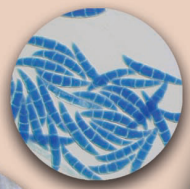
भाकृअनुप
ICAR

PhytoFuRa Publication No. II

Protocols

for Phytophthora, Fusarium & Ralstonia Research



PhytoFuRa
AN ICAR OUTREACH PROJECT ON
Phytophthora, Fusarium & Ralstonia

Outreach Project on
Phytophthora, Fusarium & Ralstonia
Diseases of Horticultural and Field Crops

PROTOCOLS

FOR PHYTOPHTHORA, FUSARIUM & RALSTONIA RESEARCH

PhytoFuRa
AN ICAR NETWORK PROJECT ON
Phytophthora, Fusarium & Ralstonia

Outreach Project on *Phytophthora, Fusarium* and *Ralstonia*
Diseases of Horticultural and Field Crops
(PhytoFuRA)

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Praveena R
Prasath D
Eapen S J
Anandaraj M

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PREFACE

Wilt pathogens *viz*., the oomycete pathogen - *Phytophthora* spp., the fungal *pathogen - Fusarium* spp. and the bacterial wilt pathogen - *Ralstonia solanacearum*, have an enormous impact on the national economy. They cause havocs to a number of horticulture and field crops of the country ranging from vegetables, fruits, spices, plantation crops, ornamentals, pulses and oil seeds. On the other hand our understanding of these pathogens has tremendously improved with the advances in sequencing and genomic techniques. To harness the power of these new generation technologies and to bring in more synergy in our efforts to deal with these pathogens, Indian Council of Agricultural Research (ICAR) launched an Outreach Project on *Phytophthora*, *Fusarium* and *Ralstonia* Diseases of Horticultural and Field Crops (PhytoFuRa) in the year 2009. Research was carried out under six thematic areas - biodiversity, diagnostics, epidemiology, genomics, host resistance, disease management and HRD. The project was operational initially in 19 centres distributed in ten states with ICAR - Indian Institute of Spices Research (ICAR-IISR) as the lead centre and subsequently during 12th Five Year Plan, three more centres were added.

The PhytoFuRa Project was one of the flagship program of ICAR with a total outlay of ₹ 34.1 crores (2009 - 2017). It dealt with a wide range of crops including apple, banana, black pepper, brinjal, chickpea, chilli, citrus, coconut, cocoa, colocasia, ginger, guava,oil seeds, pigeon pea, potato, rubber, safflower, seed spices, sugarcane and tomato. The inter-institutional collaboration under this project was unique and exemplary. The PhytoFuRa portal (http://www.phytofura.net.in) facilitated better and faster interaction and sharing of resources among the investigators and research fellows. The project was also instrumental in developing new techniques/protocols which are destined to have a high impact in the research and development of some of the crops.Many of them are modifications or improvisations of the already existing protocols. But we felt that documenting them will help students as well as the research community and avoid reinventing the wheel. There are 49 of them included in this small publication which will guide those who wish to work on these pathogens.

We take this opportunity to salute the stewardship shown by Dr. M. Anandaraj in ably leading the project since its inception and brilliantly coordinating more than 80 scientists across the country. We thank all the investigators of the project for their commendable contribution. We would like to place on record our sincere gratitude to Dr. S. Ayyappan (Former Secretary, DARE & Director General, ICAR) and Dr. Trilochan Mohapatra, the present incumbent for their valuable guidanceand keen interest in the project. The support and guidance received from former Deputy Director Generals (Horticultural Science) Dr. H.P. Singh, Dr. N.K. Krishna Kumar and Dr. A.K. Singh, the present Deputy Director General (HS) are gratefully acknowledged.The financial support for the project received from ICAR is gratefully acknowledged.

Editors

डा. आनन्द कुमार सिंह
उपमहानिदेशक (बागवानी विज्ञान)
Dr. Anand Kumar Singh
Deputy Director General (Hort. Sci.)

भारतीय कृषि अनुसंधान परिषद
कृषि अनुसंधान भवन–II,
पूसा, नई दिल्ली–110 012
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
KRISHI ANUSANDHAN BHAWAN-II,
PUSA, NEW DELHI-110 012 (INDIA)

21.11.17

FOREWORD

The pathogens, *viz*, *Phytophthora* spp., *Fusarium* spp. and *Ralstonia solanacearum* cause serious diseases in a number of agri.-horticultural corps leading to enormous economic losses in India. Our understanding of the host plant-pathogen interactions and other associated factors are crucial for developing soud disease management strategies. Fostering meaningful collaborations and pooling of resources in research programmes to enhance the efficiency and make full use of frontier technologies is need of the hour for enhancing efficiecy. Realizing this, the Indian Council of Agricultural Research (ICAR) has supported a National level Outreach Project on *Phytophthora*, *Fusarium* and *Ralstonia* Diseases of Horticultural and Field Crops (Phyto-FuRA) during 2009-17 at 19 Centers across the country covering six thematic areas *viz*. biodiversity, diagnostics, epidemiology, genomics, host resistance, disease management and the human resource development.

The project has immensely contributed to our understanding of the biology, diversity and ecology of these three pathogens of immense economic significance at National level. The concerted efforts of the collaborating institutions have largely helped in rolling out several effective field management strategies for diseases caused by these pathogens in a number of agri-horticultural crops. In addition to standzrdization of management technologies, the project has also accomplished several milestones of high academic value.

I am glad that the ICAR-Indian Institute of Spices Research, Kozhikode, the lead center has compiled and brought out three crisp publications covering technologies, protocols and scientific publications that reflect the achievement of this high impact project for the benefit of stakeholders. I take this opportunity to appreciate the efforts made by the scientists associated with planning, execution, monitoring and evaluation of the project which led to such significant outcome. I wish that the outcomes presented in this compilation will suitably be scaled up at field and help in fine tuning further research in this sector.

New Delhi
21.11.2017

(Anand Kumar Singh)

Phytophthora

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Phytophthora

Phytophthora is a major threat to several economically important horticultural crops including spices and plantation crops. The genus *Phytophthora* with over one hundred species, is one of the most destructive plant pathogen and occurs in various ecosystems including agricultural and non-agricultural systems. They attack various plant parts and are responsible for the severe economic losses of various agricultural and horticultural crops. 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. There are also some species with a narrow host range such as *P. sojae* and *P. infestans.* Due to their significance and economic importance, there has been increasing interest in the molecular genetics and genomics of *Phytophthora* species. In spite of the efforts to manage the pathogen all over the world, the pathogen is threatening to re-emerge, breaking the host resistance and other management strategies. Genetic recombination occurs not only by sexual recombination but also through parasexual recombination resulting in the emergence of new races. The pathogens belonging to this genus affect several horticultural and field crops in a wide range of agro-climatic regions. The wet monsoon period prevailing over various parts of the country provide ideal conditions for them to infect. In perennial crops like apple, citrus and 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 upon the site of infection and extent of damage. There are new diseases caused by *Phytophthora* in field crops such as cassava that threaten the cultivation of these crops.

