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AWARDS AND HONOURS

K.C. Mehta and Manoranjan Mitra Award

Diversity of *Phytophthora* affecting Horticultural crops in India

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The genus *Phytophthora* with over one hundred species is one of the most destructive among plant pathogens. Species of *Phytophthora* occur in various ecosystems including agricultural and non agricultural systems and rain forests. They attack various plant parts and are responsible for the severe economic losses of various agricultural and horticultural crops. Ever since the genus was erected after the notorious Irish famine due to late blight of potato, several plant pathogenic species have been described, the latest being *P. ramorum* causing oak wilt in pacific coast of the United States of America and *P. kernoviae* on forest trees and ornamentals in UK (Brasier *et al.,* 2005). Species of the genus *Phytophthora* were reviewed by Erwin & Ribeiro (1996) in the book '*Phytophthora* worldwide', which included 59 species with five varieties. Since then, several new *Phytophthora* species have been described from different parts of the world and now over one hundred species are described (Sikora *et al.,* 2012). Most of the species of the genus have a wider host range, for example, over 2000 plant species are thought to be susceptible to infection by *P. cinnamomi* in Australia, where this pathogen has severely altered native plant communities since its accidental introduction in the 1920s (Hardham, 2005). There are also some species with a narrow host range such as *P. sojae* and *P. infestans*. In India *P. palmivora* infects several horticultural crops such as palms, cocoa, citrus, black pepper and cassava. Due to their significance and economic importance, there has been increasing interest in the molecular genetics and genomics of *Phytophthora* species. In spite of best efforts by scientists all over the world, the pathogen is threatening to re-emerge breaching all barricade put up as host resistance and other management strategies against late blight of potato and tomato (Fry and Goodwin 1997; Kamoun, 2001). Their ability to survive has been tremendous due to the emergence of new races. The genetic recombination occurs not only by sexual recombination but also through parasexual recombination. The pathogens of this genus affect most of the cultivated crops in a wide range of agro climatic regions affecting both horticultural and field crops in India in different agro-climatic zones (Table 1). The wet monsoon period prevailing over various parts of the country provides ideal conditions for them to infect. In perennial crops like apple, citrus, black pepper the damage caused to the below ground portions such as roots and collar is expressed later in the season. The expression of symptoms depends up on the site of infection and extent of damage. Though, many crops are affected by *Phytophthora* in India, only some of the diseases are well investigated. There are new diseases caused by *Phytophthora* in field crops such as cassava that threaten the cultivation of these crops.

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Symptoms

The symptoms on the aerial parts include brown to purple lesions. When the infection is on the leaves, fruits and stem, lesions appear water soaked and on roots it is usually confined to cortical region, sometimes extending to cambium. It is soon followed by saprophytes resulting in wood discolouration as shown in fig.1.

Life Cycle

The vegetative phase of the pathogen is multinucleate coenocytic mycelium with occasional septa delimiting reproductive structures. The cell wall comprises mainly of insoluble polymers of glucans with ß, 1-3 and ß, 1-6 linkages. The protoplast is enveloped in a double layered plasma membrane with other cell organelles typical of eukaryotic cell such as nuclei, mitochondria, endoplasmic reticulum and dictyosomes. There are four unique organelles in *Phytophthora,* viz., peripheral vesicles that later relocate into the zoospores, fingerprint vesicles containing water soluble reserve polysaccharide laminarin which is made up of ß, 1-3 linkages of glucans, bullet shaped micro bodies with crystalline fibrils and microtubules in the cisternae of endoplasmic reticulum that later form flagellar hairs. The nutric requirement varies and for all species external source of thiamine is required. Glucose, sucrose and other glucose containing saccharides serve as carbon source, whereas, trehalose, cellobiose or cellose are poorly utilized. Organic acids and amino acids are unsuitable and useful only for buffering. Nitrate, ammonia or single amino acid such as asparagine are utilized as nitrogen source. Sterols are not essential for vegetative growth but required for reproduction.

Table 1. *Phytophthora* affecting various crops in India

Crop	Disease	*Phytophthora* species
Apple	Collar rot	*P. cactorum*
Areca nut	Fruit rot, bud rot	*P. arecae, P. meadii*
Betel vine	Collar rot, leaf rot	*P. capsici, P. parasitica*
Black pepper	Collar, root, leaf rot	*P. capsici, P. palmivora, P. parasitica, P. citrophthora*
Cardamom	Capsule rot	*P. meadii*
Carnation	Collar	*P. nicotianae*
Cassava	Tuber rot	*P. palmivora*
Chilli	Collar	*P. capsici*
Citrus	Root rot, gummosis fruit rot	*P. capsici, P. palmivora, P. nicotianae (=P. parasitica) P. citrophthora, P. bohemeriae, P. insolita*
Cocoa	Pod rot, canker	*P. palmivora, P. capsici*
Coconut	Bud rot	*P. palmivora*
Colocasia	Blight	*P. colocasiae*
Crossandra	Wilt	*P. nicotianae*
Cucurbits	Fruit rot	*P. capsici*
Gerbera	Wilt	*P. nicotianae*
Palmyra palm	Bud rot	*P. palmivora*
Potato	Blight	*P. infestans*
Rubber	Leaf fall	*P. heveae*
Tobacco	Black shank	*P. nicotianae*
Tomato	Blight	*P. infestans*
Strawberry	Red stele, leathery rot, Ripe rot	*P. fragariae , P.cactorum , P. nicotianae*
Vanilla	Fruit rot, stem rot	*P. meadii*

