a protein domain (PSSM). You can use this profile to scan databases for new members of the family.
DNA regulatory elements	You can turn a DNA multiple alignment of a binding site into a weight matrix and scan other DNA sequences for potentially similar binding sites.
Structure prediction	A good multiple alignment can give you an almost perfect prediction of your protein secondary structure for both proteins and RNA.
Non synonymous SNP analysis	Various gene alleles often have different amino-acid sequences. Multiple alignments can help you predict whether a Non-Synonymous Single-Nucleotide Polymorphism is likely to be harmful.

Table 9-9	Multiple Sequence Alignment Resources over the Internet	
Method	***Description***	***Address***
Tcoffee	Accurate combination of sequences and structures	www.tcoffee.org www.ch.embnet.org/software/TCoffee.html www.ebi.ac.uk/t-coffee/
Probcons	A Bayesian version of Tcoffee	probcons.stanford.edu/
MUSCLE	A fast and accurate sequence cruncher	www.drive5.com/muscle/
Kalign	A fast sequence aligner	msa.cgb.ki.se
MAFFT	A fast and accurate sequence cruncher using Fast Fourier Transforms	www.ebi.ac.uk/Tools/msa/mafft/
Dialign	Ideal for Sequences With Local Homology	bibiserv.techfak.uni-bielefeld.de/dialign/

Fig. 6: Commonly used MSA programs and their short description and website

SEQUENCE DATABASES

At the beginning of the "genomic revolution" a Bioinformatics concern was the creation and maintenance of a database to store biological information, such as nucleotide and amino acid sequences. Development of this type of database involved not only design issues, but also the development of complex interfaces whereby researchers could both access existing data as well as submit new or revised data.

Storage of macromolecular data like a gene, protein, an enzyme or a functional RNA in electronic databases is a complicated task. At present each entry into the databases is given a serial number and then stored in a relational database that knows the proper linkages between that serial number and all information pertaining to that entry. DNA sequence, protein sequence and protein structure, though well related are currently maintained as separate database projects and in unconnected data formats, because biological sequence and structure databases have evolved in parallel. The first molecular biology database was the Protein Data Bank (PDB), the central repository for X-ray crystal structures of protein molecules (at the Brookhaven National Laboratory in 1971). From 15 sets of co-ordinates in 1973, now it has about 64623 entries. Journals that publish crystallographic results now require submission to the PDB as a condition of publication.

Sequence databases generally specialize in one type of sequence data: DNA, RNA or protein. There are three major sequence data collections and deposition sites in Europe, Japan and the United States. The first DNA sequence database was the Gene Sequence Database (GSDB) at

Los Alamos National Lab in 1979. Now up-to-date gene sequence information is available from GSDB through the National Centre for Genome Resources. The European Molecular Biology Laboratory, the DNA Database of Japan and the National Institutes of Health co-operated to support the database GenBank. Numerous biological databases and information sources are available on the web.

Nucleotide sequence databases

GenBank (http://www.ncbi.nlm.nih.gov/genbank/): GenBank is the nucleotide sequence database built and distributed by the National Centre for Biotechnology Information (NCBI) at the National Institutes of Health. As on today Biotechnology contains more than 85 billion bases from over 100,000 species, and is growing exponentially and over 30,000 people per day access it. The data are obtained through direct submission of sequence data from individual laboratories, from large-scale sequencing projects, and from the US Patent and Trademark Office. A little more than half of the total sequences in the database are from *Homo sapiens.*

There are two ways to search GenBank: a text-based query can be submitted through the Entrez system at *www.ncbi.nlm.nih.gov/Entrez/* or a sequence query can be submitted through the BLAST family of programs (see http://www.ncbl.nlm.nih.gov/BLAST/). To search GenBank through the Entrez system you would select the Nucleotides database from the menu. The Entrez Nucleotides Database is a collection of sequences from several sources, including GenBank, RefSeq, and the Protein Databank, so you don't actually search GenBank exclusively. Searches of the Entrez Nucleotides database query the text and numeric fields in the record, such as the accession number, definition, keyword, gene name, and organism fields to name just a few. Nucleotide sequence records in the Nucleotides database are linked to the PubMed citation of the article in which the sequences were published. Protein sequence records are linked to the nucleotide sequence from which the protein was translated.

If you have obtained a record through a text-based Entrez Nucleotides Database search you can read the nucleotide sequence in the record. However, most researchers wish to submit a nucleotide sequence of interest to find the sequences that are most similar to theirs. This is done using the BLAST (Basic Local Alignment Search tool) programs. You select the BLAST program you wish to use depending upon the type of comparison you are doing (nucleotide to nucleotide, or nucleotide to protein sequence, etc.) and then you select the database to run the query in (any of several nucleotide or protein databases).

EMBL (http://www.ebi.ac.uk/embl/): “The EMBL Nucleotide Sequence Database constitutes Europe's primary nucleotide sequence resource. Main sources for DNA and RNA sequences are direct submissions from individual researchers, genome sequencing projects and patent applications. The database is produced in an international collaboration with GenBank (USA) and the DNA Database of Japan (DDBJ). Each of the three groups collects a portion of the total

sequence data reported worldwide, and all new and updated database entries are exchanged between the groups on a daily basis."

From the home page you can submit simple text searches to the EMBL Nucleotide Sequence Database, or to the Protein Databank (what you search when you select protein structures from the menu) or to a protein sequence database called Swall. For more complex searches, they recommend accessing the databases through the Sequence Retrieval System (SRS) server (littp://srs.ebi.ac.uk/). SRS is a database querying / navigation system, similar in function to the Entrez system. It allows you to simultaneously search across several databases and to display the results in many ways. SRS can be used to access a large number of databases, including EMBL, SWISS-PROT and the Protein Databank, depending upon the configuration of the particular SRS server you are using. The structure and content of an EMBL Nucleotide record is very similar to that of an NCBI Entrez Nucleotide database record.

DDBJ: DDBJ is the sole DNA data bank in Japan, which is officially certified to collect DNA sequences from researchers and to issue the internationally recognized accession number to data submitters. It collects data mainly from Japanese researchers, but of course accepts data and issues the accession number to researchers in any other countries and exchange the collected data with EMBL/EBI and GenBank/NCBI on a daily basis. The three data banks share virtually the same data at any given time.

Protein sequences databases

SWISS-PROT (http://us.expasy.org/sprot/): SWISS-PROT is a curated protein sequence database which strives to provide a high level of annotation (such as the description of the function of a protein, its domain structure, post-translational modifications, variants, etc.), a minimal level of redundancy and a high level of integration with other databases. The data in Swiss-Prot are derived from translations of DNA sequences from the EMBL Nucleotide Sequence Database, adapted from the Protein Identification Resource (PIR) collection, extracted from the literature and directly submitted by researchers. It contains high-quality annotations, is nonredundant, and cross-referenced to several other databases, notably the
EMBL nucleotide sequence database, PROSITE pattern database and PDB.

From the home page, a quick text search can be done by accession or ID number, description, gene name, or organism. By searching SWISS-PROT through the Sequence Retrieval System (SRS) more sophisticated searches can be performed and the format of the results can be customized. Access to SWISS-PROT (directly or via SRS) and links too many other proteomics resources are available from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) at http://ca.expasy.org/. The SWISS-PROT records are quite detailed. Be advised that other databases or search systems that import SWISS-PROT data may not always provide access to the entire SWISS-PROT record.

Entrez Protein Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein): The Protein database contains sequence data from the translated coding regions from DNA sequences in GenBank, EMBL and DDBJ as well as protein sequences submitted to PIR, SWISSPROT, PRF, and the Protein Data Bank (PDB) (sequences from solved structures). The native SWISS-PROT records usually contain more detailed annotations than will be obtained from Entrez Protein Database records derived from SWISS-PROT records. In typical Entrez fashion, results from a search of the Protein database link to PubMed, to the taxonomy database, to related sequences, and in some cases to pre-computed BLAST search results (look for BLink links).

Protein Information Resource - International Protein Sequence Database (PIRPSD) (http://pir.georgetown.edu/): In 1988 the Protein Information Resource (PIR), which is affiliated with Georgetown University Medical Center, established a cooperative effort with the Munich Information Center for Protein Sequences (MIPS) and the Japan International Protein Information Database (JIPID) to collect, publish and distribute the PIR-International Protein Sequence Database (PIR-PSD). They describe the database as "a comprehensive, non-redundant, expertly annotated, fully classified and extensively cross-referenced protein sequence database in the public domain". Text searches can be done in the title, species, author, citation, keyword, superfamily, feature and gene name fields. Gapped-BLAST sequence similarity searches are also an option. Note that both SWISS-PROT and the Entrez Protein database contain data adapted from the PIR.

Genome databases

Entrez Genome (http://www.ncbi.nlm.nih.gov/sites/genome): The whole genomes of over 6272 organisms can be found in Entrez Genomes. The genomes represent both completely sequenced organisms and those for which sequencing is in progress. All three main domains of life - bacteria, archaea, and eukaryota - are represented, as well as many viruses and organelles. Text searches can be done from the main page. Data can also be accessed alphabetically by species (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/allorg.html), or hierarchically by drilling down through a taxonomic list to a graphical overview for the genome of that organism, then to specific chromosomes, then on to specific genes. At each level are maps, pre-computed summaries, and analysis appropriate to that level, and links to related records from a variety of other Entrez databases. BLAST searches of some genomes are also possible.

Very useful pages for some of the most commonly studied species (e.g., human, mouse, fruit fly, malaria parasite) can be found on the Genomic Biology page under "organism-specific resources" (http://www.ncbi.nlm.nih.gov/Genomes/). These pages are so detailed that each could be classified as a comprehensive web site in itself. Each one brings together links to the genomic data, useful tools, related data sources and news about the genome of that species.

The Genome Database (GDB) (http://www.gdb.org/): The Genome Database is the official central repository for genomic mapping data resulting from the Human Genome Initiative. The

database contains three types of data: (1) regions of the human genome, including genes, clones, and ESTs, (2) maps of the human genome, including cytogenetic maps, linkage maps, radiation hybrid maps, content contig maps, and integrated maps (these maps can be displayed graphically via the Web), and (3) variations within the human genome including mutations and polymorphisms, plus allele frequency data. There are options to browse genes by chromosome, genes by symbol name, and genetic diseases by chromosome. There are multiple ways to search, including text-based searches for people, citations, segment names or accession numbers, and sequence searching via BLAST.

KEGG - Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/): Despite the name, this is actually a biochemical pathway database and gene catalog, not an encyclopedia in the book sense. The primary objective of KEGG is to computerize the current knowledge of molecular interactions; namely, metabolic pathways, regulatory pathways, and molecular assemblies. At the same time, KEGG maintains gene catalogs for all the organisms that have been sequenced and links each gene product to a component on the pathway. Because we need an additional catalog of building blocks, KEGG also organizes a database of all chemical compounds in living cells and links each compound to a pathway component.

Genome mapping in crop plants (http://ukcrop.net): UK CropNet was established in 1996 to apply a bioinformatics approach to the study of genome evolution in crop plants. It contains genome information for *Arabidopsis*, Barley, Brassica, Forage grasses, Millet and comparative analysis. All UK CropNet databases are freely available for anyone to download (though you will also need to download the necessary ACEDB software).

The TIGR *Arabidopsis thaliana* Database (http://www.tigr.org/tdb/e2kl/athl/): Provides access to *Arabidopsis* genomic sequence data and annotation generated at TIGR and assemblies of *Arabidopsis* ESTs from world-wide sequencing projects.