Detection of *Phytophthora nicotianae* in irrigation water by *Ypt1* gene-based nested PCR

1

Objective:

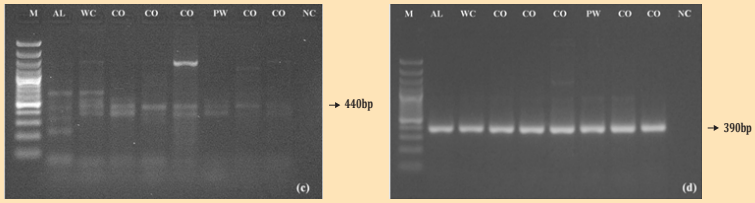
To develop species-specific detection of *Phytophthora nicotianae* in irrigation water through PCR-based technique

Brief description:

A set of universal primer pair Ypt1F/Ypt1R (outer set, Ypt1F (5′-CGACCATTGGCGTGGACTTT-3′) and Ypt1R (5′-ACGTTCT CGCAGGCGTATCT-3′) and *P. nicotianae* specific primers Pn1 /Pn2 (inner set) from regions of the RAS-related protein Ypt1, Pn1 (5′-GACTTTGTAAGTGCCACCATAC-3′) and Pn2 (5′-CTCAGCTCTTTTCCTTGGATCT-3′) were used for PCR amplification using BIORADT100TM thermal cycler. PCR reactions were carried out in a 25 µl reaction volume containing 2.5 µl of 10x PCR buffer (Fermentas Inc. Maryland, USA), 0.5 µl of 2.5 mM dNTPs, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl each of 10 µl primers (universal primers Ypt1F and Ypt1R) (Integrated DNA Technologies, USA), 0.2 µl of 20 mg/mL BSA (Fermentas Inc. Maryland, USA) and 1.25 U of Taq DNA polymerase (Invitrogen, Life Technologies Corporation, USA), 1 µL template DNA (20 40 ng/µL). The thermal cycling conditions used were: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation, annealing and elongation steps, respectively at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min and a final extension step of 72°C for 10 min. The PCR conditions used for inner primer Pn1/Pn2 were similar as described for Ypt1F/ Ypt1R, but with the exception of an annealing temperature of 66°C, 30 cycles and exclusion of BSA from the reaction mixture. DNA samples of irrigation water tested for the presence of *P. nicotianae* using the nested PCR results in multiple bands ranging from 200 bp to 1.2 kb (including desired 440 bp) in the first round of PCR amplification using the Ypt1F/Ypt1R primer pair. Subsequently, in second round of amplification of using *P. nicotianae* specific primers Pn1/Pn2, a band of ~390 bp can be observed in infected samples.

The present protocol assessed *Ypt*1 gene specific primers for detection of *P. nicotianae* in citrus irrigation water using a nested polymerase chain reaction (PCR) assay. The assay detected the pathogen in naturally infested water samples collected

Inventors
Das, A. K., Bawage, S. S., Nerkar, S. G. and Kumar, A.
ICAR-Central Citrus Research Institute, Amaravati road, Nagpur, Maharashtra - 440010



Nested PCR amplification of *Ypt1* gene

from orchard basin more rapidly and accurately than standard isolation methods. This PCR-based detection for *P. nicotianae* in contaminated irrigation water will serve as a reliable method for phytosanitary programmes in citriculture.

Application:

The developed PCR-based method would provide rapid and accurate detection of *P. nicotianae* in water samples at an adequate level of sensitivity which will allow citrus growers to adopt timely disease management strategies.

Related publication:

Das, A. K., Bawage, S. S., Nerkar, S. G. and Kumar, A. (2013). Detection of *Phytophthora nicotianae* in water used for irrigating citrus trees by *Ypt1* gene-based nested PCR. *Indian Phytopathology* 66(2): 132-134.

Detection and quantification of *Phytophthora nicotianae* in root, water and soil using real-time PCR

2

Objective:

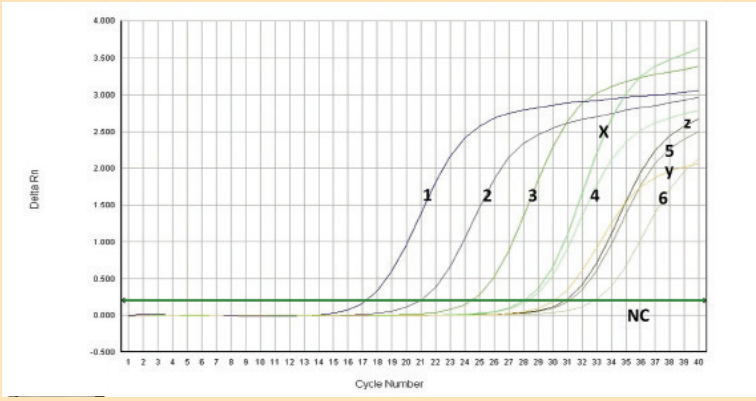
To develop species-specific detection methodology for detecting *P. nicotianae* infecting citrus through PCR-based techniques

Brief description:

A set of ITS region-based primer pair PNIC 1(5′-CAATAGTTGGGGGTCTTATT-3′)/PNIC2(5′-GTATACCGAA GTACACATTAAG-3′) specific to *Phytophthora nicotianae* was used. PCR amplifications were carried out on BIORAD T100TM thermal cycler in a 25 µl volume consisting 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM of each primer (PNIC1/PNIC2), 1 U of Taq DNA polymerase (Bioline Inc, USA) and 1 µl of template DNA. The PCR conditions used were: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation, annealing and elongation steps respectively at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min and final extension step of 72°C for 10 min. PCR amplification of genomic DNAs using the primer pair PNIC1/ PNIC2 generated 750 bp sequence for *P. nicotianae* isolates. Real-time qPCR analysis was done with ITS region primer pair (Ph.nicF/Ph.nicR) and probe (Pn.proFAM) specific to *P. nicotianae* were used. The real-time PCR amplification reaction was performed using a ABI 7300 Real Time PCR system (Applied Bio-systems, California) in a 25 µl reaction volume consisting of 200 nM (each) primer, 100 nM probe, 1x TaqMan® Universal Master Mix (Applied Biosystems, CA) and 1 µl of template DNA. The standard amplification protocol was 95°C for 15 min followed by 40 cycles at 94°C for 15 s and 60°C for 1 min. The species specific primers helped to detect *P. nicotianae* isolates with a band of 750 bp.

The sensitivity of the primers was assessed using serial dilutions of total DNA extracted from *P. nicotianae*. In the 10-fold dilutions, series of *P. nicotianae* DNA extracted from pure cultures, the detection limit was found 5.8 ng/µl - 58 fg/µl. Standard curves

Inventors
Das, A. K., Nerkar S. G., Kumar, A., Kadam, R. and Bawage, S. S.
ICAR- Central Citrus Research Institute, Amaravati road, Nagpur, Maharashtra - 440010



Sensitivity of TaqMan PCR assay assessed by 10-fold serial dilutions of *Phytophthora nicotianae* DNA. Real time amplification curve of different concentration of DNA; 1-6 (5.8 ng/ µl-58 fg / µl), x=soil DNA, y=root DNA, z= water DNA. NC: PCR negative control.

prepared showed a linear correlation between input DNA and Ct after the TaqMan PCR. The correlation coefficient (r2) of the standard curve was 0.987. *P. nicotianae* was also detected in soil, root and water samples collected from citrus orchards.

Application:

The developed PCR-based assays are robust and reliable technique to detect and quantify *P. nicotianae* in mycelial culture as well as in citrus tissues, soil and water.

Related publications:

Das, A. K., Nerkar, S., Kumar, A. and Kadam, R. (2013). Detection and quantification of *Phytophthora nicotianae* from root, water and soil samples using real-time PCR. National Citrus Meet. August 12-13, 2013, NRC for Citrus, Nagpur, India, pp.225.

Das, A. K., Nerkar, S., Kumar, A. and Bawage, S. (2016). Detection, identification and characterization of *Phytophthora* spp. infecting citrus in India. *Journal of Plant Pathology* 98(1): 55-69.

A diagnostic tool for detection of *Phytophthora nicotianae* with extracellular cystatin like cysteine protease inhibitor (*EPIC1*) gene

3

Objective:

To design extracellular cystatin like cysteine protease inhibitor (*EPIC1*) gene based PCR primers and to validate their use as a diagnostic tool for the detection of *P. nicotianae*

Brief description:

Internal Transcribed Spacer (ITS) or intergenic mitochondrial DNA spacers (mtDNA-IGS), *Ypt1* gene, *Lpv* gene, SCAR/ RAPD markers are very much in use in PCR-based molecular detection of *Phytophthora* spp. Though these genes are effective in detecting *Phytophthora* spp. but they all have their own limitations. Hence potential of the new gene, extracellular cystatin like cysteine protease inhibitor *EPIC* gene, is explored to broaden the molecular diagnostic tool box for *Phytophthora* spp. detection in infected samples. FEPIC1F/FEPIC1R was found the best suitable pair for its use in PCR-based species specific detection system.