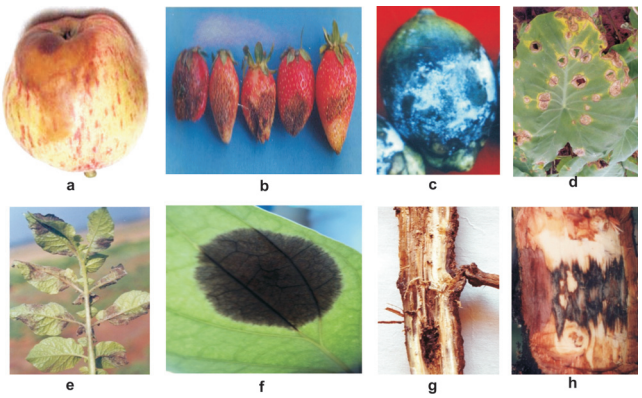

Fig. 1. Symptoms on the aerial parts, usually water soaked lesions brown to purple. On stem and roots, infection on cortical tissues reach the vascular tissues and blacken the xylem as shown in g and h: a) apple, b) strawberry, c) areca nut, d) colocasia leaf, e) potato leaf, f) black pepper leaf, g) black pepper stem and h) cocoa stem.

Some species such as *P. infestans* are highly demanding in their nutritional requirements (Buczaki, 1983; Erwin *et al*., 1983). The availability of nutrients determines the vegetative and reproductive phase of this pathogen. Under *in vitro* conditions the mycelial growth of pathogen shows various morphologic features depending up on the medium and other cultural conditions. The common features such as white cottony, stellate or chrysanthemum like floral patterns are produced by several species (Fig. 2).

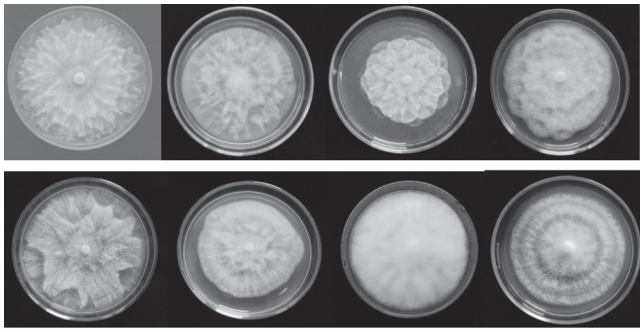

Fig. 2. Colony morphology of isolates of *Phytophthora capsici* isolated from black pepper

Asexual reproduction

The availability of nutrients and the environmental conditions trigger the switching over from vegetative to reproductive phase of the pathogen. Both asexual and sexual reproduction depends up on endogenous nutrient reserves. Almost all species reproduce asexually by the formation of sporangia. Each sporangium is produced by the elongation of the mycelium and bulging to give rise to various shapes and sizes of sporangia- borne on specialized branch of the mycelium called sporangiophores that may be branched or solitary. A typical sporangium consist of a stalk, sporangial body with an exit pore called as papilla made of mucilaginous material that dissolves while liberating the contents of sporangium during indirect germination of sporangium. The contents of the sporangium transforms into uninucleate protoplasts that differentiate into motile zoospores. From each sporangium 16 - 64 zoospores are produced depending up on the number of mitotic divisions in the sporangium. In *Phytophthora* the differentiation into individual zoospores occurs within the sporangium and individual zoospores emerge out through the exit pore through the dissolution of mucilaginous papilla, whereas the differentiation occurs outside in a vesicle in case of the genus *Pythium.* The stalk length may vary from short occluded less than 5µm as in *P. palmivora* or as long as more than 200 µm as in *P. capsici* (Fig. 3).

Based on the type of papilla the sporangia are classified as papillate with prominent papilla (*P. palmivora)*, semi-papillate (*P. citrophthora*) and non-papillate without papilla (*P. cinnamomi*). Asexual reproduction is influenced by narrow temperature ranges. The production of sporangia is dependent on temperature, light and relative humidity. The sporangia may germinate directly to give rise to mycelium or indirectly by liberating motile zoospores. Indirect germination requires change in temperature such as chilling under laboratory conditions. Such conditions are available for pathogens under natural conditions.

When the encysted zoospore germinates it may produce a mycelium when nutrients are available. If the conditions are not favorable the mycelium would produce a microsporangium to tide over the adverse conditions.

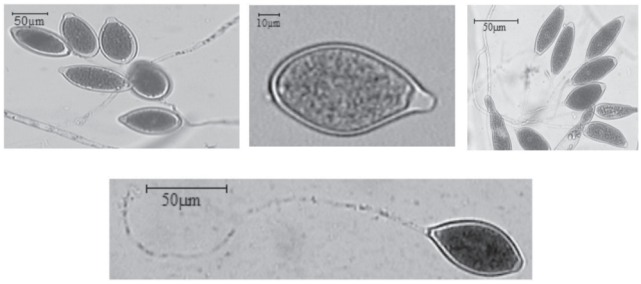

Fig. 3. Sporangia showing prominent papilla and stalk. A) Sporangia of *P. palmivora*, b) A single sporangium of *P. palmivora* showing a short pedicle, c) Sporangia of *P. capsici* with long stalks arising from sporangiophore and d) A single sporangium of *P. capsici* with long stalk.