Arabidopsis Information Resource (TAIR) (www.arabidopsis.org): The Arabidopsis Information Resource (TAIR) provides a comprehensive resource for the scientific community working with *Arabidopsis thaliana*, a widely used model plant. TAIR consists of a searchable relational database, which includes many different datatypes. The data can be viewed using our interactive MapViewer, and analyzed.

Oryzabase (http://www.shigen.nig.ac.jp/rice/oryzabase/): The Oryzabase is a comprehensive rice science database established in 2000 by rice researcher's committee in Japan. The database is originally aimed to gather as much knowledge as possible ranging from classical rice genetics to recent genomics and from fundamental information.

GOBASE - Organelle genome database (http://megasun.bch.umontreal.ca/gobase): GOBASE is a taxonomically broad organelle genome database that organizes and integrates diverse data

related to organelles. The new version focuses on the mitochondrial and chloroplast subset of data. In its third phase, GOBASE will also include information on representative bacteria that are thought to be specifically related to the bacterial ancestors of mitochondria and chloroplasts.

Comprehensive Microbial Resource (CMR)

(http://cmr.jcvi.org/cgibin/CMR/CmrHomePage.cgi): CMR is a tool that allows the researcher to access all of the bacterial genome sequences completed to date. It contains the sequence and annotation of each of the completed genomes as well as associated information about the organisms (such as taxon and gram stain pattern), the structure and composition of their DNA molecules (such as plasmid vs. chromosome and GC content), and many attributes of the protein sequences predicted from the DNA sequence (such as pI and molecular weight).

PRACTICAL PROCEDURE

Searching for an accession number in the NCBI database

To view the NCBI entry for the DEN-1 Dengue virus (which has accession NC_001477), follow these steps:

1. Go to the NCBI website (www.ncbi.nlm.nih.gov).
2. Search for the accession number.
3. On the results page, if your sequence corresponds to a nucleotide (DNA or RNA) sequence, you should see a hit in the Nucleotide database, and you should click on the word 'Nucleotide' to view the NCBI entry for the hit. Likewise, if your sequence corresponds to a protein sequence, you should see a hit in the Protein database, and you should click on the word 'Protein' to view the NCBI entry for the hit.
4. After you click on 'Nucleotide' or 'Protein' in the previous step, the NCBI entry for the accession will appear.

Querying the NCBI Database

You may need to interrogate the NCBI Database to find particular sequences or a set of sequences matching given criteria, such as:

1. The sequence with accession NC_001477
2. The sequences published in Nature 460:352-358
3. All sequences from Chlamydia trachomatis
4. Sequences submitted by Matthew Berriman
5. Flagellin or fibrinogen sequences
6. The glutamine synthetase gene from Mycobacteriuma leprae
7. The upstream control region of the Mycobacterium leprae dnaA gene
8. The sequence of the Mycobacterium leprae DnaA protein
9. The genome sequence of Trypanosoma cruzi
10. All human nucleotide sequences associated with malaria

Sequence Alignment

Early in the days of protein and gene sequence analysis, it was discovered that the sequences from related proteins or genes were similar, in the sense that one could align the sequences so that many corresponding residues match. Comparison of related protein and nucleotide sequences have facilitated many recent advances in understanding the information content and function of genetic sequences. For this reason, techniques for aligning and comparing sequences, and for searching sequences databases for similar sequences, have become cornerstones of bioinformatics. Sequence alignment is a fundamental procedure conducted in any biological study that compares two biological sequences. The biological sequences can be DNA, RNA or Protein. It is the procedure by which one attempts to infer which positions within sequences are homologous i.e., which site shares a common evolutionary history. Sequence alignment is a necessary step that allows one to study deeper questions, such as identification and quantification of conserved regions or functional motifs, profiling of genetic disease, phylogenetic analysis and ancestral sequence profiling and prediction. Sequence alignment algorithms produces a hypothesis of homology, like other hypotheses, these alignments may contain more or less error depending on nature of data.

Why do we want to align sequences?

1. Assigning functions to unknown proteins
2. Determine relatedness of organisms
3. Identify structurally and functionally important elements
4. Make predictions about the 3D structure
5. Needed if we have an unknown DNA or protein sequence:
    - To find sequences/regions of significant similarity in a sequence repository or database.
    - To identify all of the homologous sequences in a database or repository.
    - To identify motifs or domains with a sequence similarity that is significantly better than chance expectation

Principles of sequence alignment

The resemblance of two DNA sequences taken from different organisms is based on the hypothesis that all genetic material had one ancestral ancient DNA. During the course of evolution, mutations occurred, creating differences between various families of species. Most of these differences are due to local mutations, each modifying the DNA sequence in a specific manner. These local modifications are of three types Insertion of a letter or several letters to the sequence, deletion of a letter or more from the sequence, substitution or replacement of letter in the sequence by another.

Table 1. Description of terms related to sequence similarity

Homologous	Characters are similar due to common ancestry
Analogous	Characters are similar due to convergent evolution
Orthologous	Characters are homologous with conserved function
Paralogous	Characters are homologous with divergent function

Xenologous	Sequence identity due to horizontal transfer

Types of Sequence Alignments

Global alignment

It is an alignment that assumes that the two proteins are basically similar over the entire length of one another. The alignment attempts to match them to each other from end to end, even though parts of the alignment are not very convincing.

An example:

```
ALAESRGPSTKDFGKISESREFDN
  |      ||||    | |     |
 DLNQLERASTKDINMILESRGPSN
```

Local alignment

Local alignment is an alignment that searches for segments of the two sequences that match well. There is no attempt to force entire sequences into an alignment, just those parts that appear to have good similarity, according to some criterion. Using the same sequences as above, one could get:

```
----------SRGPS----------
          |||||
----------SRGPS----------
```

It may seem that one should always use local alignments. However, it may be difficult to spot an overall similarity, as opposed to just a domain-to-domain similarity, if one uses only local alignment. So, global alignment is useful in some cases. The popular programs BLAST and FASTA for searching sequence databases produce local alignments.

Scoring Matrices

Scoring matrix describes the likelihood that two residue types would mutate to each other in evolutionary time. For example, consider two protein sequences, one of which has an alanine in a given position. A substitution to another small, hydrophobic amino acid, such as valine, would be less likely to have an impact on the function of the resulting protein than a substitution to a large, charged residue such as lysine. Thus, in scoring an alignment, we might want to score positions in which an alanine is aligned with a small more favorable amino acid than positions in which an alanine is aligned with a bulky or charged amino acid like lysine. For nucleotide sequence alignments, scoring matrices are generally quite simple. BLAST – commonly used tool for aligning and searching nucleotide sequences uses a very simple matrix that assigns a score of +5 if the two aligned nucleotides are identical, and -4 otherwise.

Transition / Transversion Matrix provides a mild reward for matching nucleotides, a mild penalty for transitions – substitutions in which a purine (A or G) is replaced with another purine or a pyrimidine (C or T) replaces another pyrimidine, and more severe penalty for transversions, in which a purine is replaced with a pyrimidine (C or T) or vice versa. Several criteria can be considered when devising a scoring matrix for amino acid sequence alignments. Most common

criteria are chemical/physical similarity and observed substitution frequencies. For example, in similarity- based matrices, pairing two different amino acids that both have aromatic functional groups might receive a significant positive score, while pairing an amino acid that has a nonpolar functional group with one that has a charged functional group might result in a scoring penalty. Scoring matrices have been derived based on residue hydrophobicity, charge, electronegativity, and size. Another similarity based matrix for amino acids is based on the genetic code: A pair of residues is scored according to the minimum number of nucleotide substitutions necessary to convert a codon from one residue to the other. One problem with similarity-based matrices results from the difficulty of combining these various physical, chemical, and genetic scores into a single meaningful matrix.

PAM Matrices & BLOSUM Matrices

There are several common ways in which weights can be applied for amino acid differences. The most common and most famous way to assign weights is to use Dayhoff's PAM 250 matrix. This is a matrix of weights that is derived from how often different amino acids replace other amino acids in evolution. This was based on a data base of 1,572 changes in 71 groups of closely related proteins appearing in earlier volumes of this amazing predecessor to electronic databases. PAM matrix is used extensively in BLAST (Basic Local Alignment Search Tool) algorithm, which is extremely fast, robust and popular heuristic. PAM stands for percent accepted mutations and these were inferred from the types of changes observed in these proteins. Every change was tabulated and entered in a matrix enumerating all possible amino acid changes.

The normalization of each matrix entry is done such that the PAM matrix represents substitution probabilities over a fixed unit of evolutionary change. For PAM -1, this unit is 1 substitution. The particular PAM matrix that is most appropriate for a given sequence alignment depends on the length of the sequences and on how closely the sequences are believed to be related. It is more appropriate to use the PAM -1 matrix to compare sequences that are closely related, whereas the PAM-1000 matrix might be used to compare sequences with very distant relationships. In practice, the PAM-250 matrix is a commonly used compromise.

Another popular scoring matrix, BLOSUM (for BLOcks SUbstitution Matrix) is a commonly used scoring matrix for sequence alignment. It gives a score for each pair of amino acids based on how likely we will observe such a pair in alignments of truly conserved blocks of amino acids. A higher score indicates that such pair of amino acids is often seen to be aligned to each other when aligning functionally similar proteins with each other.

Like the PAM matrices, various BLOSUM matrices can be constructed to compare sequences with different degrees of relatedness. The significance of the numbering for BLOSUM matrices, however, can be thought of as the inverse of the PAM numbers. The lower numbered PAM

matrices are appropriate for comparing more closely related sequences, while lower numbered BLOSUM matrices are used for more distantly related sequences.

Database searching for similar sequences

When we have a sequence, and we want to find other sequences similar to it in a database, we do not really need the full alignment of this sequence against all others. All we want is a value, **a score** that will tell us how similar our probe sequence is to the every other sequence. This score should be **sensitive** (so that as many of the true homologs are found) and **specific.** There is a simple rule-of-thumb: A database hit having a **sequence identity of 25% or more (protein lengths 200 residues or more) is almost certainly a true hit**, if one uses reasonable parameter settings for the common programs BLAST or FASTA. There are cases where this is not true, for example when the sequences have a high amount of low-complexity regions (Ser-Thr-rich regions, and such), but this can usually be dealt with by applying a low-complexity filter.

But what to do about hits with lower degree of identity? The basic problem is how to judge whether a score is significant or not. Could a given score be the result of pure chance? The various search programs (BLAST, FASTA) attempt to answer this question by computing an **expectation value**. This is an estimate of the likelihood that a given hit is due to pure chance, given the size of the database. This calculation uses probability theory and other statistical assumptions. It should be as low as possible. If the value is close to 1 (say, 0.01) rather than 0.0 or 1.0e-45, then the hit is suspect.

Pairwise Sequence alignment

One of the cornerstones of modern bioinformatics is the comparison or alignment of protein and nucleotide sequences. In bioinformatics, a sequence alignment is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns.

Pairwise sequence alignment is the process of comparing two sequences by searching for a series of individual characters that are identical and same order in the sequences. The degree to which two species or populations share identities is indicated by similarity. Pairwise sequence alignment methods are used to find the best-matching pairwise (local) or global alignments of two query sequences. Pairwise alignments can only be used between two sequences at a time, but they are efficient to calculate and are often used for methods that do not require extreme precision (such as searching a database for sequences with high similarity to a query). One way of quantifying the utility of a given pairwise alignment is the 'maximum unique match', or the longest subsequence that occurs in both query sequence. Longer MUM sequences typically

reflect closer relatedness. If two sequences share a stretch of nearly identical nucleotides or amino acids, it is not necessary that they are direct descended from a common ancestor; but a very high level of similarity is a strong indication of homology.