PCR reactions were executed in a total volume of 25 µl containing 1x PCR buffer (Fermentas Inc. Maryland, USA), 0.2 mM of each dNTPs (Fermentas Inc. Maryland, USA), 2 mM $MgCl_2$ (Fermentas Inc. Maryland, USA), 0.5 µM of each (F*EPIC1*F/ F*EPIC1*R) primers (Integrated DNA Technologies, Coralville, USA), 1 U of Taq DNA polymerase (Invitrogen, Life Technologies Corporation, USA) and 1 µl template DNA (20 to 40 ng/ µl). The thermal cycling conditions used were initial denaturation at 94°C for 3 min followed by 25 cycles of denaturation, annealing and elongation steps, respectively at 94°C for 30 s , 58°C for 30 s and 72°C for 1 min and a final extension step of 72°C for 10 min. F*EPIC1*F/F*EPIC1*R primers were tested successfully on *P. nicotianae* infected citrus leaf, stem and root tissues. No cross reactivity of primers was observed with six other *Phytophthora* spp., *viz*., *P. palmivora*, *P. citrophthora*, *P. boehmeriae*, *P. lacustris, P. insolita, P. tropicalis* and three other fungi *viz*., *Pythium* sp., *Colletotrichum* sp. and *Alternaria* sp. Detection by these primers was done effectively up to 100 pg μL^{-1} of DNA isolated from pure culture of *P. nicotianae.*

Inventors
Das, A. K. and Nerkar, S. G.
ICAR-Central Citrus Research Institute, Amaravati Road, Nagpur, Maharashtra - 440010

Application:

EPIC1 PCR assay was found to be a robust and reliable technique to detect *P. nicotiana*e in infected citrus planting materials and thus would be useful in restricting *P. nicotianae* mediated destruction in citrus.

Related publication:

Nerkar, S. G. and Das, A. K. (2016). Extracellular cystatin-like protease inhibitor (*EPIC1*) gene-based PCR primers for specific detection of *Phytophthora nicotianae* infecting citrus. *Plant Pathology Journal* November : 01 - 10.

Development of SCAR markers for species-specific detection and identification of *Phytophthora nicotianae*

4

Objective:

To develop species-specific PCR primers outside the intergenic sequences of ribosomal genes, using sequence characterized amplified region (SCAR) markers to detect *Phytophthora nicotianae*

Brief description:

Sequence characterized amplified region (SCAR) markers are one of the most effective and accurate tools for detection and identification of fungi. SCAR markers were developed for the species-specific detection and identification of *Phytophthora nicotianae*. A primer pair was designed from the sequence characterized amplified region (SCAR) obtained by screening 20 RAPD primers (OPA1 to OPA20). The polymorphic band associated only with *P. nicotianae* was sequenced and specific primers (SCAR 4F/SCAR 4R) were designed. PCR reactions were carried out in a volume of 25 µl, consisting of 1x PCR buffer (Thermo Fisher Scientific Inc., USA), 2 mM $MgCl_2$ (Fermentas Inc. Maryland, USA), 0.2 mM each dNTP (Fermentas Inc. Maryland, USA), 0.5 µM of each SCAR primer (SCAR 4F/SCAR 4R) (Integrated DNA Technologies, Coralville, USA), 1 U of Taq DNA polymerase (Bioline USA Inc, Taunton, MA) and 1 µl of template DNA (20-40 ng/µl). Amplifications were performed on a BIORAD T100TM thermal cycler using the following cycling protocols: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation, annealing and elongation steps, respectively at 94°C for 45 s, 58°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 10 min. SCAR primer pair developed for *P. nicotianae* gave the result with amplicon size of 330 bp.

Application:

The developed SCAR-based PCR assay proved to be a reliable, simple and rapid technique to detect *P. nicotianae* in cultures as well as in infected plant materials.

Inventors

Das, A. K., Gawande, N. D., Nerkar, S. G., Thakre, N. and Kumar, A.

ICAR-Central Citrus Research Institute, Amaravati Road, Nagpur, Maharashtra - 440010

5

A multiplex PCR assay for simultaneous detection of two *Phytophthora* species, *P. nicotianae and P. palmivora*

Objective:

To develop a multiplex PCR assay for simultaneous detection of *Phytophthora nicotianae* and *P. palmivora*

Brief description:

P. nicotianae and *P. palmivora* cause *Phytophthora* rot of citrus and a multiplex PCR technique for simultaneous detection and quantification of the two pathogens was developed. Primers were designed specific for *P. nicotianae* (Pn1/Pn2) and *P. palmivora* (Pal1s/Pal2a) based on *Ypt1* gene and ITS region, respectively. PCR reactions were carried out in a 25 µl volume consisting of 1x PCR buffer (Fermentas Inc. Maryland, USA), 0.2 mM of each dNTPs (Fermentas Inc. Maryland, USA), 2 mM $MgCl_2$ (Fermentas Inc. Maryland, USA), 0.5 µl of each (Pal1s/Pal2a) primers and 1 µl of each (Pn1/Pn2) primers (Integrated DNA Technologies, Coralville, USA), 1 U of Taq DNA polymerase (Invitrogen, Life Technologies Corporation, USA), 1 µl of template DNA and sterile distilled water was added to make up the final volume. The thermal cycling conditions used were: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation, annealing and elongation steps, respectively at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min and a final extension step of 72°C for 10 min.

Multiplex PCR assay with primer pairs Pn1/Pn2 and Pal1s/Pal2a amplified amplicons of size 400 bp and 650 bp in case of mixed infection of *P. nicotianae* and *P. palmivora.*

Application:

The developed multiplex PCR assay is a sensitive and robust technique to detect *P. nicotianae* and *P. palmivora* simultaneously in mixed infections occurring in citrus nurseries and field.

Inventors
Das, A. K., Nerkar, S. G., Kadam, R. and Kumar, A.
ICAR-Central Citrus Research Institute, Amaravati Road, Nagpur, Maharashtra - 440010

Detection of *Phytophthora infestans* using SCAR marker

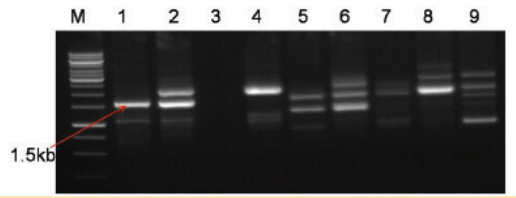
6

Objective:

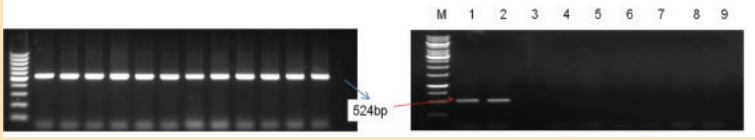
Detection of *P. infestans* from host tissues

Brief description:

Late blight of potato caused by *Phytophthora infestans* is one of the most devastating disease of potatoes not only in India but throughout world were potato is cultivated. The timely detection and appropriate identification of *Phytophthora infestans* associated with potato plants or tubers are important in formulating the management strategies for plant diseases. PCR reactions was performed in a total volume of 25 µl, containing 1x Taq DNA buffer with 1.5 mM $MgCl_2$, 5U/ µl Dream Taq DNA Polymerase, 200 mM of each dNTPs, 10 pmol of each forward and reverse primer, 10 ng/µl template DNA, milliQ water (variable) per reaction. The PCR conditions are as follows: denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58-60°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. Amplified PCR products (524 bp) were separated by electrophoresis on 1.8%



PCR amplification of different pathogens with OPA 12. Isolates Lane 1 & 2 - *P.infestans* (A1 & A2 strain), 3-blank, 4- *P. colocasiae*, 5-*P. cactorum*, 6-*P. palmivora*, 7-*P.capsici*, 8-*F. solani*, 9-*R. solani*



PCR amplification with SCAR PITH2 F/PITH2 R primer.