Other oomycetes pathogens like downy mildews release their zoospores in the early hours of the morning (02.00h) when the temperatures are relatively cooler. In case of *P. arecae* (now referred to as *P. meadii*) sporangia are produced on the infected fruits only when there is alternate sunshine and rain and the humidity is between 95- 98%, if the humidity is 100% the infected fruits are covered only by mycelium. This has an effect on the spread of the disease (Anandaraj *et al.,* 1991). The zoospores are one of the specialized reproductive structures of oomycetes pathogens. It is a naked bit of protoplasm containing all the necessary information and infrastructure to start a new infection process. Since they lack a cell wall, there is a constant inflow of water that is expelled by the action of the vacuoles. This phase of the life cycle is most explosive as abundant zoospores are produced but at the same time this is the most vulnerable stage. Several metabolites and peptides produced by the antagonistic agents directly act on the wallless plasma membrane leading to the disintegration of zoospores. The typical zoospore is kidney shaped with a lateral groove through which a pair of flagella is inserted. The forward one is tinsel type with fine hairs and the posterior one is smooth. Both help in motility. The anterior end is narrower than the posterior end and the zoospores are longer than their width. The zoospores being motile move in search of the host plants. They are positively rheostatic moving against water currents, negatively geotropic and chemotactic. Species of *Phytophthora* are known to move at 100-200 µm/second. *P. cryptogea* is reported to move 2.5-3.5 cm and *P. cinnamomi* 6cm in flooded soil (Tyler, 2002). Once near the host it is influenced by host factors and deploys several mechanisms to reach the infection court. The host recognition by *Phytophthora* is reported to be brought about by attraction of zoospores by physical, chemical and electrical means and by trophic movements of zoospores as well as hypha. Another important factor that influences the movement of zoospores is the root exudates by host plants. The movement of zoospores, their encystment on root surface, germination and penetration is guided by zoospore taxis involving chemicals, electrostatic force around roots and the root exudates. The presence of iso-flavone compound accelerates the movement of zoospores around roots. In addition, aggregation of zoospores is reported to be species specific and dependent on calcium ions (Erwin *et al*., 1983, Erwin and Ribeiro, 1996).

Host recognition and pathogenesis

One of the major events during pathogenesis is recognition of host by the pathogen and the pathogen by the host. This precedes signal transduction and activation of defenses. At molecular level this is brought out by the interaction of products of pathogen avirulence (*Avr*) genes and the host resistance (R) genes in a well orchestrated manner as propounded by Flor's (1955) gene for gene hypothesis. A single dominant gene (*Avr*) in the pathogen sends out a product that is recognized by the dominant gene (R) product of the host resulting in activation of defense genes. The genetics of avirulent *Avr* genes and resistant R genes is well understood in the pathosystem involving *Phytophthora*, especially soybean- *P. sojae*; tomato and potato–*P. infestans*; tobacco- *P. parasitica*; strawberry-*P. fragariae*; cowpea- *P. vignae*; pigeon pea- *P. cajani;* pepper (capsicum)-*P. capsici.*

Host recognition of pathogens is brought about by elicitors of pathogens. These include carbohydrates, proteins and other small molecules. Cell wall fragments from several *Phytophthora* species comprising of 1, 3 and 1, 6 glucosides have been reported to have hepta-glucan binding activity in soybean plasma membrane. A sterol binding protein was produced by *Phytophthora* that has 98 amino acid conserved sequence. The elicitins triggered defense not only protect against *Phytophthora* but also against bacterial pathogens. A common feature of many different types of plant pathogens is the secretion of a variety of extracellular effectors or elicitor molecules into the plant apoplast (Von't Slot and Knogge, 2002). Many of these proteins, called elicitors elicit plant defense responses and, in particular, a form of programmed cell death called the hypersensitive response (HR). *Phytophthora* species ubiquitously secrete a unique class of highly conserved effector molecules named elicitins. Elicitins are widespread in *Phytophthora* species and closely related to *Pythium* species (Panabieres *et al*., 1997). Elicitins are low molecular weight proteins (10 kDa) secreted into liquid minimal medium (Ribeiro, 1978). Two classes of elicitins have been identified; alpha-elicitins which are acidic and inducing only necrosis, whereas beta-elicitins are basic which induce distal necrosis (Kamoun *et al*., 1993; Nespoulous *et al*., 1992). Acidic elicitins (capsicein and parasiticein) are reported from *P. capsici* and *P. parasitica* respectively, while basic-elicitin (cryptogein and cinnamomin) are reported from *P. cryptogea* and *P. cinnamomi*, respectively.

Expressed sequence tags (ESTs) are sequenced portions of messenger RNA. In recent years, EST projects have been initiated for numerous plant and animal species, and have generated a vast amount of sequence information that can be used for gene discovery, functional genetic studies and marker development (Pashley *et al.,* 2006). EST databases represent a valuable resource for the identification of genes in organisms with uncharacterized genomes and for development of molecular markers. These are useful in genetic and evolutionary studies because they are located in transcribed genes and a putative function can often be inferred from homology searches (Moccia *et al.,* 2009). Large-scale cDNA sequencing and EST analyses have been a rapid method to identify novel cDNAs which afford to identify genes of various physiological functions (Uno *et al.,* 2008; Wang *et*

al., 2007). There are several expressed sequence tags reported for *P. parasitica, P. sojae, P. infestans, P. cryptogea,* and *P. cinnamomi*. These proteins have an N-terminal extension and are rich in serine-threonine. Certain elicitins such as alpha elicitins are reported to spread throughout the plant system and beta elicitins are reported to bring about systemic spread of defense system. Once the recognition is completed the zoospore withdraws its flagella, encysts by secreting cell wall with the available arsenal of enzymes and raw materials and produces a germ tube that resembles a typical appresorium and infection peg that is involved in dissolution of host cell wall and penetration as in case of other fungi.

The other asexual reproductive structures that help in survival of the pathogens during adverse conditions are thickened mycelium and thick walled chlamydospores (Anandaraj, 1997). The dormant structures such as encysted zoospores, chlamydospores and oospores are activated to grow by the stimuli obtained from the host root.