Multiple sequence alignment

Pairwise alignments are fundamental and useful, but there are some problems with them. For instance, when using one of the popular sequence searching programs (FASTA, BLAST) which perform pairwise alignments to find similar sequences in a database, one very often obtains many sequences that are significantly similar to the query sequence. Comparing each and every sequence to every other may be possible when one has just a few sequences, but it quickly becomes impractical as the number of sequences increases. What we need is multiple sequence alignment, where **all similar sequences can be compared in one single figure or table**. The basic idea is that the sequences are aligned on top of each other, so that a coordinate system is set up, where **each row is the sequence** for one protein, and **each column is the 'same' position** in each sequence. Each column corresponds to a specific residue in the 'prototypical' protein.

As with pairwise alignment, there will be gaps in some sequences, most often shown by the dash '-' or dot '.' character. Note that to construct a multiple alignment, one may have to introduce gaps in sequences at positions where there were no gaps in the corresponding pair wise alignment. This means that multiple alignments typically contain more gaps than any given pair of aligned sequences. As new nucleotide and protein sequences become available, by determining how these sequences are 'related' to known proteins, one can make predictions of their structural, functional, and evolutionary features. The 'relation' between sequences can be level of sequence similarity or a common feature or domain in the sequences or associated structure etc.

Uses of Multiple Sequence Alignment are plenty. Some of them are listed below:

- Detecting similarities between sequences (closely or distantly related)
- Detecting conserved regions / motifs in sequences
- Detection of structural homologies; patterns of hydrophobicity/ hydrophilicity , gaps etc thus assisting improved prediction of secondary and tertiary structures and loops and variable regions
- Predict features of aligned sequences like conserved positions which may have structural or functional importance
- Making patterns or profiles that can be further used to predict new sequences falling in a given family
- Computing consensus sequence
- Inferring evolutionary trees / linkage - Phylogenetic Analysis etc.
- Deriving profiles or Hidden Markov Models that can be used to remove distant sequences (outliers) from protein families

PRACTICAL PROCEDURE

General Pairwise sequence alignment

1. Retrieve two sequence of interest from NCBI in FASTA format
2. Go to the Blast home page at https://blast.ncbi.nlm.nih.gov/Blast.cgi and select Nucleotide BLAST
3. Paste sequence 1 and 2 in prescribed space
4. Submit & analyze results

Database searching for sequence similiarity

1. Retrieve the sequence of interest from NCBI in FASTA format
2. Go to the Blast home page at https://blast.ncbi.nlm.nih.gov/Blast.cgi and select Protein BLAST
3. Paste sequence
4. Select the database to be searched against
5. Set parameters like e-value (if required)
6. Submit & analyze results

Multiple sequence alignment

1. Download the sequences from NCBI in FASTA format in text format
2. Go to the MUSCLE home page at http://www.ebi.ac.uk/Tools/msa/muscle/
3. Paste the sequences in FASTA format or select the file containing sequences
4. Select the output option
5. Click submit and analyze the result in BIOEDIT

Primer Design and Validation

Polymerase chain reaction (PCR) is an enzymatic reaction whose efficiency and sensitivity largely depend on the efficiency of the primers that are used for the amplification of a concerned gene/DNA fragment. Selective amplification of nucleic acid molecules initially present in minute quantities provides a powerful tool for analyzing nucleic acids. In silico method helps in designing primers. There are various programs available for PCR primer design. For designing the primer, DNA template sequence is required that can be taken from any of the available sequence databases, e.g., RefSeq database. The in silico validation can be carried out using BLAST tool and Gene Runner software, which check their efficiency and specificity. Thereafter, the primers designed in silico can be validated in the wet lab. After that, these validated primers can be synthesized for use in the amplification of concerned gene/DNA fragment.

Good primer design is essential for a successful PCR reaction. There are many factors to take into account when designing the optimal primers for your gene of interest. Here are some tips to consider when designing primers.

1. In general, a length of 18–30 nucleotides for primers is good.
2. Try to make the melting temperature (T_m) of the primers between 65°C and 75°C, and within 5°C of each other.

3. If the T_m of your primer is very low, try to find a sequence with more GC content, or extend the length of the primer a little.
4. Aim for the GC content to be between 40 and 60%, with the 3' of a primer ending in C or G to promote binding.
5. Typically, 3 to 4 nucleotides are added 5' of the restriction enzyme site in the primer to allow for efficient cutting.
6. Try to avoid regions of secondary structure, and have a balanced distribution of GC-rich and AT-rich domains.
7. Try to avoid runs of 4 or more of one base, or dinucleotide repeats (for example, ACCCC or ATATATAT).
8. Avoid intra-primer homology (more than 3 bases that complement within the primer) or inter-primer homology (forward and reverse primers having complementary sequences). These circumstances can lead to self-dimers or primer-dimers instead of annealing to the desired DNA sequences.
9. If you are using the primers for cloning, we recommend cartridge purification as a minimum level of purification.
10. If you are using the primers for mutagenesis, try to have the mismatched bases towards the middle of the primer.
11. If you are using the primers for a PCR reaction to be used in Invitrogen™ TOPO™ cloning, the primers should not have a phosphate modification.

Also keep in mind that most oligonucleotide synthesis reactions are only 98% efficient. This means that each time a base is added, only 98% of the oligos will receive the base. This is not often critical with shorter oligos, but as length increases, so does the probability that a primer will be missing a base. This is very important in mutagenesis or cloning reactions. Purification by HPLC or PAGE is recommended in some cases.

Oligonucleotide length	**Percent with correct sequence**
10 bases	$(0.98)^{10} = 81.7\%$
20 bases	$(0.98)^{20} = 66.7\%$
30 bases	$(0.98)^{30} = 54.6\%$
40 bases	$(0.98)^{40} = 44.6\%$

Primer length

Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR. In general, oligonucleotides between 18 and 24 bases are extremely sequence specific, provided that the annealing temperature is optimal. Primer length is also proportional to annealing efficiency: in general, the longer the primer, and the more inefficient the annealing. With fewer templates primed at each step, this can result in a significant decrease in amplified product. The primers should not be too short,

however, unless the application specifically calls for it. As discussed below, the goal should be to design a primer with an annealing temperature of at least 50°C.

The relationship between annealing temperature and melting temperature is one of the "Black Boxes" of PCR. A general rule-of-thumb is to use an annealing temperature that is 5°C lower than the melting temperature. Thus, when aiming for an annealing temperature of at least 50°C, this corresponds to a primer with a calculated melting temperature(T_m) ~55°C. Often, the annealing temperature determined in this fashion will not be optimal and empirical experiments will have to be performed to determine the optimal temperature. This is most easily accomplished using a gradient thermal cycler like Eppendorf's Mastercycler gradient.

Melting Temperature (T_m)

It is important to keep in mind that there are two primers added to a PCR reaction. Both of the oligonucleotide primers should be designed such that they have similar melting temperatures. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer with the higher T_m will mis-prime at lower temperatures and the primer with the lower T_m may not work at higher temperatures.

The melting temperatures of oligos are most accurately calculated using nearest neighbor thermodynamic calculations with the formula:

$T_m^{primer} = \Delta H\ [\Delta S + R \ln (c/4)] - 273.15°C + 16.6 \log_{10} [K^+]$

where H is the enthalpy and S is the entropy for helix formation, R is the molar gas constant and c is the concentration of primer. This is most easily accomplished using any of a number of primer design software packages on the market. Fortunately, a good working approximation of this value (generally valid for oligos in the 18–24 base range) can be calculated using the formula:

$T_m = 2(A+T) + 4(G+C)$.

Specificity

Primer specificity is at least partly dependent on primer length. It is evident that there are many more unique 24 base oligos than there are 15 base pair oligos. That being said, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

Because *Taq* polymerase is active over a broad range of temperatures, primer extension will occur at the lower temperatures of annealing. If the temperature is too low, non-specific priming may occur which can be extended by the polymerase if there is a short homology at the 3' end. In general, a melting temperature of 55°C –72°C gives the best results (Note that this corresponds to a primer length of 18–24 bases using Wallace's rule above).

Complementary Primer Sequences

Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back", partially double-stranded structures, can occur which will interfere with annealing to the template.
Another related danger is inter-primer homology. Partial homology in the middle regions of two primers can interfere with hybridization. If the homology should occur at the 3' end of either primer, primer dimer formation will occur which, more often than not; will prevent the formation of the desired product via competition.

G/C content and polypyrimidine (T, C) or polypurine (A, G) stretches

The base composition of primers should be between 45% and 55% GC. The primer sequence must be chosen such that there is no PolyG or PolyC stretches that can promote non-specific annealing. Poly A and Poly T stretches are also to be avoided as these will "breath" and open up stretches of the primer-template complex. This can lower the efficiency of amplification. Polypyrimidine (T, C) and polypurine (A, G) stretches should also be avoided. Ideally the primer will have a near random mix of nucleotides, a 50% GC content and be ~20 bases long. This will put the T_m in the range of 56°C – 62°C.

3'-end sequence

It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming. We have already explored the problem of primer homologies occurring at these regions. Another variable to look at is the inclusion of a G or C residue at the 3' end of primers. This "GC Clamp" helps to ensure correct binding at the 3' end due to the stronger hydrogen bonding of G/C residues. It also helps to improve the efficiency of the reaction by minimizing any "breathing" that might occur.
There are several web-based services or stand-alone software provided to the public for primer design, such as PRIDE, PRIMER MASTER, PRIMO, Primer3, Primer3Plus, Prime and Web Primer (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer), and Primer Design Assistant (PDA). Users can define the parameters listed in the menu of these tools and then get several pairs of primers for the target template sequence. However, most of them only take a single sequence query.

Primer validation

Designed primers can be validated using FAST PCR. In FAST PCR program check the primers length, quality, GC content and percentage. *In silico* PCR is used to check the primer amplification and product size. After checking the quality, primers are validated in wet lab condition for its amplification and compare the product size of both *in silico* wise and wet lab condition

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Practical procedure

Detecting Conserved regions using Muscle

1. Access Muscle homepage at http://www.ebi.ac.uk/Tools/clustalw2/index.html

2. Paste the sequences in text box and Click the Run button.
3. Open the alignment in an alignment editor
4. Note the first conserved region.

Predicting primer using Primer3Plus

1. Open the primer3Plus homepage at http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi
2. Paste the sequences in text box or the file containing the sequences can be uploaded
3. Go to the general setting tab and set the parameters, then Click the Pick Primer button
4. Select the primers that satisfy the set conditions and click on Send to Primer3Manager
5. Go to primer3plus to check whether the primer satisfies the primer properties, if it satisfies the GC% and Tm range
6. In primer3Plus change the task option to Primer_Check and paste the identified primer in the box named primer to check then click check primer
7. note down length, TM and GC%
8. Click on order selected primers and save the result to a notepad

Analysis of primers designed using Oligocalc

1. Open OligoCalc at http://www.basic.northwestern.edu/biotools/oligocalc.html
2. Enter the conserved nucleotide sequence and click Calculate button
3. Note down the primer properties, if it satisfies the GC% and Tm range
4. Click the check self-complementarity button.
5. Check if there is any hairpin and primer dimmer (Autodimer) formation.
6. Choose another conserved region for reverse primer, such that it will have a reasonable amplicon size in between.
7. Repeat step 1-5 for the reverse primer.

Fast PCR- In silico PCR

1. Go to in silico PCR option in Fast PCR and paste the forward and reverse primers in fasta format in the tab (predesigned primers) and paste sequences in (sequences) tab then go to run.
2. Note down the product size and check whether forward and reverse primer is binding to all sequences.