PCR amplification with PITH2 F/ PITH2 R primer.

Inventors
Touseef Hussain, Mehi Lal, B. P. Singh and Sanjeev Sharma
ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

agarose gel and captured under gel documentation system. The SCAR primers PITH2 F/PITH2 R was developed and produced a single diagnostic DNA amplification product consisting of 524 bp from all the *P. infestans* isolates. SCAR markers can detect the presence of *P. infestans* in infected tubers and infested soil samples and the SCAR primer pair did not amplify DNA from other *Phytophthora* spp. and potato fungal pathogens.

Application:

To detect the presence of *P. infestans* in potato seed material and naturally infested soil samples.

Development of *POL* gene RNAi construct of *Phytophthora infestans*

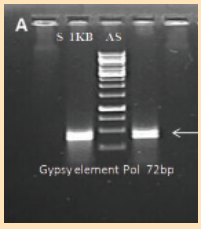
7

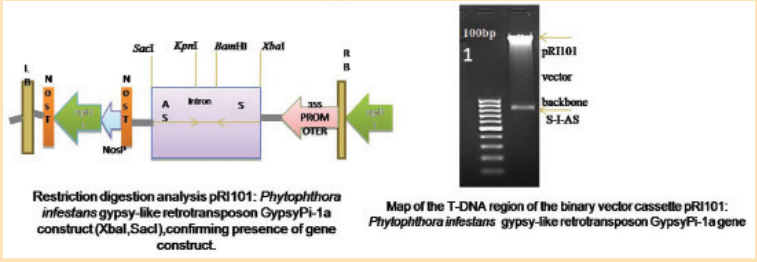
Objective:

To develop putative transformants in potato

Brief description:

The dynamism in *Phytophthora infestans* is mainly due to the movement of transposable elements. Nearly 33% of the genome is covered by transposable long terminal repeats LTR/Gypsy-Ty3 element. Gypsy-Ty3 is well characterized and is independent transposable element and code for its own tranposase enzyme which can be targeted for silencing in *P. infestans.* The strategy adopted was to construct inverted repeat cDNA fragment of *Phytophthora* Gypsy like element *GypsyPi-1*, a gene intervened by potato granule bound starch synthase (GBSS) intron (iIR-GypsyPi-1a). Transcription of iIR-GypsyPi-1a gene construct in the transgenic potato background would generate hairpin (hp) Gypsy element Pol transcript, which will induce formation of siRNA causing silencing of *Pol* gene expression. A 307 bp cDNA sequence of Pol was included for construction of sense and anti-sense fragment of iIR-GypsyPi-1a. The iIR-GypsyPi-1a gene construct under the control of constitutive promoter CaMV 35S on MCS of binary plant transformation vector pRI101. A total of 300 internodal stem cuttings of Kufri Bahar (LB





Restriction digestion analysis pRI101: *Phytophthora infestans* gypsy-like retrotransposon GypsyPi-1a construct (XbaI,SacI),confirming presence of gene construct.

Map of the T-DNA region of the binary vector cassette pRI101: *Phytophthora infestans* gypsy-like retrotransposon GypsyPi-1a gene

Patil, V. U., Singh, B. P., Sanjeev Sharma, Sundaresha, S. and Chakrabarti, S. K.
ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

Inventors

susceptible cultivar) have been used for *Agrobacterium*-mediated genetic transformation and to develop putative transformants.

Application:

To understand pathogen dynamism and development of late blight resistant varieties.

Simultaneous detection of *Fusarium* spp. and *Phytophthora infestans*

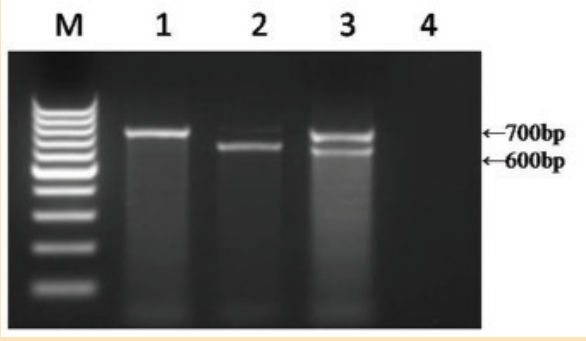
8

Objective:

To detect *Fusarium* spp. and *Phytophthora infestans* simultaneously in a single PCR

Brief description:

PCR reaction was performed in a total volume of 25 µl, containing of 1x GreenTaq buffer (with 1.5 mM $MgCl_2$), 200 µl of each dNTPs, both pathogen-specific primers 10 µl (F+R), Taq DNA polymerase enzyme (5 U/µl), 20 ng/µl template DNA and remaining milliQ water. The PCR reaction was performed in thermal cycler (Eppendorff Mastercycler). The PCR conditions are as follows: denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. Amplified PCR products (600 bp for *Phytophthora infestans* and 700 bp for *Fusarium* spp.) can be separated by electrophoresis on 2.0 % agarose gel.



PCR detection of *Fusarium* spp. and *Phytophthora infestans.*

Application:

Simultaneous detection of *Fusarium* spp. and *Phytophthora infestans* in a single PCR.

Inventors

Mehi Lal, Touseef Hussain, B. P. Singh, S. K. Kaushik and Sanjeev Sharma

ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

9

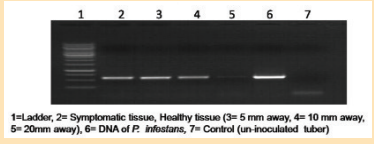
PCR-based diagnosis to detect and quantify *Phytophthora infestans* in potato tubers

Objective:

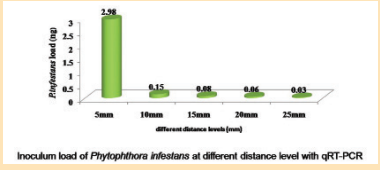
To detect latent infection of *Phytophthora infestans* in potato seed material

Brief description:

The rapid NaOH tissue assay was used with primer set INF FW2 and INF REV for detection of *P. infestans* from artificially infected potato tubers, seed tubers (storage), infected potato leaf, pure culture of *P. infestans*. A single PCR product approximately 600 bp in size was detected. DNA from healthy tubers did not amplify with these primers and could not be detected by ethidium staining on agarose gel. The standardized PCR protocol could detect upto a level of 10 fg of genomic DNA. *P. infestans* was detected in artificially infected tubers (cv. Kufri Jyoti) by the PCR assay and ethidium bromide staining of agarose gels at four and six days post-inoculation but not at two days post-inoculation, before the occurrence of visible symptoms. Moreover, amplified products of expected size were obtained from tuber lesions and symptomless areas at various distances from the tuber lesions.



1=Ladder, 2= Symptomatic tissue, Healthy tissue (3= 5 mm away, 4= 10 mm away, 5= 20mm away), 6= DNA of *P. infestans*, 7= Control (un-inoculated tuber)



Inoculum load of *Phytophthora infestans* at different distance level with qRT-PCR

PCR based diagnosis to detect and quantify *Phytophthora infestans* in potato tubers

Application:

Highly sensitive and robust technology for detection of latent infection in potato seed material by quarantine agencies and farmers.