Sexual reproduction

The sexual reproduction involves fusion of antheridia (male) and oogonia (female) reproductive cells. The species may be homothallic producing both sex organs or heterothallic which require the presence of opposite mating types called as A1 and A2 mating types either physically or through their metabolites and some refer this as induction types. As the sexual reproduction requires external sources of sterols that lead to the synthesis of hormones it is highly dependent on external conditions (Erwin *et al.,* 1983). Once the required hormones are available sexual reproductive structures are produced. The oospores have thick walls made up of insoluble ß-glucans that serves as food reserves for the germination of oospores. There are two types of oogonia namely paragynous where the anthridium and oogonium lie side by side and amphigynous where the anthridium appear as a collar below the oogonium (Fig. 4). During the developmental stages of amphigynous condition the anthredial initial enlarges first followed by oogonial initial that pierces through the anthridial initial and grows faster and enlarges leaving the anthridium behind (Erwin *et al*., 1983).

Parasexual recombination

The genetic changes occurring in *Phytophthora* through other than sexual fusion was thought to be brought about by somatic fusion that is difficult to prove experimentally. Based on allozyme studies it is proposed that the changes occurring could be through mutations and mitotic recombination.

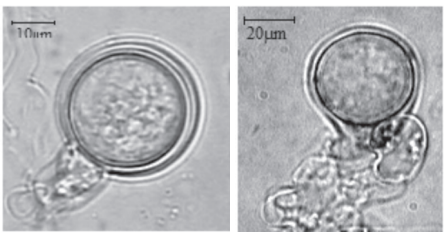

Fig. 4. Oospores of *Phytophthora.* a. Amphigynous- *P. capsici,* b. Paragynous- *P. cactorum*

Mutations occurring in populations of single lineages both for virulence and fungicide resistance have been characterized for *P. infestans* and *P. sojae.* The number of propagules produced by *Phytophthora* per lesion and the selection pressure imposed by the host and environment provides ample scope for mutation (Goodwin, 1997).

Taxonomic position of *Phytophthora*

For long this genus has been traditionally included under the kingdom fungi, under the class oomycetes and studied with other plant pathogenic fungi. The genus though filamentous and absorptive in nutrition as other fungi, they were considered unique because of several characters. Primarily, based on their cell wall composition that is mainly of β-1-3 and 1-6, glucans unlike chitin in fungi. The storage polysaccharide is mycolaminarin as in some algae and they depend on external source of thiamine and ß–hydroxy sterols. The vegetative phase is diploid and meiosis occurs only in the gametangia. They also differ in their asexual reproductive structures, nutritional requirements and their sensitivity to fungicides. The oomycetes pathogens have long been considered as unique group (Buczaki, 1983) because of their special characteristics under the kingdom fungi. Modern molecular techniques especially based on the data generated on the smaller sub unit of ribosomal DNA (SSU r DNA) have paved the way for phylogenetic studies. The oomycetes are considered to have had a separate ancestor more related to brown algae than true fungi and placed under a separate kingdom Staminipila among eukaryotes (Forster *et al*., 1990; Alexopoulos, 1996; Mueller *et al.,* 2004). For finer resolution of divergence among various clades of eukaryotes, data generated on multiple genes are used. Four protein coding sequences namely α- tubulin, β- tubulin, actin and elongation factor 1- α (EF1- a) genes in addition to r-DNA were used to study the divergence. Based on this data it is opined that the photosynthetic straminipiles namely brown algae, diatoms and golden brown algae are closer to the oomycetes than to the true fungi (Baldauf *et al*., 2000). Hence, they are no longer considered as 'fungi' but many scientists consider them as pseudo fungi or fungus like.

Identification and diversity analysis of *Phytophthora*

Previous identification of *Phytophthora* was purely based on the morphological aspects and other growth-temperature relationships (Brasier and Griffin, 1979; Hansen *et al.,* 1986). Along with this, other criteria such as breeding systems and gametangial structure, were used to provide insights into behaviour and adaptation. *Phytophthora* species have traditionally been divided into six morphological groups based on features of the sporangium, antheridium, and reproductive behavior, although these characteristics are sometimes ambiguous (Newhook *et al*., 1978; Stamps *et al*., 1990; Waterhouse, 1963). Owing to the high variability and overlaps in morphology within and between species of *Phytophthora* (Erwin and Ribeiro, 1996), identification of some *Phytophthora* species based on morphological criteria is often difficult, unreliable and time-consuming. Hence, other easier more reliable methods like rDNA was suggested (Drenth *et al.,* 2006).

ITS rDNA

Molecular detection methods then came into the scene and aided in the identification of *Phytophthora.* A phylogenetic analysis of 50 described *Phytophthora* taxa, based on ITS1 and ITS2 rDNA sequences, clustered the taxa into eight main lineages designated Clades 1–8 (Cooke *et al.,* 2000). Clades 1–5 mainly comprised *Phytophthora* with papillate caducous sporangia and an aerial mode of dispersal, while the *Phytophthora* in Clades 6–8 were mainly non-papillate with a largely soil-borne root-infecting habit. Several species specific markers were designed from the ITS region by several workers (Kong *et al.,* 2004; Chowdappa *et al.,* 2003b), for detection and identification of *Phytophthora* sp. However in some cases the ITS sequences are not sufficiently variable, making the design of primers to identify and detect closely related taxa very difficult or impossible. For example, the important *Phytophthora* pathogens such *as P. nemorosa, P. ilicis, P. psychrophila* and *P. pseudosyringae* have very similar ITS regions sequences and the design of effective and robust specific primer sets is very challenging (Martin and Tooley, 2003).