Blast to check species specific

1. Go to Blast site – http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome
2. Paste Forward and Reverse primers in the following manner. So that the blast will be set to search short nucleotide sequences.

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INTRODUCTION TO CHEMI-INFORMATICS, MOLECULAR DOCKING AND COMPUTER AIDED DRUG DESIGNING

Prathiba

Cheminformatics (also known as chemoinformatics, chemioinformatics and chemical informatics) is the use of computer and informational techniques applied to a range of problems in the field of chemistry. These *in silico* techniques are used, for example, in pharmaceutical companies in the process of drug discovery. These methods can also be used in chemical and allied industries in various other forms.

Cheminformatics is a field of information technology that focuses on the collection, storage, analysis, and manipulation of chemical data. The chemical data of interest typically includes information on small molecule formulas, structures, properties, spectra, and activities (biological or industrial). Cheminformatics originally emerged as a vehicle to help the drug discovery and development process, however cheminformatics now plays an increasingly important role in many areas of biology, chemistry, and biochemistry.

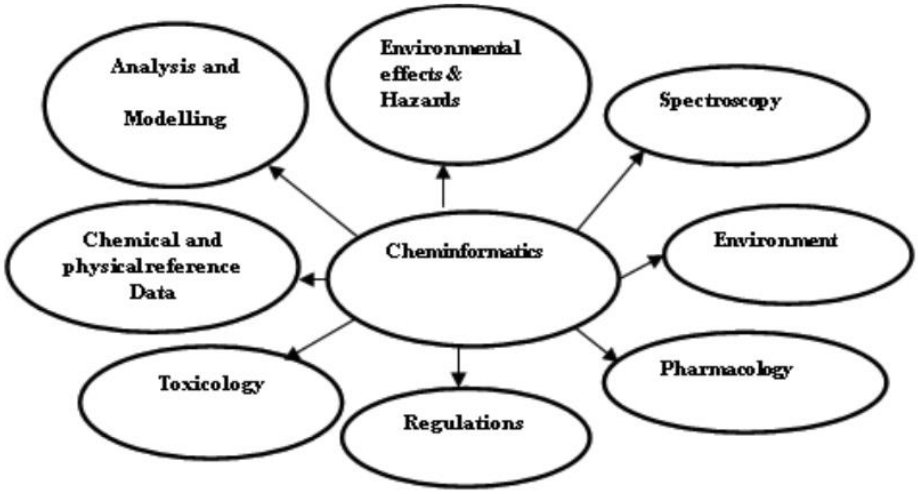
The term chemoinformatics was defined by F.K. Brown in 1998:
Chemoinformatics is the mixing of those information resources to transform data into information and information into knowledge for the intended purpose of making better decisions faster in the area of drug lead identification and optimization.
The overview of cheminformatics studies is mentioned as follows:

- Chemical data collection, analysis and management.
- Data representation and communication.
- Database design and organization.
- Chemical structure and property prediction (including drug-likeness).
- Molecular similarity and diversity analysis.
- Compound or library design and optimization.
- Database mining.
- Compound classification and selection.
- Qualitative and quantitative structure-activity or property relationships.
- Information theory applied to chemical problems.
- Statistical models and descriptors in chemistry.
- Prediction of in vivo compound characteristics.

Need and importance of cheminformatics

Cheminformatics plays a key role to maintain and access enormous amount of chemical data, produced by chemist (more than 45 million chemical compounds are known and the number may increase in million every year,) by using a proper database. Also, the field of chemistry needs a novel technique for knowledge extraction from data to model complex relationships between the

structure of the chemical compound and biological activity or the influence of reaction condition on chemical reactivity. Cheminformatics has wider range of application and figure shows influence if cheminformatics in some specific research areas.



Need for cheminformatics

Three major aspects of Cheminformatics are;

i) Information Acquisition, is a process of generating and collecting data empirically (experimentation) or from theory (molecular simulation)

ii) Information Management deals with storage and retrieval of information and

iii) Information use, which includes Data Analysis, correlation, and application to problems in the chemical and biochemical sciences

Applications of cheminformatics

The range of applications of cheminformatics is rich indeed; any field of chemistry can profit from its methods. The following lists different areas of chemistry and indicates some typical applications of cheminformatics:

a) Storing data generated through experiments or from molecular simulation Retrieval of chemical structures from chemical database (Software libraries).

b) Prediction of physical, chemical and biological properties of chemical compounds.

c) Elucidation of the structure of a compound based on spectroscopic data.

d) Structure, Substructure, Similarity and diversity searching from chemical database.

e) High Throughput Screening (HTS) is the integration of technologies (laboratory automation, assay technology, micro plate based instrumentation, etc.) to quickly screen chemical compounds in search of a desired activity.

f) Docking - Interaction between two macromolecules.

g) Drug Discovery.

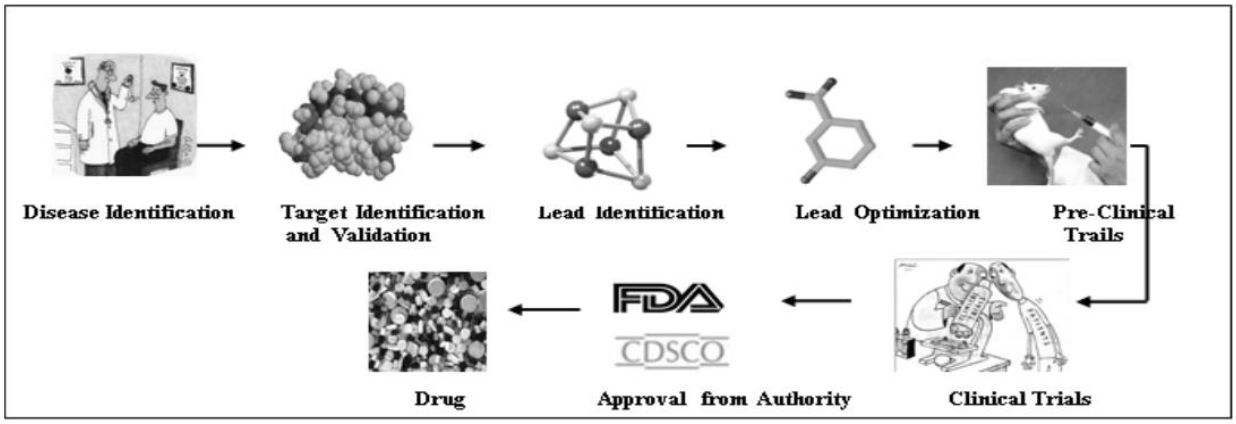
h) Molecular Science, Materials Science, Food Science (nutraceuticals), Atmospheric chemistry, Polymer chemistry, Textile Industry, Combinatorial organic synthesis (COS)

The primary application of cheminformatics is in the storage, indexing and search of information relating to compounds. The efficient search of such stored information includes topics that are dealt with in computer science as data mining, information retrieval, information extraction and machine learning.

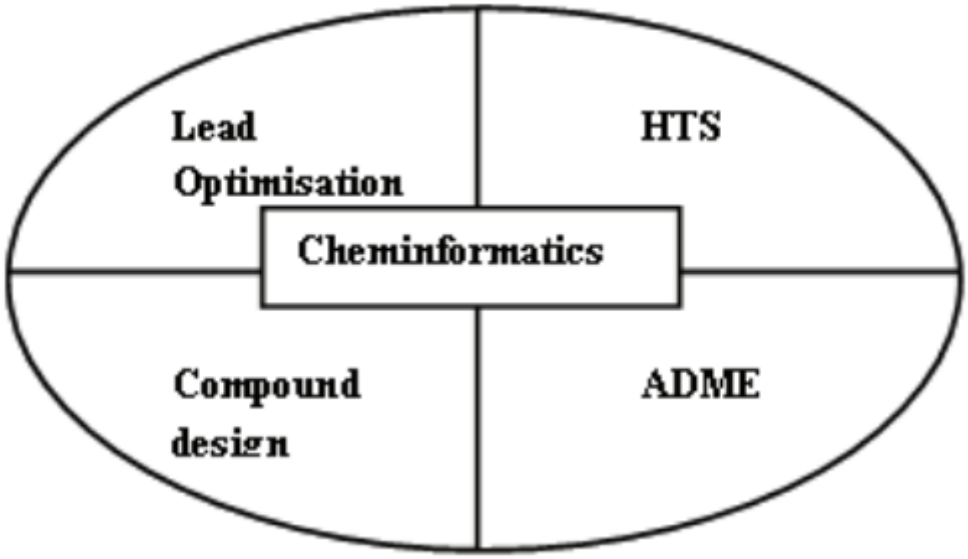
Role of Cheminformatics in Modern Drug Discovery

Recent chemical developments for drug discovery are generating a lot of chemical data which is referred as information explosion. This has created a demand to effectively collect, organize, analyze and apply the chemical information in the process of modern drug discovery and development. The drug discovery process is aimed at discovering molecules that can be very rapidly developed for effective treatments to meet medical needs. The entanglement of chemistry and information management started in the mid of 1970s, applying in the area of prediction of protein structure, Fourier transform of X-ray crystallography, enzyme and chemical kinetics, analyze various types of spectroscopy data and binding of chemical compounds. During early 1980s, computer technology is considered as the core component by the medical chemist to solve chemical problems. For example, collecting crystal structures of small molecules in Cambridge Structural Database (CSD) provides a fertile resource for geometrical data on molecular fragments for calibration of force fields and validation of results from computational chemistry.

The need of storing macromolecular data results in Protein Data Base (PDB). The needs and refinement on these approaches result in several tools and upgrading the process of solving the problems. The traditional drug discovery process starts with a particular Disease, Identification of target, and Identification of molecule effective against target and Preclinical testing. Identification of target and synthesis the molecule to increase their suitability takes more amounts of time and cost (in millions) which is done in 'WET LAB'. This is the area where chemical informatics play its major role in discovery process of the drug. The development process starts with human clinical trials, approval from authority and delivers the product in the market. This process takes about 10-15 years to discover, develop and bring drug to the market. The modern pharmaceutical drug discovery and development pipeline process starts with Disease selection, Target identification, Lead identification, Lead Optimization, Pre -clinical trial testing, Clinical trial testing, Approval and circulation (Drug in market). In traditional drug discovery phase, the process which cost more time and money is replaced with lead identification and lead optimization process in modern drug discovery system. Each phase has an interaction component that transfers data, knowledge and information to one another.