Inventors
Sanjeev Sharma, Touseef Hussain, Singh, B. P. and Chakrabarti, S. K.
ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

ELISA-based diagnosis of *Phytophthora infestans*

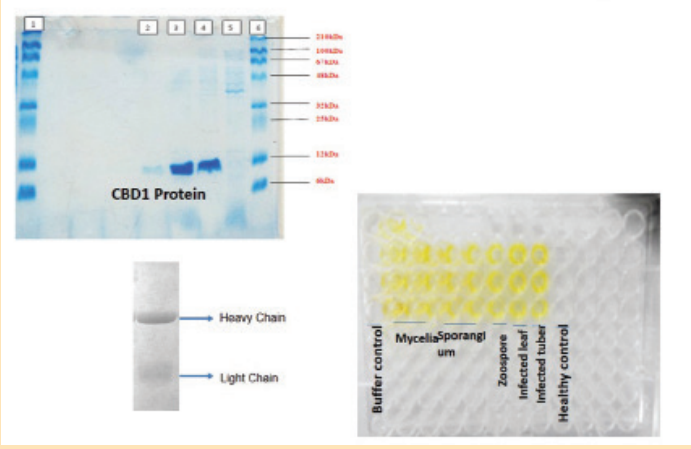
10

Objective:

To diagnose late blight pathogen in standing crop of potato

Brief description:

Phytophthora infestans cell wall-based protein gene (392 bp) has been isolated and cloned into pETSUMO expression vector. Positive clones were confirmed through colony PCR by using vector specific T7 promoter primer and revealed the 650 bp fragment size and confirmed the integration of the gene. Protein was extracted and purified using G-Bioscience protein extraction kit. The purified protein was detected through SDS PAGE, which revealed the ~12 KDa protein size and has been customized for developing polyclonal antibody. Polyclonal antibody of cell wall bound cellulose binding protein (CBD) of *P. infestans* was raised in rabbits, purified, analyzed by SDS PAGE (90%) and subjected to validation through DAC-ELISA. The recombinant CBD1 antiserum was evaluated by measuring its titre and efficacy in detecting *P. infestans* using DAC-ELISA. The expression of CBD1 was detected 2 fold over buffer control in mycelium, sporangium and zoospores as well as in infected leaf samples. Data indicated that, the produced recombinant antiserum was efficient and accurate in determining negative and positive results in ELISA tests.



ELISA based diagnosis of *Phytophthora infestans*

Inventors
Sundaresha, S., Sanjeev Sharma, Singh, B. P., Sadhana Sharma Vandana Thakur and Jeevalatha, A.
ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

Therefore, this antiserum is suitable for certification programmes of potatoes due to its low cost, high specificity, feasibility and its endless supply from recombinant bacterial clones carrying the CBD genes for this oomycete.

Application:

Rapid and accurate detection and diagnosis of late blight pathogen in standing crop and in seed lots by quarantine agencies and farmers.

RNAi technology for the management of late blight in potato

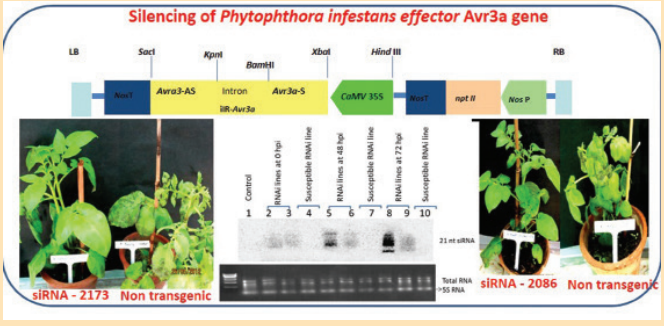
11

Objective:

To develop late blight resistant potato varieties

Brief description:

RNA interference (RNAi) has proved to be powerful genetic tool for silencing genes in plants. Host-induced gene silencing of pathogen genes has provided a gene knockout strategy for a wide range of biotechnological applications. The RXLR effector *Avr3a* gene is largely responsible for virulence of oomycete plant pathogen *Phytophthora infestans.* An attempt was made to silence the *Avr3a* gene of *P. infestans* through RNAi technology. The *P. infestans* inoculation resulted in lower disease progression and a reduction in pathogen load, as demonstrated by disease scoring and quantification of pathogen biomass in terms of Pi08 repetitive elements, respectively. Transgenic plants induced moderate silencing of *Avr3a* and the presence and/or expression of small interfering RNAs, as determined through Northern hybridization, indicated siRNA targeted against *Avr3a* conferred moderate resistance to *P. infestans.* The single effector gene did not provide complete resistance against *P. infestans.* Although the *Avr3a* effector gene could confer moderate resistance, for complete resistance, the cumulative effect of effector genes in addition to *Avr3a* needs to be considered. The host-induced RNAi is an effective strategy for functional genomics in oomycetes.



Application:

Development of late blight resistant varieties.

Inventors

Pattanayak, D., Sundaresha, S., Singh, B. P. , Sanjeev Sharma Suman Sanju and Aditi Thakur.

ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

12

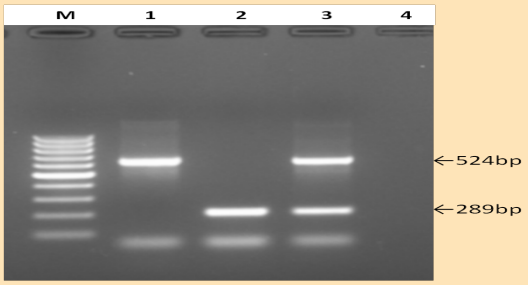
Simultaneous detection of early and late blight of potato

Objective:

To detect early and late blight diseases of potato simultaneously in a single PCR

Brief description:

PCR reaction was performed in a total volume of 25 µl, consisting of 2.0 µl 10x GreenTaq buffer (with 1.5 mM $MgCl_2$), 2.0 µl dNTPs (2.5 mM), 2.0 µl both pathogen specific primers 10 µl (F+R), 0.2 µl Taq DNA polymerase enzyme (5 U) (Fermentas), 2.0 µl template DNA and 9.8 µl double distilled water. The PCR reaction was performed in thermal cycler (Eppendorff Master cycler). The PCR conditions are as follows: denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. Amplified PCR products (524 bp for *Phytophthora infestans* and 289 bp for *Alternaria solani*) were separated by electrophoresis on 2.0 % agarose gel and picture was captured under gel documentation system.



Simultaneous detection of *Phytophthora infestans and Alternaria solani*

Application:

Simultaneous detection of early and late blight pathogens of potato in a single PCR.

Related Publication:

Touseef Hussain, B. P. Singh, S.K Kaushik, Mehi Lal and Anubha Gupta. (2014). PCR protocol for quick and combined detection of early and late blights of potato. National Seminar on Emerging Problems of Potato, 12 November 2014, CPRI, Shimla. p169.

Inventors
Touseef Hussain, B. P. Singh, S. K. Kaushik, Mehi Lal and Anubha Gupta
ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

A rapid and efficient method for *in vitro* production of *Phytophthora colocasiae* zoospores

13

Objective:

To mass produce *Phytophthora colocasiae* zoospores for artificial inoculation

Brief description:

Taro leaf blight caused by *Phytophthora colocasiae* presents the single biggest constraint for taro cultivation globally. To undertake screening of taro accessions and cultivars against leaf blight under *in vitro* conditions, it is important to produce *Phytophthora colocasiae* zoospores.

A methodology was developed to mass produce *Phytophthora colocasiae* zoospores for artificial inoculation. Mycelial plugs (5 mm diameter) were excised from actively growing cultures of *P. colocasiae* on carrot agar (CA) medium. The plugs were placed on the adaxial side of the taro leaf discs (5 cm; cv. Sree Kiran, leaf blight susceptible) floating on 50 mL sterile water in 200 mm sterile Petri dishes. The lids of the Petri dishes were lined with moistened filter paper to maintain high humidity and incubated at 25°C in the dark for 3-4 days. Following incubation, the infected leaf portions were excised, placed with adaxial side upwards in 6 well plates with 2 mL sterile water and slowly immersed in water. Then, the plates were chilled at 4°C for 15 min and thereafter at 37°C for another 30 min. Zoospores are produced in sterile water maintained in the wells and the suspension can be used for screening taro accessions. The protocol developed can be used for the rapid and easy production of *Phytophthora colocasiae* zoospores.