Mitochondrial genes

Mitochondrial genes are another set of genes used for identification and phylogenetic analysis of *Phytophthora.* TrnG–TrnY region (mitochondrial genome region between gene trnG (gcc) and gene trnY (gua)), Atp9–Nad9 (mitochondrial genome region between gene Atp9 and gene Nad9), Cox2–Cox1 region (mitochondrial genome region between gene Cox2 and gene Cox1) and TrnY–Rns region (mitochondrial genome region between gene trnY (gua) and gene Rns) etc. were analysed by Schena and Cooke (2006). Among the analyzed regions the TrnG–TrnY was the least variable and therefore unsuitable as a target region for the design of species specific diagnostics. Higher levels of sequence diversity were found in the Atp9–Nad9 region and the occurrence of intraspecific variability in the Atp9–Nad9 region is reported for *P. infestans* and closely related species (Wattier *et al*., 2003). Cox2–Cox1region was found to be more appropriate for identification, taxonomic and phylogenetic studies. This region can be amplified easily and aligned as the total length is quite similar in all *Phytophthora*s. This region has a combination of conserved and more variable portions. A general disadvantage of mitochondrial DNA is the very high AT/GC ratio. Generally, the mitochondrial DNA is more difficult to amplify and requires higher concentration of $MgCl_2$ compared to genomic DNA. Another potential complication of using mitochondria based marker system for species identification comes in the case of species hybrids (Delcan and Brasier, 2001). The mitochondrial genome is maternally inherited; therefore, depending upon which species functioned as the maternal parent the species-specific primers pair will amplify a diagnostic band indicating the presence of a particular species when, in fact, it is a hybrid. This was observed in natural hybrids of *P. nicotianae* and *P. cactorum*, all of which had the mitochondrial DNA restriction fragment length polymorphism of *P. nicotianae* (Man in'tVeld *et al*., 1998).

The phylogenetic trees generated from the TrnG–TrnY, Cox2–Cox1 and Atp9–Nad9 regions showed clustering of taxa, which was concordant with that determined by the analysis of the ITS regions (Cooke *et al*., 2000). This data indicated that the short variable TrnG–TrnY, Cox2–Cox1 and Atp9–Nad9 regions are poorly suited for broad scale phylogenetic analysis but can be utilized to improve the resolution of ITS in indiscriminating subgroups of more closely related *Phytophthora* species. The TrnY– Rns region was too variable to align accurately and cannot be utilized for a broad scale phylogenetic analysis, but this region will be very powerful for reconstructing the phylogenetic history of a newly originated species in relation to their geographic origin. Such phylogeographic analyses will aid in the reconstruction of pathways of global pathogen spread.

Nuclear-encoded genes

The nuclear-encoded ribosomal RNA genes (rDNA) provide attractive targets to design specific primers since they are highly stable, can be amplified and sequenced with universal primers, occur in multiple copies, and possess conserved as well as variable sequences (White *et al*., 1990). The phylogenetic analysis using rDNA-IGS (intergenic spacer region of the rDNA) region sequences matched closely to the based on ITS analysis (Cooke *et al*., 2000) with clades 1–5 grouping together and the non-papillate taxa in clades 7 and 8 at a basal position in the tree.

Another example of nuclear genes used for taxonomic studies of *Phytophthora* is ras-related protein (*Ypt1*) gene. They are highly polymorphic in nature and contains conserved coding regions flanking very variable introns. The Ypt1 gene enables the differentiation of closely related species such as *P. pseudosyringae, P. nemorosa, P. psychrophila,* and *P. ilicis* that have almost identical ITS regions. This region is free from intraspecific variation that could cause problems for diagnostic assays. Compared to other available target sequences the Ypt 1 gene has the enormous advantage to enable the design of all specific primers in a limited DNA region, but it is having the disadvantage of being a single copy gene (Chen and Roxby, 1996).

SSR markers for diversity analysis

The genus *Phytophthora* with over one hundred species continue to prove a threat to plant productivity on a global scale. Each species has a wide variability in relation to virulence, morphology and sensitivity to chemicals. Diversity has been studied using various markers including genetic markers. Microsatellites are stretches of DNA consisting of tandemly arranged units of 1-6 bp in length, characterized by relative abundance, hypervariability, locus specific, co-dominant and multi allelic nature and also called as simple sequence repeats (SSRs), which are ubiquitous in the coding and non-coding regions of prokaryotes and eukaryotes. Variation arises in the number of tandem repeats, which can be detected by PCR with primers designed from the conserved flanking sequence. Microsatellites (SSRs) are powerful markers in the study of *Phytophthora* population biology, epidemiology, ecology, genetics and evolution. SSRs are suited for population-genetic studies, since they enable quantification of putative heterozygotes which enables

estimation of naturally occurring outcrossing. Silvinia *et al*. (2011) used 219 isolates of *Phytophthora* sojae to compare three microsatellite analysis methods. Two capillary electrophoresis methods, the Applied Biosystems 3730 Genetic Analyzer and the CEQ 8000 Genetic Analysis system, detected an average of 2.4-fold more alleles compared to gel electrophoresis with a mean of 8.8 and 3.6 alleles per locus using capillary and gel methods, respectively. The two capillary methods were comparable, although allele sizes differed consistently by an average of 3.2 bp across isolates.