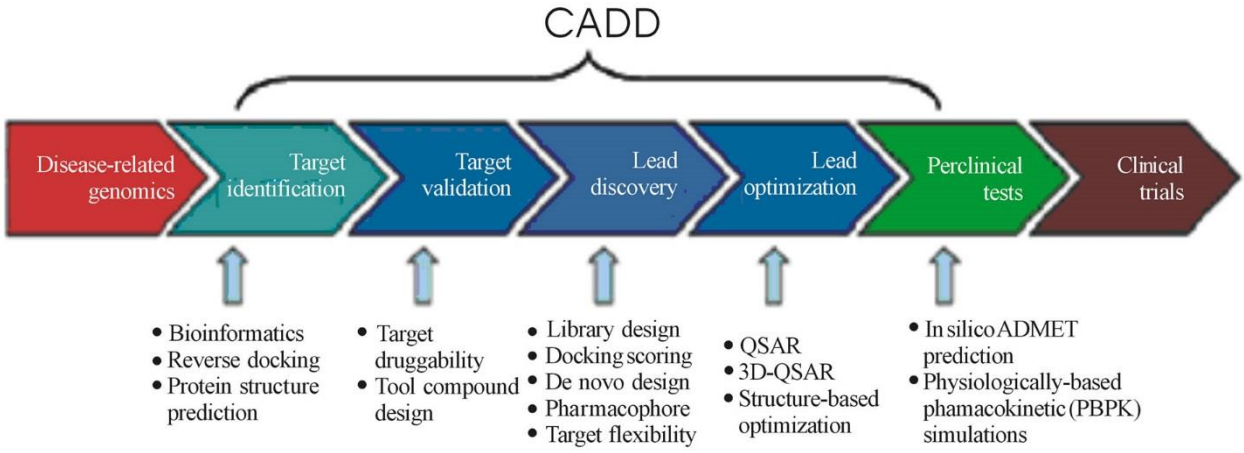
Modern Drug Discovery and Development Life Cycle



COMPUTER-AIDED DRUG DESIGN

Drug design, often referred to as rational drug design or simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target. The drug is most commonly an organic small molecule that activates or inhibits the function of a biomolecule such as a protein, which in turn results in a therapeutic benefit to the patient. In the most basic sense, drug design involves the design of molecules that are complementary in shape and charge to the biomolecular target with which they interact and therefore will bind to it. Drug design frequently but not necessarily relies on computer modeling techniques. This type of modeling is sometimes referred to as computer-aided drug design. Finally, drug design that relies

on the knowledge of the three-dimensional structure of the biomolecular target is known as structure-based drug design.



Types

There are two major types of drug design. The first is referred to as ligand-based drug design and the second, structure-based drug design.

Ligand-based

Ligand-based drug design (or indirect drug design) relies on knowledge of other molecules that bind to the biological target of interest. These other molecules may be used to derive a pharmacophore model that defines the minimum necessary structural characteristics a molecule must possess in order to bind to the target. In other words, a model of the biological target may be built based on the knowledge of what binds to it, and this model in turn may be used to design new molecular entities that interact with the target. Alternatively, a quantitative structure-activity relationship (QSAR), in which a correlation between calculated properties of molecules and their experimentally determined biological activity, may be derived. These QSAR relationships in turn may be used to predict the activity of new analogs.

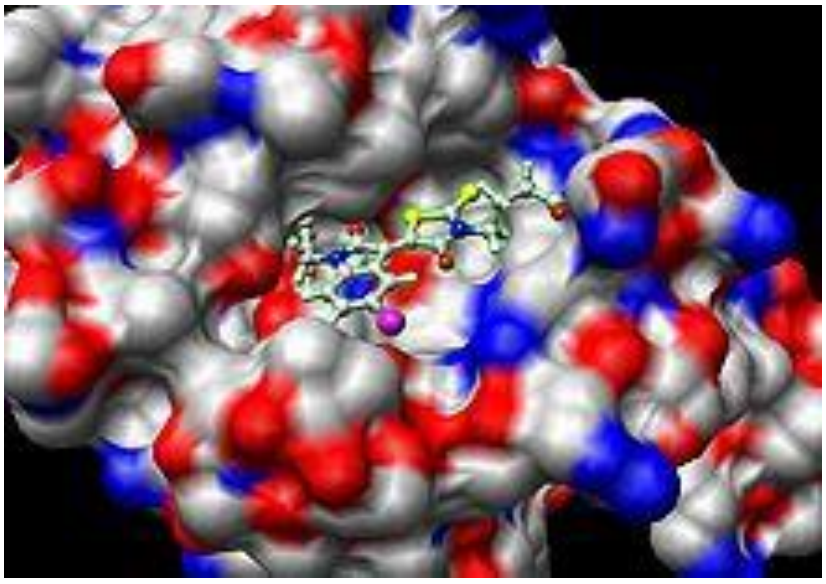
Structure-based

Structure-based drug design (or direct drug design) relies on knowledge of the three dimensional structure of the biological target obtained through methods such as x-ray crystallography or NMR spectroscopy. If an experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive graphics and the intuition of a medicinal chemist. Alternatively various automated computational procedures may be used to suggest new drug candidates.

DOCKING

Molecular docking is a well-established computational technique which predicts the interaction energy between two molecules. This technique mainly incorporates algorithms like molecular dynamics, Monte Carlo stimulation, fragment based search methods which are mentioned in details in later part.

Molecular docking studies are used to determine the interaction of two molecules and to find the best orientation of ligand which would form a complex with overall minimum energy. The small molecule, known as ligand usually fits within protein's cavity which is predicted by the search algorithm. These protein cavities become active when come in contact with any external compounds and are thus called as active sites.



Types of docking

- **Lock and Key or Rigid Docking** – In rigid docking, both the internal geometry of the receptor and ligand is kept fixed during docking.
- **Induced fit or Flexible Docking** - In this model, the ligand is kept flexible and the energy for different conformations of the ligand fitting into the protein is calculated. Though more time consuming, this method can evaluate many different possible conformations which make it more reliable.

Mechanics

Molecular docking can be divided into two separate sections.

1) Search algorithm – These algorithms determine all possible optimal conformations for a given complex (protein-protein, protein-ligand) in a environment i.e. the position and orientation of both molecules relative to each other. They can also calculate the energy of the resulting complex and of each individual interaction.

The different types of algorithms that can be used for docking analysis are given below.

- Molecular dynamics
- Monte Carlo methods
- Genetic algorithms
- Fragment-based methods
- Point complementary methods
- Distance geometry methods
- Systematic searches

2) Scoring function – These are mathematical methods used to predict the strength of the non-covalent interaction called as binding affinity, between two molecules after they have been docked. Scoring functions have also been developed to predict the strength of other types of intermolecular interactions, for example between two proteins or between protein and DNA or protein and drug. These configurations are evaluated using scoring functions to distinguish the experimental binding modes from all other modes explored through the searching algorithm.

For example:

• Empirical scoring function of Igemdock

Fitness = vdW + Hbond + Elec

• Binding Energy

ΔG bind = ΔGvdw + ΔGhbond + ΔGelect + ΔGconform + ΔG tor + ΔG sol

Application

- Hit identification – docking combined with a scoring function can be used to quickly screen large databases of potential drugs *in silico* to identify molecules that are likely to bind to protein target of interest.
- Lead optimization – docking can be used to predict in where and in which relative orientation a ligand binds to a protein (also referred to as the binding mode or pose). This information may in turn be used to design more potent and selective analogs.
- Bioremediation – Protein ligand docking can also be used to predict pollutants that can be degraded by enzymes.

Docking models

Over the years biochemists have developed numerous models to capture the key elements of the molecular recognition process. Although very simplified, these models have proven highly useful to the scientific community

The Lock and Key Theory

As far back as 1890 Emil Fischer proposed a model called the "lock-and-key model" that explained how biological systems function. A substrate fits into the active site of a macromolecule, just like a key fits into a lock. Biological 'locks' have unique stereochemical features that are necessary to their function.

The Induced-Fit Theory

In 1958 Daniel Koshland introduced the "induced-fit theory".

The basic idea is that in the recognition process, both ligand and target mutually adapt to each other through small conformational changes, until an optimal fit is achieved.

The Conformation Ensemble Model

In addition to small induced-fit adaptation, it has been observed that proteins can undergo much larger conformational changes. A recent model describes proteins as a pre-existing ensemble of conformational states. The plasticity of the protein allows it to switch from one state to another.

Docking classification

Molecular docking classifies biomolecules into three categories: small molecules (also called 'ligands'), proteins, and nucleic acids

The most important types of docking systems are: proteinligand, protein-protein and nucleic acid-protein. The interactions between a small molecule and a protein are by far much better understood than those between a protein and a nucleic acid.

Docking packages

Non-Commercial

AutoDock(Art Olsen, David Goodsell, Scripps)

UCSF DOCK (Kuntz Group)

Commercial

Glide (Schrodinger)

GOLD (CCDC)

FlexX(BiosolveIT)

ICM (Molsoft)

Surflex(Tripos)

Docking methodology

Protein structure preparation

In this step the 3D structure of the receptor should be downloaded from PDB; and modified. This should include removal of the water molecules from the cavity, stabilizing charges, filling in the missing residues, generation the side chains etc. according to the parameters available. After modification the receptor should be biological active and stable.

Identification of the active site

After the receptor is built, the active site within the receptor should be identified. The receptor may have many active sites but the one of the interest should be selected. Most of the water molecules and heteroatoms if present should be removed.

Ligand preparation

Ligands can be obtained from various databases like ZINC, PubChem or can be sketched using tools like Chemsketch.

Grid generation

Docking

This is the last step, where the ligand is docked onto the receptor and the interactions are checked. The scoring function generates scores depending on which the ligand with the best fit is selected.

Druglikeness may be defined as a complex balance of various molecular properties and structure features which determine whether particular molecule is similar to the known drugs. These properties, mainly hydrophobicity, electronic distribution, hydrogen bonding characteristics, molecule size and flexibility and of course presence of various pharmacophoric features influence the behavior of molecule in a living organism, including bioavailability, transport properties, affinity to proteins, reactivity, toxicity, metabolic stability and many others.

Drug design, often referred to as rational drug design or simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target. The drug is most commonly an organic small molecule that activates or inhibits the function of a biomolecule such as a protein, which in turn results in a therapeutic benefit to the patient. In the most basic sense, drug design involves the design of molecules that are complementary in shape and charge to the biomolecular target with which they interact and therefore will bind to it. Drug design frequently but not necessarily relies on computer modeling techniques. This type of modeling is sometimes referred to as computer-aided drug design. Finally, drug design that relies on the knowledge of the three-dimensional structure of the biomolecular target is known as structure-based drug design. In addition to small molecules, biopharmaceuticals and especially therapeutic antibodies are an increasingly important class of drugs and computational methods for improving the affinity, selectivity, and stability of these protein-based therapeutics have also been developed.

Lipinski Rule of Five

Lipinski's rule of five also known as the Pfizer's rule of five or simply the rule of five (RO5) is a rule of thumb to evaluate druglikeness or determine if a chemical compound with a certain pharmacological or biological activity has chemical properties and physical properties that would make it a likely orally active drug in humans. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most orally administered drugs are relatively small and moderately lipophilic molecules.

The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism, and excretion ("ADME"). However, the rule does not predict if a compound is pharmacologically active.

Lipinski's rule states that, in general, an orally active drug has no more than one violation of the following criteria:

Components of the rule:

- No more than 5 hydrogen bond donors
- No more than 10 hydrogen bond acceptors
- A molecular mass less than 500 daltons
- An octanol-water partition coefficient
- log P not greater than 5

Protein Data Bank (PDB)

The Protein Data Bank (PDB) is a crystallographic database for the three-dimensional structural data of large biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray crystallography, NMR spectroscopy, or, increasingly, cryo-electron microscopy, and submitted by biologists and biochemists from around the world, are freely accessible on the Internet via the websites of its member organisations (PDBe, PDBj, and RCSB.

1) Open the RCSB PDB website http://www.rcsb.org/pdb/home/home.do
2) Enter the protein name in the search box (Protein kinase 1URF)
   If you are giving "protein kinase" you will get many results. But if you want specific structure give the ID also "Protein kinase 1URF"
3) Click on PDB format.

PUBCHEM

PubChem is a database of chemical molecules and their activities against biological assays. The system is maintained by the National Center for Biotechnology Information (NCBI), a component of the National Library of Medicine, which is part of the United States National Institutes of Health (NIH).