Application:

Mass production of zoospores for artificial inoculation within a short period.

Related publication:

Nath, V. S., Shyni, Hegde, V. M., Misra, R. S., Veena, S. S., Raj, M. and Jeeva, M. L. (2016). A rapid and efficient method for *in vitro* screening of taro for leaf blight disease. *Journal of Phytopathology* 164(7-8): 520–527.

Inventors

Nath V. S., Shyni, Hegde V. M., Misra R. S., Veena S. S., Raj M. and Jeeva M. L.

ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala - 695017

14

A rapid and efficient method for *in vitro* screening of taro for leaf blight caused by *Phytophthora colocasiae*

Objective:

To screen taro germplasm lines for leaf blight caused by *Phytophthora colocasiae*

Brief description:

Taro leaf blight caused by *Phytophthora colocasiae* presents the single biggest constraint for taro cultivation globally. To accelerate breeding and selection for disease resistance to leaf blight, it is important to develop bioassays which could differentiate resistant and susceptible cultivars efficiently.

Taro leaves of 2-3 month old plants were detached and surface sterilized using 0.8% sodium hypochlorite for 1 min followed by rinsing three times in sterile distilled water. Five taro leaf discs (5 cm) floating on sterile distilled water in 200 mm Petri plates were inoculated with 50 µL of zoospore suspension ($5x10^5$ zoospores/ml) of *P. colocasiae* and incubated at 25°C in the dark for 4 days. The leaf discs were visually examined daily and lesion development was recorded. Test plants can be classified into four categories (0-4) based on lesion diameter. 0-1 cm: highly resistant; 1-2 cm: moderately resistant; 2-3 cm: moderately susceptible and 3-4 cm: highly susceptible. Taro accessions could be efficiently classified into various resistance groups based on a 0-4 score and screening assay results were consistent with the field evaluation scores of taro accessions. The rapid, simple and repeatable method for *in vitro* screening can be used to screen large numbers of taro cultivars for leaf blight caused by *P. colocasiae.*

Application:

The protocol developed is easy, quick, reliable and can be adopted with limited amount of leaf samples, highly reproducible and shows consistency with the field disease incidence scores.

Related publication :

Nath, V. S., Shyni, Hegde, V. M., Misra, R. S., Veena, S. S., Raj, M. and Jeeva, M. L. (2016). A rapid and efficient method for *in vitro* screening of taro for leaf blight disease. *Journal of Phytopathology* 164(7-8): 520-527.

Inventors
Nath V. S., Shyni,B., Hegde V. M., Misra R. S., Veena S. S., Raj M. and Jeeva M. L.
ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala - 695017

AFLP fingerprinting of *Phytophthora colocasiae*

15

Objective:

To standardize AFLP protocol for *Phytophthora colocasiae* and to study the genetic diversity among *P. colocasiae* isolates

Brief description :

Among several efficient methods for revealing genetic variability within and among *Phytophthora* spp., AFLP technique is the method of choice as it is a robust, reliable molecular marker assay and the number of polymorphisms detected per reaction is much higher than that revealed by restriction fragment linked polymorphisms (RFLP) or the PCR-based randomly amplified polymorphic DNA (RAPD).

Each selective AFLP reaction was carried out in a total volume of 25 µL, containing 0.5 µL TaqI selective primer (200 ng), 0.4 µL EcoRI selective primer (200 ng), 2.5 µL 10x PCR buffer, 0.8 µL dNTPs (2.5 mM), 0.8 µL Taq DNA polymerase (1 U/µL), 2.5 µL pre-amplification products and 17.5 µL double deionized water. The PCR reactions were performed with: 2 min at 95°C; 30 s denaturing at 94°C, 30 s annealing at 65°C and 2 min elongation at 72°C, followed by reduction of the annealing temperature in each cycle by 0.7°C for 12 cycles. The annealing temperature was maintained at 56°C for the remaining 23 cycles. To the amplification product, an equal volume of formamide loading buffer was added. The amplification product was denatured at 95°C for 5 min and then electrophoresed on a 6% denaturing polyacrylamide gel at a constant power of 90 W for approximately 90 min until the forward-running dye reached the end of the gel and the AFLP gels were silver stained. Inter and intra-specific genetic diversity among *P. colocasiae* isolates collected from same field was assessed using amplified fragment length polymorphism (AFLP) marker.

Application:

Can be employed for understanding the genetic diversity among and within the populations of *P. colocasiae.*

Related publications:

Nath, V. S., Sankar, M. S., Hegde, V. M., Jeeva, M. L., Misra, R. S., Veena, S. S. and Raj, M. (2013). Genetic diversity of *Phytophthora colocasiae* isolates in India based on AFLP analysis. *Biotech* 3(4): 297-305.

Inventors

Nath V. S., Sankar M. S., Hegde V. M., Jeeva M. L., Misra R. S., Veena S. S. and Raj M

ICAR- Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala - 695017

16

Total RNA isolation from taro and *Phytophthora colocasiae*

Objective:

To isolate good quality RNA from taro and *Phytophthora colocasiae*

Brief description:

Taro leaf blight caused by *Phytophthora colocasiae* presents the single biggest constraint for taro production globally. Understanding the molecular mechanisms underpinning infection will be of great asset in managing this devastating disease. Conventional protocols for the isolation of total RNA from the infected leaf sample results in a mixture of RNA representing both pathogen and host origin.

The harvested fungal mycelia/plant material was ground to a fine powder in liquid N_2, then 2 mL TRIzol Reagent (Invitrogen) was added and the sample was further ground. The sample was split into two equal volumes and transferred to 2 mL Eppendorf tubes. After 5 min incubation at room temperature, 300 µL of chloroform was added, the tube shaken vigorously for 15 s and allowed to stand at RT for 3 min. The sample was then centrifuged at 13,000 g for 15 min at 4°C. The supernatant from the final extraction step was transferred to a clean 1.5 mL Eppendorf tube and the RNA precipitated with 500 µL isopropanol at -20°C for 2 h or longer (over night when small RNAs are to be recovered). Precipitated RNA was collected by centrifugation at 13,000 g for 15 min at 4°C, the pellet washed with 1 mL of ice cold 75% ethanol and air dried briefly at RT. The RNA pellets were resuspended in 20 µL of nuclease free water and the two duplicate tubes combined. The protocol developed will help to isolate good quantity and quality of RNA from both taro and *P. colocasiae.*

Application:

The protocol yields good quality RNA in high quantities.

Related publication:

Nath, V. S., Rajitha, M., Darveekaran, S. S., Hegde, V. M., Jeeva, M. L., Misra, R. S., Veena, S. S. and Raj, M. (2015). Identification of *Phytophthora colocasiae* genes regulated during infection on taro *(Colocasia esculenta). Physiological and Molecular Plant Pathology* 89: 78-86.