Lees *et al*. (2006) developed SSR markers to characterize a wider collection of 90 *P. infestans* isolates from the UK and six other countries along with isolates from the closely related species *P. mirabilis*, *P. ipomoea* and *P. phaseoli* collected from around the world. Amongst the 90 isolates of *P. infestans* examined, considerable SSR diversity was observed with 68 different genotypes and an average of 3·9 (range 2–9) alleles per locus. When other *Phytophthora* species were genotyped, all loci were successfully amplified and the majority was polymorphic, indicating their usefulness for studying related taxa.

Zhendong *et al*. (2004) identified 415 SSRs of *P. sojae* after searching 5800 ESTs. The most frequent repeats were trinucleotide repeats (50.1%) and the least frequent were tetranucleotide repeats (8.2%). Forty primer pairs were designed and tested on 5 strains of *P. sojae*. Of the 33 functional primer pairs, 28 primer pairs produced characteristic SSR bands of the expected size, and 15 primer pairs (45.5%) detected polymorphism among 5 tested strains of *P. sojae*. Based on the polymorphisms detected with 20 EST-SSR markers, the 5 tested strains of *P. sojae* were clustered into 3 groups (Zhendong *et al*., 2004). Random amplified microsatellites (RAMS) and repetitive extragenic palindromic (REP) DNA fingerprinting analysis of 118 isolates of *P. capsici* from black pepper by Troung *et al.* (2010) showed that the population was genetically more diverse where two mating types were found, although the overall genetic diversity was low with most of the isolates belonging to one clonal group. Analysis of forty-six *P. capsici* isolates based on RAMS and REP fingerprinting revealed that isolates from black pepper were genetically distinct from isolates recovered from chilli (Troung *et al*., 2010). Twenty-two isolates from chilli clustered into two clonal groups at a DICE similarity level of >85%, whereas twenty-four isolates from black pepper were separated from these chilli isolates at a similarity level of <50%. In general, the genetic diversity among isolates of *P. capsici* from black pepper was greater than that of the chilli isolates. The study indicated that the *P. capsici* population infecting chilli and black pepper in Vietnam consists of two separate genetic strains, adapted to chilli and black pepper, despite their morphological similarity and host cross-infectivity (Troung *et al.,* 2012).

Multigene approach

Recent studies have used multiple loci from both the nuclear and mitochondrial genomes (Donahoo *et al*., 2006; Ivors *et al*., 2004; Kroon *et al*., 2004; Villa *et al*., 2006). Schena and Cook (2006) successfully used intergenic region of mitochondrial DNA (mt-IGS), intergenic spacer region of rDNA (r-DNA IGS) and ras related protein *Ypt*1 to develop "molecular tool box" for detection and characterization of *Phytophthora* species.

Molecular methods used for detection and identification of *Phytophthora* are constantly evolving and the latest described by Sikora *et al.* (2012) involves a padlock probe (PLP)-based multiplex method employing TaqMan polymerase chain reaction assay that could differentiate closely related species and can be used in diagnostics.

Another method of mutation screening by targeted induced local lesions in genomes (TILLING) has now been added to the repertoire of molecular biological protocols available for the genus *Phytophthora*. Several mutants were identified for two different genes in *P. sojae*, one encoding a necrosis inducing protein *PsojNIP* and the other a phospholipase D, *PsPXTM-PLD* (Francine and Mark, 2006).

A Multi-locus phylogeny of *Phytophthora* utilizing markers derived from complete genome sequences of *P. ramorum* and *P. sojae* was done by Blair *et al.* (2008). Loci were sought that would be informative across the genus and/or within clades or species complexes. Seven loci (28S ribosomal DNA, 60S ribosomal protein L10, Beta-tubulin, Elongation factor 1 alpha, enolase, heat shock protein 90 and TigA gene fusion Protein) that were successfully amplified and sequenced across the genus were chosen for more comprehensive analysis. These loci included portions of seven protein-coding genes and the 5′ portion of the 28S ribosomal DNA. These genes do not contain introns, and are conserved throughout eukaryotes.

Inconsistencies in phylogenies have been found within the genus *Phytophthora* depending on the molecular region, and the analysis method used (Kroon *et al.,* 2004; Martin and Tooley, 2003). The studies based on a single gene would not give a clear picture about the ancestry and phylogeny of the organism, so it is better to study multi-locus phylogeny.

Whole genome sequencing for taxonomic studies

Whole genome sequencing or complete genome sequencing is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time. The information gathered from sequencing will provide the raw data for the field of bioinformatics, where computer science and biology live in symbiotic harmony to derive meaningful knowledge from DNA sequences, which can address various problems of taxonomy (Cristianini and Hahn, 2006; Gibbs *et al.,* 2004).

New species discovery

Phytophthora foliorum sp. nov., causing leaf blight of azalea (Donahoo *et al.,* 2006); *P. gallica* sp. nov., causing oak decline (Jung and Nechwatal, 2008), *P. gemini* sp. nov., from the halophilic plant Zostera marina (Man in't Veld *et al.,* 2011); *P. himalsilva* sp. nov. from the remote forests in Nepal (Vettraino *et al.,* 2011); *P. inundata* sp. nov., from trees and shrubs in wet or flooded soils (Brasier *et al.,* 2003); *P. kernoviae* sp. nov., causing bleeding stem lesions on forest trees and foliar

necrosis of ornamentals in UK (Brasier *et al.,* 2003); *P. parsiana* sp. nov., a high-temperature tolerant species (Mostowfizadeh-Ghalamfarsa *et al.,* 2008); *P. pseudosyringae* sp. nov., causing root and collar rot of deciduous tree species in Europe (Jung *et al.,* 2003); *P. quercetorum* sp. nov., from oak forest soils (Balci *et al.,* 2008), etc. are some of the recently reported species.