1. Open Pubchem site https://pubchem.ncbi.nlm.nih.gov/
2. Enter the compound name in the search box (Eg: Ursolic acid)
3. Click on Ursolic acid.
4. Save in SDF format.

CORINA

CORINA Classic (the classic command-line version of CORINA) is a fast and powerful 3D structure generator for small and medium sized, typically drug-like molecules. Its robustness, comprehensiveness, speed and performance makes CORINA Classic a perfect application to convert large chemical datasets or databases.

CORINA Classic matured through a series of versions during the past decades and has become the recognized world-wide gold standard in industry and academia to generate 3D molecular models of high quality.

1. Open online demo corina (https://www.mn-am.com/online_demos/corina_demo)
2. Submit to see the structure
3. Click on PDB to see the SMILES converted into pdb format.

Drug likeness Tool (DruLiTo)

Druglikeness is a qualitative concept used in drug design for how "druglike" a substance is with respect to factors like bioavailability. It is estimated from the molecular structure before the substance is even synthesized and tested.

1. Click on 'browse' button and select the molecule either in .mol or .sdf format (Eg. Betulinic Acid)
2. Click on calculate properties which calculates all the descriptors
3. In drug likeness rule box, the users can even customize the rules as per the customize button
4. Click on Lipinski's Rule
5. Analyze the results.

VISUALIZATION TOOL-RASMOL

Commands for rasmol:

backbone on
backbone off
background blue
background black
dots 100
dots 0
hbonds on
hbonds off
hbonds 100
hbonds 0
ribbons on
ribbons off
ribbons 100
ribbons 0
show information
show sequence
show symmetry
spacefill temperature
spacefill 100
spacefill 500
spacefill 0
ssbonds on
ssbonds off
select sulphur
spacefill on
select polar
select hydrophobic
select acidic
select basic
select helix
select sheet
select turn

IPR ISSUES IN rDNA RESEARCH

T E Sheeja

Establishment of an IPR system

The Convention establishing the World Intellectual Property Organization (WIPO) defines Intellectual Property as under: "Literary artistic and scientific works; performances of performing artists, phonograms, and broadcasts; inventions in all fields of human endeavour; scientific discoveries; industrial designs; trademarks, service marks, and commercial names and designations; protection against unfair competition; and all other rights resulting from intellectual activity in the industrial, scientific, literary or artistic fields". IP, protected through law, like any other form of property can be a matter of trade, that is, it can be owned, bequeathed, sold or bought. IP is the foundation of knowledge-based economy, and is intangible and non-exhaustible but time-bound and is limited to countries where the applicant has made registration for protection. These laws have been collectively referred to as Intellectual Property Rights (IPR). In simple terms IPR refers to a group of laws intended to provide legal protection for an intellectual creation.

India as a member of the World Trade Organisation (WTO) is obliged to comply with the Agreement on Trade Related Aspects of Intellectual Property Rights (TRIPS). This requires that member countries provide for Intellectual Property Rights (IPRs) in one form or the other in all fields of technology, including agriculture. In India, the administration of IPR is through the office of Controller General of Patents, Designs and Trademarks (CGPDTM) located at Mumbai. Presently seven types of IPRs are recognized *viz.*, copyright, trademarks, geographical indications, industrial designs, patents, integrated circuits and trade secrets. These can be classified into two broad categories. 1. Copyright and related rights and 2. Industrial property rights, which include IPRs like patents, trademarks, geographical indications, and industrial designs. IPRs that do not fit into this division are termed as *sui generic*, meaning "one of its kind" covers the plant breeder's rights.

Copyright is the exclusive right to do or authorize others to do certain acts in relation to (i) literary, dramatic, musical and artistic works (ii) cinematograph film and (iii) sound recordings. The nature of acts varies according to the subject matter. Basically, it is the right to copy or reproduce the work in which copyright subsists. The term of copyright is life of the author of work plus 60 years with certain exceptions. India is a signatory of the Berne convention and universal copyrights convention, for protection of copyrights, which enables copyright protection in any country in the world.

Patents are the most important IPR today for agricultural goods and services and provide protection to patentable plants and animals and their related biotechnological processes. Research results in any field of technology, whether processes or products, which are new,

inventive (non-obvious) and useful (industrially applicable) and are patentable under Patent Act, constitute patentable IP.

Plant Variety Protection (PVP) provides incentives for R&D in agricultural technologies. It also improves range of availability of plant materials and also improves innovation. However, it is suspected that PVP increases inequality in developing countries between breeders protected by PVP and farmers and agricultural workers who do not receive any protection for their knowledge (Srinivasan, 2005). Plant patents are unique to breeders of USA, where there is an option to apply for a plant patent on their novel new genetic material. The advantage of a plant patent over PVP variety protection in the USA is that the patent grants much wider protection than the PVP, though period of protection is shorter.

Trademarks are also applicable to agricultural products. It helps clear distinction of the goods and services of one from the other, thereby helping the consumer from being deceived. Once in use, trademarks confer a perpetual right, unlike plant breeder's rights or patents, and this is another major advantage of using one. In essence, this means that once plant breeder's rights have expired, the trademarked brand can continue in perpetuity. Renewal is usually every 10 years (Smiler and Erbisch, 2004). Though it is a popular way of protection, the major drawback is that the registration may have to be renewed from time to time.

Geographical indications are a category of commercial marks and include appellations of origin, associated with products originating from a particular geographical area, where the essential characteristics of the product are attributed to its place of origin. Distinct GIs provide protection to all producers within a locality and differs from other IPR in that it has no time limits.

IPRs are important not only because India as a member of TRIPS is obliged to abide by the conditions of an international agreement but also because they offer possible mechanisms for stimulating research, improving access to technology and promoting business growth, to benefit the farming community. In the present scenario, it has become necessary to do so since the past experience on issues like turmeric and basmati has established the fact that unprotected research results in the public domain can lead to unacknowledged use and exploitation for commercial gains by external agencies within and outside the country. This is true especially in case of research in frontier sciences, such as agro-biotechnology.

Biotechnology and IPR

Biotechnology is a synergy of biological sciences and technology based industrial art. Biotechnology includes any technique that uses living organisms or parts of organisms to make or modify products to improve plants or animals or to develop microorganisms for specific uses (Congress of the United States, office of Technology Assessment, 1990). In Biotechnology the basic material is a biological material or process or product with an industrial application.

However, the recent developments like rDNA, cell fusion and monoclonal antibody technology has raised fundamental social and moral issues and hassles in IP rights. With current challenges and market demands intellectual property rights has become a key factor for the success and survival of Biotech industry.

In Biotechnology, currently protection of IP is facilitated through protection of plant varieties and patents, in the form of "trade secrets" (hybridization conditions, cell lines, customer lists, merchandising plans etc), copyrights (DNA sequence data which may be published) and trademarks that ensure biotechnology products better marketing opportunities (laboratory equipments bears trademarks well known to workers in this field, vectors useful in rDNA research).

Patents

As far as biotech/rDNA research is concerned patent is the most important systems of IPR. As per the Indian guidelines, life forms of plant and animals except microorganisms are not patentable in India. Also a method or process of agriculture and horticulture is non patentable. Similarly terminator gene technology is non patentable. Though organs and tissues are not patentable, biotechnological materials such as recombinant DNA, plasmid and process of manufacturing thereof are patentable. Gene and DNA sequences without having disclosed their function are not patentable. However, methods for rendering plants free of diseases or putting an additive value to a plant can be claimed for patenting. In pursuance to the TRIPS Agreement Article 27.3(b) plants and animals were left out of the compulsions of strict patent regime.

The most promising use from rDNA technology is the breakthrough of natural species barriers by moving genes from one species to another which is impossible naturally. GM crops have been developed using traits like resistance to insect pests and plant diseases, delayed fruit ripening, better taste, nutrition, elimination of saturation of oils, allergens etc. and for improved agronomic traits like resistance to abiotic and biotic stresses. Other breakthroughs involve genetic use restriction technologies (GURT) such as terminator genes. Although these technologies restrict unauthorized use, they are shrouded in controversies and ethical and humanitarian conflicts as it restricts the farmer's rights on the seeds they grow. Due to these reasons such methods were withdrawn from the future plant breeding programmes. However, it is evident that in order to protect and encourage in biotechnology investment it is utmost essential to grant some sort of protection to these technologies and increasingly biotechnology is receiving some form of protection, mainly occurring in USA and other industrialized nations. In 1985 the US Board of Patent Appeals allowed patent protection for asexually, sexually or *in vitro* derived plants. Similar to genetically engineered microorganisms, genetically altered plants were allowed patent by the US PTO and it was decided that normal US utility patents could be granted for other types of plants also eg. genetically modified plants. It was later affirmed by USPTO that plant utility patents could be granted to sexually reproduced plants in an infringement lawsuit for sexually

reproduced corn hybrids by Pioneer Hi-Bred International Inc. The court held that newly developed plant breeds fall within the patentable subject matter and neither the PPA nor the PVPA limits the scope of its coverage. Among transgenic plants, herbicide resistant cotton, canola, soybean etc. insect-resistant potato, cotton, maize etc have been patented. In Japan also plant patents are allowed. Though the EPO has granted patent to a transgene conferring herbicide resistance for Plant Genetic Systems, Belgium, a patent claimed by Monsanto on a '*Nap Hal'* traditional land race of wheat with good biscuit making qualities was revoked confirming the ruling that "plant varieties cannot be patented". Whilst patents are granted in many countries for plants and microorganisms, it has been the issue for animals which has been most controversial.

Protection of plant varieties

As per the mandate of the TRIPS agreement the plant varieties need to be protected. In pursuance of the agreement India has enacted "Protection of Plant Varieties and Farmers Rights (PPV and FR) Act 2001", a *sui generis* system of plant variety protection. This law is unique since it has also the aspects of the Farmers rights under the gambit of law. The model based on which the act was framed is the UPOV Act, an International Convention (Convention of the Union for the Protection of New Varieties of Plants". As per this at, a plant variety qualifies for protection when it meets three essential criteria i) distinctiveness ii) Uniformity iii) stability and the variety should be new in commercial sense. The application for its protection can be filed in the country where it developed or in any other UPOV member country.

The Indian PPV and FR Act provide both breeders as well as farmer's rights. Accordingly protection may be provided to new varieties, extant varieties (already in cultivation or of common knowledge) or farmers' varieties. Novelty feature is included in case of new varieties. It provides protection to essentially derived variety and also elaborates provisions for the protection of farmers' rights. Transgenic varieties are considered under essentially derived variety. Agri or Plant Biotechnology has witnessed major breakthroughs in transgenics for various plant traits. Efforts have been made to improve crop yield by transferring more than one gene. Drought and other biotic stresses is another area of transgenic development. Similarly efforts are on for developing crops with increased nutritional quality like golden rice, improved taste, texture and appearance of food, reduced dependence on agrochemicals like the Bt transgenics. Crops have also been genetically engineered to acquire tolerance to broad spectrum herbicides eg. Round Up ready crops. Agribiotechnology is being applied for novel uses other than food for example, oilseed can be made to produce fatty acids for detergents, substitute fuels and petrochemicals. Plants have been engineered to produce insulin and vaccines. Advantages of such edible vaccines are enormous once they qualify the clinical trials.