Inventors
Nath V. S., Rajitha M., Darveekaran S. S., Hegde V. M., Jeeva M. L., Misra R. S., Veena S. S. and Raj M
ICAR- Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala - 695017

PCR-based diagnosis of *Phytophthora colocasiae*

17

Objective:

To detect and quantify *Phytophthora colocasiae* in soil and planting material

Brief description:

Developed a conventional and real-time PCR technique exploiting three target regions *viz.,* RAS related protein-*Ypt1* (PCSP-RL F- GGTGTGGACTTTGTGAGTTTCAG, PCSP-RL R- AAGGGAGTTGGCACAACCATT), G protein alpha subunit - *GPA1 (*PCSP-G F-TTGGTGGCGTGTAGTCTGTG, PCSP-G R-AGCTTCCGGTTGATGGTAGC) and Phospho ribosyl anthranilate isomerase - *TRP1* (PCSP-T F- AGCGCCTTAACGCTCCCT , PCSP-T R- GAGCCCTTGAACCACTTGGG) for the detection and quantification of *P. colocasiae* in infected field and seed material. The assays were performed in a volume of 25 µL, which included 100 ng of template DNA, 100 µM each deoxynucleotide triphosphate, 10 ng of each primer, 1.5 mM $MgCl_2$, 19 Taq buffer (10 mM Tris HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 1 U of Taq DNA polymerase (Merck GeNei, India). The optimized PCR regime includes denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 1 min and a final extension at 72°C for 8 min.

The primer pairs successfully amplified *P. colocasiae* isolates from different regions of India in the optimized PCR condition and yielded an amplicon of approx. 206, 248 and 581bp for RAS, TRP and GPA1 genes respectively

Application:

The assay proved to be a robust and reliable technique for detecting *P. colocasiae* in taro planting material and for assessing the distribution of pathogen within fields.

Related publications:

Nil

Inventors

Nath, V. S., Hegde, V. M., Jeeva, M. L., Misra, R. S., Veena, S. S., Raj, M., Unnikrishnan, S. K. and Sankar, D. S.

ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala – 695017

18

A technique for screening castor and safflower for resistance against *Phytophthora* sp.

Objective:

To screen and identify resistance sources in castor and safflower against *Phytophthora* leaf blight

Brief description:

Seedling blight caused by *Phytophthora* is a serious disease of germinating seed, affecting cotyledonary leaves and growing points, causing plant mortality resulting in loss of plant stand. An agar culture disc inoculation technique was developed to screen and select resistance sources to *Phytophthora* leaf blight disease in castor and safflower. Healthy mature castor/safflower leaves of 20 days old plants were selected and the reverse side of the leaves was inoculated with *Phytophthora* agar discs (5 mm) taken from actively growing edges of the culture. Agar bit was covered with small wet cotton and sealed with cellophane. *Phytophthora* used in this study has to be grown on carrot agar at 18^{o}C in the dark for 5 days. Leaf blight symptoms appear within 24-48 h after inoculation and the leaves can be examined for lesion size and disease severity.

Application:

Screening of germplasm and cultivars for identification of resistance sources for introgression of resistance genes in to elite back ground.

Inventors
R. D. Prasad and V. Dinesh Kumar.
ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad - 500030

Development of a protocol for isolation of *Phytophthora cactorum* from soil

19

Objective:

To isolate *Phytophthora cactorum* causing collar rot in apple from soil and to quantify the population in soil

Brief description:

Collar rot of apple caused by *Phytophthora cactorum* is the most severe disease in nursery as well as under orchard conditions. Crown or collar rot causes extensive death of apple trees in many orchards and often occurs on trees between 3 and 8 years of age. The disease often affects low areas of orchards having heavy, poorly drained soils, but it can affect all orchard sites if trees are first infected in the nursery. Soil was collected upto 10 cm depth, sieved by passing through 22.5 mm sieves and air dried at room temperature for 24 days. Ten grams of soil in Petri plate was moistened with 3.8 mL of pond water and then incubated at 22±1°C for 96 h. The plate was flooded by adding 12.5 mL pond water. These covered plates were then chilled at 68°C for 2 h in the refrigerator. The pre-soaked (6 h) and surface sterilized sunnhemp seeds (5-6 No.) were put in the plate as bait and then incubated in light at 22±1°C. After 45 days the germinating radicles with brown lesions near the tip were cut in small pieces, surface sterilized and transferred onto Petri plates containing specific corn meal agar medium amended with pimaricin, ampicillin, rifampicin and PCNB (CMA-PARP) and incubated at 22±1°C. The growth of *P. cactorum* was observed after 48-72 h. The methodology provides quantitative data of *P. cactorum* populations in apple soils under different conditions.

Application:

The method developed is consistent and reliable and can be adopted to detect the *P. cactorum* population in apple orchard soils.

Related publication:

Negi, H. S. (2014). Studies on diversity analysis on collar rot pathogen (*Phytophthora cactorum*) of apple and its management, Ph.D. Thesis, Dr. Y.S. Parmar University of Horticulture and Forestry.

Inventors
Sharma, I. M. and Negi, H. S.
Dr.Y.S.Parmar University of Horticulture and Forestry, Kullu, Himachal Pradesh - 173230

20

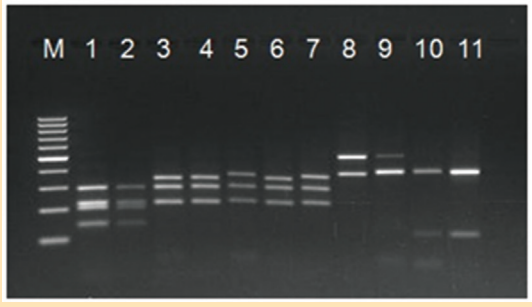
ITS-RFLP detection of *Phytophthora* in black pepper

Objective:

To detect different species of *Phytophthora* in black pepper

Brief description:

The internal transcribed spacer (ITS) region of *Phytophthora* isolates was amplified using the primers ITS-6 and ITS-4 to obtain an amplicon ~900 bp size. The ITS-RFLP profiling showed different patterns for each species. The four *Phytophthora* species *viz*., *P. capsici, P. citrophthora, P. palmivora* and *P. nicotianae* were easily identified by the ITS-RFLP profiling. *P. capsici* isolates of black pepper showed two distinct banding patterns and based on which *P. capsici* isolates could be grouped into group 1 and 2. Group 1 showed bands at approximately 150, 200, 220 and 300 bp whereas, group 2 showed bands of 220, 300 and 350 bp size. *P. citrophthora* isolate showed bands at approximately, 220, 300 and 370 bp size. *P. palmivora* isolates, produced bands at 350 and 480 bp whereas, *P. nicotianae* isolates showed bands at 100 and 350 bp.



ITS-RFLP pattern of *Phytophthora* isolates from black pepper. M: 100 bp ladder; Lane1 & 2: *P. capsici* group 1; Lane 3, 4, 6 & 7: *P. capsici* group 2, Lane 5: *P. citrophthora*, Lane 8 & 9: *P. palmivora* and Lane 10 & 11: *P. nicotianae*

Application:

Can be used for species-specific detection of *Phytophthora* infecting black pepper.

Inventors
Anandaraj, M., Vinitha, K. B. and Suseela Bhai, R.
ICAR-Indian Institute of Spices Research, Marikunnu PO,Kozhikode, Kerala - 673012

A real-time PCR based technology for detection and quantification of *Phytophthora capsici* infecting black pepper

21

Objective:

To detect the presence of *Phytophthora capsici* in soil and to quantify the population in soil

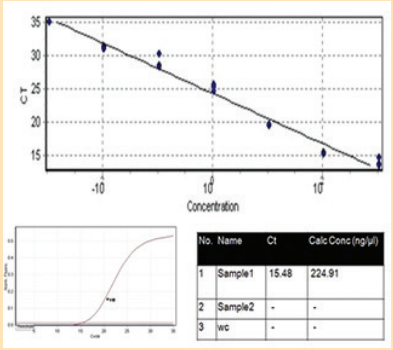
Brief description:

A real-time PCR (qPCR) based method was developed for the detection and quantification of *P. capsici* in soil. Primers for the assay were designed from RAPD-SCAR region (Acc. FN298514.1) of *P. capsici.* For standardization of the assay, genomic DNA isolated from *P. capsici* isolate 05-06 (positive) and *P. nicotianae* isolate 02-21 (negative) were used along with a water control. The qPCR products were verified using 2% agarose gel electrophoresis. The assay could detect *P. capsici* in positive sample without showing any amplification in negative sample and water control, there by proving the sensitivity of the assay in detecting *P. capsici.*

The assay could be used for detecting the presence of *P. capsici* in soil. To quantify *P. capsici* population in the soil, a standard curve need to be constructed using 10 fold dilutions of genomic DNA (isolated from *P. capsici* isolate 05-06) from 1 to 10^{-7} in triplicates.