Indian scenario

Phytophthora foot rot, caused by *Phytophthora capsici*, is one of the most serious threats to the production of black pepper (*Piper nigrum* L.) throughout black pepper growing regions of the world including India (Anandaraj, 2005). The pathogen infects the roots, stems, leaves and fruit at any stage of plant growth. *P. capsici* is heterothallic and requires both A1 and A2 mating types for sexual reproduction (Sarma *et al.,* 1988). These two mating types are reported to coexist in several areas of black pepper cultivation in Indonesia (Manohara *et al.,* 2004) and Malaysia (Kueh and Sim, 1992). The epidemic development of black pepper foot rot depends on environmental conditions, drainage, soil moisture, soil fertility, cultivar and cultural practices (Anandaraj, 2000 and 2011).

P. capsici, was first described by Leonian (1922) as the blight pathogen on chilli (*Capsicum annuum* L.) and considered to be host specific. *Phytophthora* isolates obtained from black pepper were previously classified as *Phytophthora palmivora* based solely on morphological characters (Holliday and Mowat, 1963; Alconero *et al.,* 1972; Turner, 1973). The black pepper isolates were grouped under *P. palmivora* MF4 based on the classification of Zentmyer *et al.* (1977). The species was re described by Al-Hedaithy and Tsao (1979) and Tsao (1991) to include other hosts reclassified as *P. capsici* on the basis of morphological characters (Tsao, 1991). Based on the morphological characters and host range, Aragaki and Uchida (2001) classified a sub group of *P. capsici* in to *P.tropicalis.* But the isolates in India share the characters of both *P. capsici* and *P. tropicalis* in rDNA analysis (Sheji *et al.,* 2009) and host range as they infect both *Piper nigrum* and *Capsicum annuum*. In India, cocoa is affected by three distinct species viz., *P. palmivora, P. capsici and P. citrophthora.* Based on protein electrophoresis Chowdappa and Chandramohanan (1995) concluded that the isolates of *P. palmivora* and *P. capsici* form distinct groups whereas *P. citrophthora* formed two subgroups. Chowdappa *et al.* (2003a, 2003b) working with *P. capsici* from plantation crops like black, pepper, betel vine and cocoa suggested the use of ITS regions of rDNA for taxonomic purposes and for assessing intra-specific population variation in *P. capsici*. ITS sequences for *Phytophthora* isolates from betel vine (*Piper betle*), brinjal (*Solanum melongena*), guava (*Psidium guajava*), roselle (*Hibiscus subdariffa*), black pepper (*Piper nigrum*), sesame (*Sesamum indicum*), taro (*Colocasia esculenta*), chilli (*Capsicum annuum*), pointed gourd (*Trichosanthes dioica*), papaya (*Carica papaya*) were reported by Roy *et al.* (2009) and opined that *P. nicotianae* the most prevalent species on betel vine besides, *P. capsici* and *P. palmivora* was not recorded in eastern India.

The Indian Council of Agricultural Research, New Delhi has launched an outreach project on *Phytophthora*, *Fusarium* and *Ralstonia* diseases of horticultural and field crops (PhytoFuRa), in the year 2008-2009 operational in 17 centres distributed in nine states. Among them eight centres *viz.* Indian Institute of Spices Research, Kozhikode; Central Potato Research Institue, Shimla; Central Plantation Crops Research Institute, Kasaragod; Central Tuber Crops Research Institute, Trivandrum; National Research Centre for Citrus, Nagpur; ICAR Research complex, Umiam; YS Parmar University of Horticulture and Forestry, RC, Kullu and National Bureau of Agriculturally Important Insects, Bangalore are involved in the research in *Phytophthora*. The research under this project includes six thematic areas *viz*. biodiversity, diagnostics, epidemiology, genomics & bioinformatics, host resistance and disease management (Annual report PhytoFuRa, 2011).

The studies on *Phytophthora* isolates infecting black pepper, revealed the existence of four different species *viz*., *P. capsici, P. palmivora, P. parasitica* and *P. citrophthora.* These isolates varied much in their virulence and were classified into three groups *viz.* less virulent, moderately virulent and highly virulent isolates. *P. palmivora* was known to be the major pathogen causing bud rot/ fruit rot disease of coconut, but recently other species such as *P. capsici, P. nicotianae* and *P. meadii* were also reported from India. Similarly in the case of cocoa, besides *P. palmivora* and *P. capsici*, other species such as *P. meadii* and *P. citrophthora* were also found to be involved. In the case of citrus, other than *P. nicotianae* and *P. palmivora,* three species *viz*., *P. citrophthora, P. salixsoil* and *P. insolita* were found to be associated. *P. insolita* was isolated from water accumulated under the canopy of a Nagpur mandarin tree from Nagpur region for the first time in India (Data unpublished).

The morphological characterization of the *Phytophthora* isolates from different hosts such as apple, black pepper, cocoa, coconut, etc. showed high diversity among them. The *Phytophthora* isolates from black pepper showed different types of colony morphology and different types of sporangial morphology. *P. palmivora* isolates from coconut showed different types of colony morphology and types of sporangial morphology. *P. nicotianae* isolates from citrus showed 11 different colony types on V8 agar and 7 different types on PDA whereas *P. palmivora* isolates showed 3 and 5 patterns in V8 agar and PDA respectively.