Biological compounds

DNA, RNA and protein are not living in themselves but occurring naturally. The ability to isolate genes and produce the proteins they encode has enormous commercial impact. The

availability and scope of patent protection on genes and genome related technologies is considered vital for survival and success of the biotechnology industry. As per USPTO DNA sequences are considered chemical compounds and are patentable as compositions of matter. The explanation given is that an isolated and purified DNA molecule that has the same sequence as a naturally occurring gene is different from the naturally occurring compound as it is processed through purifying steps that separate the gene from other molecules naturally associated with it and hence is eligible for patent protection. If a patent application discloses only nucleic acid molecular structure for a newly discovered gene and no utility for the claimed isolated gene, the claimed invention is not patentable since one of the requirements of a patent is utility. In case of DNA patents EPO requires that the specific industrial application of a DNA sequence or a partial DNA sequence of a gene must be disclosed in the patent application. In Japan, utility means industrial applicability as prescribed in the Japanese Patent Law, which states that any invention which is industrially applicable may be patented. DNA fragments, genes and recombinant proteins are considered to be chemicals by the JPO. The industrial applicability of conventional type chemicals require that at least one use be described in the specifications as filed.

Issues in rDNA technology-case studies

It is already mentioned that the most promising use from rDNA technology is the breakthrough of natural species barriers by moving genes from one species to another which is impossible naturally. The grant of IPR to transgenics probably is the most controversial issue in biotechnology in the last few decades. The transgenic seed industry was set of in the early 1990, initially focusing on soybean, corn and cotton and the main players were companies like Monsanto, Pioneer, Syngenta, Dow and Bayer. The industry mainly was involved in innovation involving genetic transformation technologies and genomics, genetic traits like insect resistance and herbicide tolerance and seeds containing genetic traits. Once the industry started facing misuse without the inventor being benefitted from his efforts, the need for IP protection was felt. Many instances have been reported wherein transgenic seeds have been either purchased from black market or saved by farmers from the previous years crop. However, the grant of IP protection to plant varieties and living organisms faced stiff opposition from various facets of the society. The major concerns were that the protection and registration of IP rights over transgenic crops may create monopolization over such seeds or methodologies in regard to such crops or over the crops themselves. If created, such monopolies will directly affect the rights of farmers and may also adversely affect the earth's biodiversity. The monopoly also restricts access to GM technology. Farmers will have to buy climate tolerant seeds for every crop season and will not be allowed to sell or store or exchange seeds for replanting. Patents have been granted for DNA sequences from nutmeg, cinnamon, rubber, jojoba and cocoa. However, when farmers buy those seeds an agreement need to be signed that prohibits them from using or replanting the seeds for the following season which means that the seeds cannot be stored and must be bought again for replantation. Thus adoption of transgenic crops will lead farmers to depend on multinational corporations to buy the crops, which is contrary to the farmers right to save, re-sow, share or sell

the variety of crops. There are also other associated hazards like the case of unintentional infringement by ignorant farmers. Patented GM crops are significantly expensive than conventional or hybrid crops and the farmers have to constantly procure from the seed producers. This would also reduce the range of local and native seeds and local traditions.

Moreover GM crops are alleged with ethical and moral issues also. The socio economic issues of GM crops and its patenting extend to the growing power of multinational corporations over traditional farming. If IP rights protection is offered to technology for developing transgenic crops, it should balance the public rights and environmental issues with the monopolistic rights. There should be proper enforcement of laws relating to the protection of farmers rights and interests simultaneously giving due credit to right holders and patentees. Another major concern when it comes to IP protection is the transaction costs for gaining freedom to operate. In actual, the IP transaction costs are independent of market size and the cost is exorbitant as the number of technologies increase. The problem is compounded when we have more than one gene in a crop. This in a way scares off investment in R&D projects, unless expected returns are promising.

Several issues have arisen in the past and some of the patents claimed by other countries could be revoked like in the case of turmeric, tamarind and neem which are the plants whose medicinal properties are known to us for decades. Indian Scientists have developed rice varieties producing enhanced levels of carotene and iron and also they were successful in introducing beta carotene genes into indigenous varieties, rice conferring drought resistance etc. However, Indian rice in foreign markets witnessed tough challenge as a consequence of grand of patent to Texmati, with label claims that it is superior or equal to basmati. Based on a petition from Govt of India the claims for Texmati were surrendered. DNA finger printing techniques of plants would protect the genetic resources and can establish profiles of origin of the genetic material in the event of award or patent dispute.

Biotech sector is emerging as a high growth sector though witnessing similar issues as are prevalent in other global economies such as USA and patenting of biological organisms, cloning, genomics, bioinformatics have become important aspect areas. Safeguarding of proprietary technologies through a robust intellectual property regime needs to be balanced against social welfare due to the unorganized small holder system in Indian farming sector. Thus, effective development and management of proprietary DNA portfolio is vital to the success of biotech companies. DNA patent is no longer a mere property, but is now the core of modern biotech companies. Biotechnological inventions were earlier interpreted in different ways by different patent offices of the world but discussions and unification of ideas have emerged in many cases. In the near future, these will also be solved and common grounds will be laid in the context of present TRIPS regulations. In Indian context while granting IP protection to transgenics, it should be kept in mind that anti competitive behavior and abuse of dominance by corporate players in this industry need to be checked. Indigenous businesses agricultural processes and

techniques practiced in our country since generations need to be protected. When it comes to seed licensing absolute control of the producer need to be discouraged and free flow of technology and its usage rights by other players of the market need to be encouraged for positive growth and returns. Moreover, there is a need to accelerate development of innovative biotech products.

PROTEOMICS – THE FUNCTIONAL GENOMICS APPROACH

P Umadevi

Bridging the gap

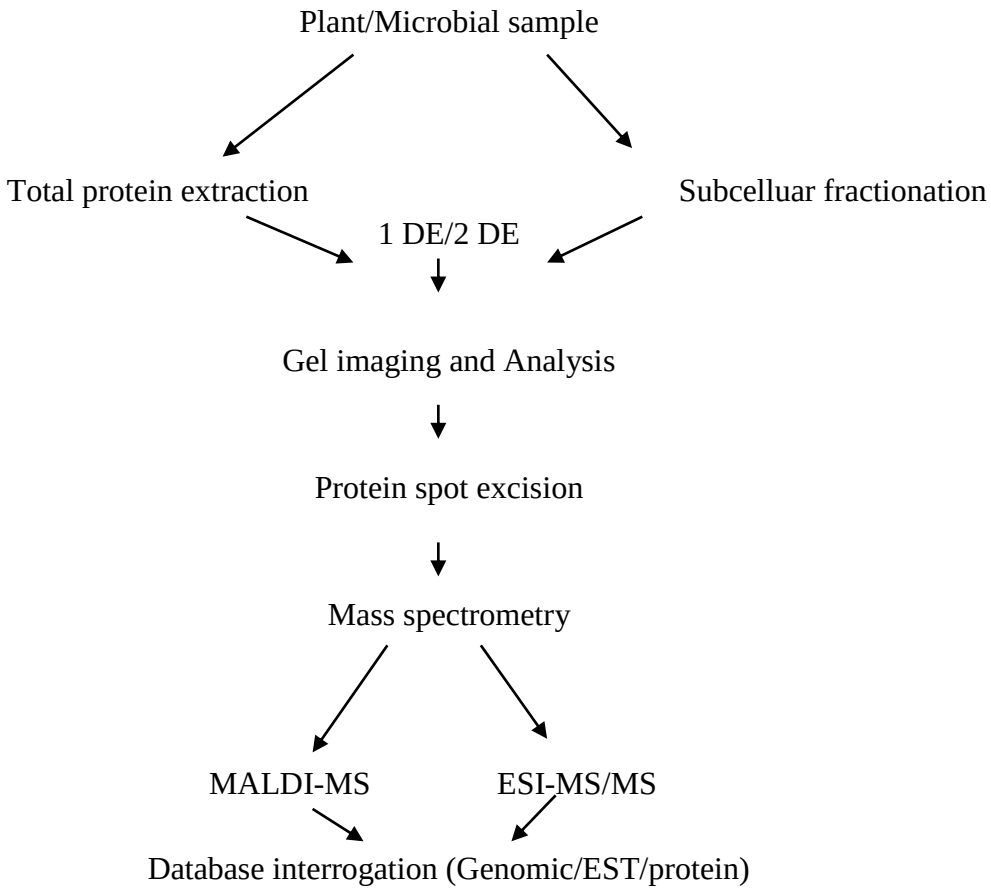
Proteomics is the study of protein properties viz., expression level, post-translational modification, interactions etc. The emergence of the concept proteomics is novel with the focus to deal with the issues concerning the function and regulation of the sequenced genes from whole genome and cDNA sequencing projects (Wasinger *et al.* 1995).

A key requirement in understanding the functional elements of the genome is accurate annotation of protein-coding genes. Most gene structures in newly sequenced organisms are based on computational predictions, often unsupported by experimental evidence. The use of proteomics data to experimentally validate gene annotations has recently become an increasingly valuable. Maillet *et al.* (2007) used combination of 2-DE, MALDI-TOF-MS and PMF which allowed the identification of numerous proteins with incorrect or incomplete ORF annotations in the current E. coli K12JM109 genome databases. Several inconsistencies in genome annotation were verified experimentally using proteomics. Merrihew *et al.* (2008) used mass spectrometry-based approach for gene annotation of any organism with the demonstration using nematode *Caenorhabditis elegans.* c peptide spectra yielded total of 429 new coding sequences. *Another syudy by Pawar et al. (2014) used mass spectrometry derived peptide data to correct the existing gene models with the development of bioinformatics approach to reanalyze the genome annotation in protozoan parasite Leishmania donovani* In any sequenced whole genome, at least 30% to 50% of predicted gene products would have no known homologues or show too little sequence homology (less than 30% identity) to known proteins to make a reliable functional annotation. Recent advancement in proteomics offers promising platforms to characterize unknown genes.

Proteogenomics in black pepper

Black pepper is an important Spice crop in tropical countries. With the absence of whole genome sequence/ EST data on this crop, our lab undertook both 2 D gel electrophoresis leaf proteins coupled with LTQ-Orbitrap mass spectrometry platform and the label free proteomics platform to bring out information on black pepper genes. Using this approach we have developed a peptide based database (Piperpep) (Umadevi et al 2016) which shows the applicability in functional genomics even in orphan crops where there is no genome sequencing attempts.

The steps involved in proteomics



Role of Bioinformatics

The data mining, integrated data management and network modeling of data from peptide mass fingerprinting data are the bioinformatics application in proteomics. Among these the data mining is a key player in proteomics. Using software the data from MS results can be compared with the set of theoretical protein sequence in the databases. In our lab we have successfully demonstrated the proteomics assisted identification of antimicrobial peptides from black pepper (Umadevi et al 2018) with the experimental utilization of bioinformatics tools for the peptide mass peak data.

Limitations of proteomics

The common problem noticed in the plant proteomics is the difficulty in obtaining good plant extract for subsequent electrophoresis separation (Umadevi & Anandaraj 2015). The interferences in plant extract such as high content of secondary metabolites, phenolics, polysaccharides and low protein content can cause inefficient protein separation. Incompletely separated spots, running difference between gels, differences in software algorithms and

therefore analysis tendencies. The main reason for low protein identification was due to the limited plant protein databases (Katam *et al.* 2010).

Black pepper proteomics

In our lab we had elucidated the role of temperature stress & the associated host proteins for the symptom expression of Piper mottle mosaic virus in black pepper (Umadevi et al 2016), developed the satellite map for the defense reaction against *Phytophthora* (Anandaraj & Umadevi 2016). The drought responsive proteins and ABA responsive proteins identified in black pepper (Krishnamurthy et al 2016) will be the important candidates towards identifying and developing drought tolerant varieties in future.

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ELEMENTS OF CRITICAL THINKING FOR A CAREER IN SCIENCE

Lijo Thomas

A career in science requires acquisition of several skills in the realm of knowledge and skills. One particular skill that is the corner stone of a successful pursuit of a career in science is the skill for critical thinking. A reflective and analytical style of thinking based on logical thought process is termed as critical thinking. A person with the ability for critical thinking can distinguish himself in any of the career paths he/she chooses, but it is especially true in case of academic careers in science and related fields. In the present world of increasing complexity arising from advances in communication, computation and technology, the process and skill set of critical thinking is even more important.

More than a passive skill, critical thinking can be viewed as a methodology that helps an individual to develop a deep understanding of the concepts, analyse observations, scrutinize data generated and evaluate results from diverse activities related to science learning and research. A personnel develops the capacity for assess and appraise each scientific experiment by making reasoned judgments based on relevant criteria. When a science student starts to make reasoned judgments about what to do and arrive at valid conclusions in relation to scientific concepts and contexts, then it is a certain sign of ability for critical thinking. They also develop the ability to consistently avoid guess work and/or mechanical thinking by using valid scientific criteria in decision making.

Ideally, a scientific mind does not accept results and observations in its face value. Each observation should generate a host of questions which explores the observation and the context of observation in further depth. Why is the observation/result like this? Is their sufficient evidence to postulate a cause-effect relationship? How good is that evidence? Are the arguments supporting my hypothesis valid? Are the arguments and logic used to explain the event biased? Is it verifiable and repeatable? What are the possible alternative explanations? These sort of critical questioning and thinking moves a mere observation or collection of data points into the realms of scientific inference and reasoning.

Learning the skill of critical thinking

Like any skill set, critical thinking needs to be taught and cultivated. Unlike many soft skills and creative skills, the skill for critical thinking cannot be transferred quickly through theory classes. The skill has to be nurtured consciously from an early age and allowed to develop over time. The theoretical part of critical thinking can be taught but the process of critical thinking itself has to be experienced and practised over a long period of time before it becomes a second nature to an individual. The ability to evaluate information, identify biases, examine the logic of arguments, and evaluate evidence would allow people from all walks of life to make better decisions. This means that the critical thinking skill, need not be seen as a skill useful only for the people in the

domain of science. Each life transaction can be made more effective through informed choices and better decisions through the use of critical thinking skill.

There are several benefits of developing critical thinking for a student of science. At one level, it helps to facilitate a deeper engagement and understanding of the area of study. The process of critical enquiry which develops as a part of the critical thinking enables this process of deeper engagement resulting in better understanding of concepts and process. The critical thinking ability also makes the students more independent in their thinking and creates confidence. The use of tools of critical thinking enables them to monitor their own progress independently of any external evaluation process.

Some of the peculiarities of the human though process also need to be understood to fully consciously avoid certain pitfalls in the thought process. A few of them are also illustrated in the following section. This is followed by discussion of some of the cognitive biases which hinder the efficient thought process. There are several hindrances for pursuing a clear logical thought process for each and every situation. This arises, either from limitations of the human capabilities or due to cognitive biases. In this section we will also examine certain cognitive biases which occlude our ability for critical thinking. The following discussion merely highlights some of the cognitive biases and is not an exhaustive discussion. The biases/effects discussed are used for their illustrative purpose so as to highlight the possibilities for our thought process to be impaired by certain situations. These particular biases have been chosen considering the possibility of encountering such situations/effects in a science career.

The two systems of thinking

The work of Daniel Kahneman and others has generated several critical insights on our cognitive processes. Essentially, our process of thinking can be divided into two systems. System 1, which produces the fast, intuitive reactions and instantaneous decisions that govern most of our lives and System 2: which is responsible for the deliberate type of thinking involved in focus, deliberation, reasoning or analysis – such as calculating a complex math problem, exercising self-control, or performing a demanding physical task. System 1 is an automatic, fast and often unconscious way of thinking. It is autonomous and efficient, requiring little energy or attention, but is prone to biases and systematic errors whereas System 2 is an effortful, slow and controlled way of thinking. It requires energy and can't work without attention.

Examples of System 2 thinking would include How to decide which college to attend, which house to buy, or whether to change careers? These are the types of decisions that engage System 2. They require attention and slow, effortful, considered responses. Even though you might have gut feelings about each college, house or career path, you would likely try to supplement them with a much more thoughtful and rational approach. This might involve collecting as much information about each option as possible, asking your friends, family or colleagues, or making a

list of pros and cons for each option. This is an example of thinking done by the System 2. System 1 is capable of making quick decisions, based on very little information. Examples of this type of thinking would be understanding a simple sentence, a simple problem like 2 + 2 = ? approximately measure the distance etc. These fleeting impressions, and the many other shortcuts called heuristics you've developed throughout your life, are combined to enable System 1 to make these decisions quickly, without deliberation and conscious effort. Understanding the nature of these two systems of thinking is important to consciously modify and train the thinking process

The Cognitive Reflection Test (CRT) is a three-question test designed to measure the ability of respondents to activate metacognitive processes and switch to System 2 (analytic) thinking where System 1 (intuitive) thinking would lead them astray. Each CRT question has a correct analytical (System 2) answer and an incorrect intuitive (System 1) answer. One of the CRT question is given below.

"The cost of a bat and a ball is $1.10."

The bat cost $1.00 more than the ball.

What is the cost of the ball ?

An intuitive (impulsive) answer that the ball costs $0.10 does spring to mind by subtracting $1.00 from $1.10. However, should this be the case, the total cost of the bat and ball would be $1.20, which is incorrect. Hence, the right answer is $0.05

The CRT, developed by Shane Frederick is designed to measure respondent's ability to activate metacognitive processes allowing them to switch from System 1 thinking to System 2 thinking. In other words, it is the disposition to resist reporting the response that first comes to mind.

Description of selected cognitive biases

Availability bias:

The availability heuristic suggests that the people treat the ease with which a value or event comes to mind as informative regarding its likelihood or frequency. We create a picture of the world using the examples that most easily come to mind. This is illogical, because in reality things don't happen more frequently just because we can conceive of them more easily. In addition, people prefer information that is easy to obtain, be it economic data or recipes. The decisions made based on the easily available information rather than on more relevant but harder to obtain information can often be wrong. The availability bias is one of the most common biases. As a researcher, you should be careful to accumulate data from all possible sources of information while evaluating evidences for or against a particular hypothesis. You can counter the availability bias by deliberately interacting with people whose experiences and expertise are different than yours. One example of availability bias in real world would be corporate board meetings, where the members discuss what is provided on the agenda and not anything else which could be equally or more import to the wellbeing of the firm.

False causality fallacy or the illusory correlation

You incorrectly connect two unrelated observations in a cause-and-effect relationship based on proximity in time or space or other shared attributes. A false causality argument generally looks like this "Event A happened. Event B happened after A. Therefore, A caused B. The false cause fallacy is sometimes summarized as "correlation is not causation" . Consider this example from German physics professors Hans-Peter Beck-Bornholdt and Hans-Hermann Dubben.

In one city, a study revealed that in each blaze, the more firefighters called out to fight it, the greater the fire damage. The mayor then imposed an immediate hiring freeze and cut the firefighting budget.

In this case the mayor seems to have fallen prey to false causality fallacy. We can see that it was not the number of firefighters called out that determined the extent of damage. The bigger the blaze, the more firefighters were called out. A failure to identify the cause and effect can lead to many erroneous results and judgements. An alert mind should be on the look out for spurious correlation, where random independent variables might have some correlation and false causality, where correlation does not mean any cause effect relationship.

Another example from the book "The art of Thinking Clearly" on fallacy of false causality: Scientists found that long periods in the hospital affected patients adversely. This was music to health insurers' ears, who, of course, are keen to make stays as brief as possible. But, clearly, patients who are discharged immediately are healthier than those who must stay on for treatment. This hardly makes long stays detrimental.

Decision-making cognitive bias: The ambiguity effect

Try to solve this problem before reading on. Jack is looking at Anne, but Anne is looking at George. Jack is married but George is not. Is a married person looking at an unmarried person?

Yes No Cannot be determined

More than 80 per cent of people answer this question incorrectly. The correct answer is, yes, a married person is looking at an unmarried person. Most of us believe that we need to know if Anne is married to answer the question. But think about all of the possibilities. If Anne is unmarried, then a married person (Jack) is looking at an unmarried person (Anne). If Anne is married, then a married person (Anne) is looking at an unmarried person (George). Either way, the answer is yes. To figure this out, most people have the intelligence if you tell them something like "think logically" or "consider all the possibilities." But unprompted, they won't bring their full mental faculties to bear on the problem. The ambiguity in the data supplied combined with the failure to consider all the possible scenarios lead a majority of the respondents to a false conclusion. This holds an important lesson for us. The failure to consider all alternatives can lead to an erroneous judgement.

Authority bias

Authority bias is the tendency to attribute greater accuracy to the opinion of an authority figure (unrelated to its content) and be more influenced by that opinion. In most of the societies, a diverse and widely accepted system of authority allows the development of sophisticated structures for the production of resources, trade, expansion and social control. Most of the societies try to inculcate the habit of accepting authority. Though inherently good for the society, an independent thinking mind involved in scientific pursuit should be aware of the existence of such a bias and should evaluate his/her response to opinions of seniors and attitudes towards dogmatic concepts encountered during the course of scientific enquiry.

Failure to visualize non-linear growth

A piece of paper is folded in two, then in half again, and again and again. How thick will it be after fifty folds? Write down your guess before you continue reading.

Are you ready? Well, if we assume that a sheet of copy paper is approximately 0.004 inches think, then its thickness after fifty folds is a little over seventy million miles. This equals the distance between the earth and the sun, as you can check easily with a calculator.

Linear growth we understand intuitively. However, we have no sense of exponential (or percentage) growth. This is because human beings seldom had the opportunity to experience non-linear growth or phenomenon. Our ancestors' experiences were mostly of the linear variety. This lack of capacity to understand the non linearity can pose problems in our judgement. We will look at another example where non-linear data is encountered. The example is from Harvard Business review.

"Imagine you're responsible for your company's car fleet. You manage two models, an SUV that gets 10 miles to the gallon and a sedan that gets 20. The fleet has equal numbers of each, and all the cars travel 10,000 miles a year. You have enough capital to replace one model with more-fuel-efficient vehicles to lower operational costs and help meet sustainability goals.

Which upgrade is better?

1. Replacing the 10-MPG vehicles with 20 Miles Per Gallon (MPG) vehicles.
2. Replacing the 20-MPG vehicles with 50 MPG vehicle.

Intuitively, option B seems more impressive—an increase of 30-MPG is a lot larger than a 10-MPG one. And the percentage increase is greater, too. But B is not the better deal. In fact, it's not even close.

Shockingly, upgrading fuel efficiency from 20 to 100-MPG still wouldn't save as much gas as upgrading from 10 to 20-MPG. But choosing the lower-mileage upgrade remains counterintuitive, even in the face of the visual evidence. It just doesn't feel right. If you're still having trouble grasping this, it's not your fault. Decades of research in cognitive psychology show that the human mind struggles to understand nonlinear relationships. Our brain wants to make simple straight lines."

The study concludes that "In recent years a number of professions, including ecologists, physiologists, and physicians, have begun to routinely factor nonlinear relationships into their

decision making. This will increase their ability to choose wisely—and to help the people around them make good decisions too"

The section above selectively discusses certain common biases and fallacies which are encountered on a routine basis. There are several such kinds of cognitive biases and fallacies that affect our judgement. The process of clear thinking involves avoiding such pitfalls of cognitive biases as well as developing a mind which remains alert to presence of logical fallacies in our arguments and thought process.