Monitoring of *P. infestans* population for prevalence of physiological races in the states of Himachal Pradesh, Uttar Pradesh, Punjab, Karnataka, Bihar, West Bengal, Assam, Meghalaya, Tamil Nadu, Rajasthan and Uttarakhand revealed that the pathogen population in these states consisted of complex races ranging from 9-11 genes. Frequency of occurrence of 11 genes was 100% in all the states except Uttar Pradesh and West Bengal. Comparison of *P. infestans* population for metalaxyl sensitivity in different parts of the country revealed that there has been a marked increase in metalaxyl resistant population. Eighty three percent isolates of Tamil Nadu and 80% isolates of Meghalaya exhibited tolerance to 400 ppm while 100% isolates of Bihar, 67% of Rajasthan, 60% of Assam, 56% of Karantaka and 27% of

HP showed tolerance to 300 ppm. Rest of the isolates showed tolerance to 200 ppm.

Among *P. infestans* the A2 mating type has displaced the A1 population in temperate highlands while in sub-tropical plains, A1 is still dominating. The *Phytophthora* isolates from citrus and *P. palmivora* isolates from coconut were predominantly of A2 mating type whereas in case of *P. capsici* and *P. colocasiae* isolates, majority were of A1 mating type.

Genetic diversity and fingerprinting of *Phytophthora* isolates using different molecular markers such as SSR, AFLP etc. suggested that *Phytophthora* isolates are at rapid pace of evolution with high level of diversity among isolates. Studies on mDNA haplotyping revealed that Indian population of *P. infestans* is composed of Ia and Ib and the population of new mt DNA haplotype Ia is on the rise (Data Unpublished).

Under this project, for the first time under ICAR a native isolate of *Phytophthora* infecting black pepper was sequenced by Indian Institute of Spices Research (IISR), Kozhikode, Karnataka, India using Next generation sequencing platform, Illumina - Solexa GA II in collaboration with Joint Genome Institute. The sequence had a total size of about 64.05 Mb with the read nucleotide composition of A: 23.04%, C: 26.43%, G: 27.13%, T: 23.40%. On assembling the sequence data with Joint Genome Institute's *P. capsici* as the reference genome, percentage coverage of 44.1013 was seen. Whole genome comparison with reference genome revealed 330,410 SNP sites and 27,192 InDels in paired end reads and 240, 424 InDels in single end reads (Data unpublished).

Taxonomic position of *P. capsici* infecting black pepper

The foot rot disease of black pepper was first reported in India as early as 1902 (Butler, 1906). Even though isolation of *Phytophthora* sp. in black pepper was reported from Karnataka (Venkata Rao, 1929), the authentic report that it is caused by *Phytophthora* came from Samraj and Jose (1966) who named it as *P. parasitica* var. *piperina* (Muller, 1936). Its taxonomic position remained controversial for quite some time, naming it as *P. palmivora* (Holliday and Mowat, 1963), as an atypical strain of *P. palmivora* (Turner, 1969) solely based on its morphological characters. Later on it was placed in one of the four morphological groups of *P. palmivora*, *P. palmivora* MF4 (Sarma *et al.,* 1982; Anandaraj and Sarma, 1990). However, it was resolved that *Phytophthora* of black pepper is *Phytophthora capsici* Leonian emend. Alizadeh and Tsao (Tsao, 1991).

Variation among *P. capsici* isolates has been studied using protein profiles, isozymes, RAPD and mtDNA analysis. The studies of Erselius and Shaw (1982), in *P. capsici* isolates detected few differences among the isolates. Based on isozyme analysis, the *P. capsici* isolates from black pepper, were classified into two groups, CAP1 and CAP2 (Oudemans and Coffey, 1991), which later resolved into CapA and CapB, respectively (Mchau and Coffey, 1995). The mitochondrial DNA analysis showed high degree of variation among isolates from various geographic locations and host plants (Forster *et al.,* 1990).

The studies conducted with 130 black pepper isolates maintained in the National repository of *Phytophthora* at IISR, Kozhikode revealed the occurrence of two distinct groups. The ITS-RFLP analysis of these isolates showed a clear variation in their banding pattern, thereby dividing them into two distinct groups. This was further supported by the sequence analysis of the ITS region.

CONCLUSIONS

A range of phenotypic and genotypic tests are applied to understand the mechanisms, processes and rates of *Phytophthora* evolution, but each has limitations and new methods are sought. Recent progress in *P. infestans*, *P. sojae* and *P. ramorum* genomics is providing the raw data for such methods and biomolecular markers are currently being developed that have tremendous potential in the study of *Phytophthora* population biology, epidemiology, ecology, genetics and evolution (Cooke and Lees, 2004). Overall, the genome sizes of fungi do not exceed 40 Mb and they are mostly haploid. In contrast, the genomes of oomycetes studied so far are all larger than 45 Mb and often double that size or more and they are diploid (Judelson and Blanco, 2005; Kamoun, 2003). The recent tools of functional genomics such as genome sequence data, DNA microarrays and proteomics are leading to better understanding of host pathogen interactions. Proteomic analysis reveals the proteins involved in early defense signaling due to biotic or abiotic stress (Katrina and Somerville, 2002),

Based on the biology and evolution of *Phytophthora infestans* and other related *Phytophthora* pathogens, Grunwald and Flier (2005) concluded that Mexico is the center of origin not only of the potato late blight pathogen *P. infestans*, but also of several related *Phytophthora* species including *P. mirabilis*, *P. ipomoeae*, and possibly *P. phaseoli*. Similarly, the studies conducted so far with isolates of P. caps--- maintained in the National repository of *Phytophthora* in India revealed the occurrence of two distinct groups. The ITS-RFLP analysis of these isolates showed a clear variation in their banding pattern, thereby dividing them into two distinct groups. The studies done so far, highlight the need for re-describing the species *P. capsici* infecting black pepper. The genome sequencing study underway will help in achieving this objective.

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