Application:

The assay can be used for detecting and quantifying the presence of *Phytophthora capsici* in soil.



Inventors

Anandaraj, M., Vinitha, K. B. and Suseela Bhai, R.

ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

22

SSR markers based diversity analysis in *Phytophthora capsici* infecting black pepper

Objective:

To analyse the genetic diversity of *Phytophthora* isolates from black pepper using SSR markers

Brief description:

Phytophthora isolates were grown as stationary cultures in Ribeiro's liquid medium (for four days) at room temperature. The mycelia was harvested by filtration through sterile Whatman No.1 filter paper and ground in a mortar with extraction buffer. DNA was extracted with phenol : chloroform : isoamyl alcohol (25:24:1, v/v), precipitated with ice-cold isopropanol, then washed the pellet with 70% ethanol and re-suspended in Tris-EDTA buffer. The quantity and integrity of the DNA was checked by resolving on a 0.8% (w/v) agarose gel and stained with ethidium bromide. For DNA quantification, 1 µl of DNA sample was diluted 50 times using nuclease free water and the absorbance was read at 260 nm using Biophotometer plus. Analyzed the quality of DNA by measuring the absorbance ratio of DNA at 260 nm and 280 nm. SSR primers were designed from whole genome of *P. sojae*, *P. ramorum* and *P. infestans* and carried out the amplification reactions in a final volume of 25 µl containing 40 ng of genomic DNA, 10 picomol of each primer, 100 µM of dNTPs, 1 unit of Taq polymerase in 1x PCR reaction buffer and 15 mM $MgCl_2$.

Application:

The protocol can be used to analyse the genetic diversity of *Phytophthora* isolates infecting black pepper.

Related publication:

Cissin, J. Suseela Bhai, R. Vinita, K. B. Nirmal Babu, K. and Anandaraj, M. (2016). Cross species amplification of microsatellite loci from *Phytophthora* spp to assess genetic diversity among the *Phytophthora* isolates from black pepper. *Journal of Spices and Aromatic Crops* 25(2): 104-112.

Inventors
K. Nirmal Babu, Cissin, J. Suseela Bhai, R. Vinita, K. B. and Anandaraj, M.
ICAR-Indian Institute of Spices Research, Marikunnu PO,Kozhikode, Kerala - 673012

Detection and relative quantification of resistant gene analogues from *Piper nigrum* and *P. colubrinum* using real time PCR

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Objective:

To detect and quantify *Piper* resistant gene analogues using real time PCR

Brief description:

qRT-PCR was carried out in Rotor Gene Q real time PCR with three step melt. The reaction mixture (20 µl) of qRT-PCR consisted of 10 µl of 5x SYBR green master mix, 1 µl each of forward primer (10 pM) and reverse primer (10 pM), 5 µl of RNA equivalent cDNA (10 ng) and 3 µl of nuclease free water. The qPCR conditions were: initial denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for 10 sec, extension at 72°C for 10 sec and melting at 62-99°C. Monitored the expression of *Piper* RGAs at 0, 0.5, 1, 2, 4, 8, 12, 16, 24, 48 and 72 hpi, respectively in challenge inoculated black pepper plants. No template control (NTC) to monitor potential contamination within the real-time PCR reagents and noRT (cDNA made without reverse transcriptase) was used as negative control to ensure that only cDNA is amplified by the specific primers and devoid of genomic DNA contamination. Performed a dissociation curve analysis with stepwise increase of 1°C per cycle from 62-99°C at the end of the reaction to confirm single PCR product. Used black pepper glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the reference gene to study gene expression in black pepper and actin gene in the case of *P. colubrinum*.

Application:

The protocol can be used to study the expression pattern of resistant gene analogues in black pepper and *Piper colubrinum*.

Inventors

K. Nirmal Babu, Surabhy E.J and Anandaraj M.

ICAR- Indian Institute of Spices Research, Marikunnu PO,Kozhikode, Kerala - 673012

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A detached leaf technique to evaluate fungicides against *P. palmivora*

Objective:

To evaluate fungicides against *Phytophthora palmivora* under *in vitro* conditions

Brief description:

The coconut palm leaflets with broad lamina of uniform length surface sterilized with 0.1 per cent mercuric chloride solution and immediately washed three times with sterile distilled water. The wound at the basal portion of the leaflet formed as a result of detaching it from petiole was sealed with paraffin wax. The whole leaflet was then folded thrice without causing any injury to get a uniform length of about 25 cm. Plastic sip straw of 5 mm diameter was cut into 15 mm length, sterilized using 99 per cent ethanol and one end of each piece was fixed on the abaxial surface of leaflets using paraffin wax so as to make a tubular column on the leaf surface. The fungicides were separately mixed with sterile distilled water to prepare the desired concentration. Prior to loading the tubular column with fungicide solution, the leaf surface within the tubular column was injured lightly with entomological pin and immediately loaded with 125 µL solution of each fungicide and125 µL of water in the case of control. The inoculum was obtained from 7 days old sporulating culture of *P. palmivora* on carrot agar. The inoculum consisting of mycelium and sporangia scrapped out from an area of 5 mm diameter of fungal colony was mixed with either fungicide solution or water (control) in the sip straw fixed on leaf lamina. All the leaflets, soon after inoculation were transferred to plastic trays lined with moist cotton which served as humid chamber. Each tray was covered with a plastic sheet and incubated at 26°C for 5 days. The inoculated leaves were examined for infection and the lesion size was measured horizontally.

Detached leaf technique to evaluate fungicides

Application:

A simple *in planta* assay technique to evaluate fungicides against *P. palmivora,* less expensive technique, can be used for large scale screening of germplasm collection and to test comparative virulence of different species/strains of the pathogen.

Inventors
Sharadraj K. M. and Chandra Mohanan, R.
ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala - 671124

PCR and real time PCR based genus-specific diagnostic assay for *Phytophthora* spp.

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Objective:

To develop a genus-specific diagnostic assay for *Phytophthora* spp. through polymerase chain reaction (PCR) and real-time PCR based protocol

Brief description:

The present protocol developed to detect *Phythophthora* spp. using polymerase chain reaction (PCR) assay would facilitate genus-specific detection of the pathogen in spices and plantation crop based cropping systems.

Total DNA isolated from different *Phytophthora* spp. was amplified in PCR using genus specific primers (Phyto PCRF 5' TGAACTGTACTTCTCTTTGCTCG 3' and Phyto PCRR 5' GAGATGCGCA CCGAAGTGCA 3') with initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 62°C for 30 s, extension at 72°C for 1 min and a final extension period at 72°C for 7 min. The 25 µL PCR reaction mixture was prepared with 12.5 µL master mix (TaKaRa Taq™ DNA Polymerase, USA), 1.0 µL each of forward and reverse primers (10 pM), 1.0 µL of genomic DNA and 9.5 µL of sterile water. PCR amplified products were electrophoretically separated on 1.2% agarose gel containing for 60 min at constant 100 V. The PCR reaction resulted in the amplification of ~309 bp amplicon in all the *Phytophthora* species but not in other non-targeted pathogens.

SYBR green-based real-time PCR was performed (Rotor-Gene® Q, Qiagen, Germany) using *P. capsici* DNA. The primers forward (Phyto_qPCRF 5' CTGAACAGGCGCTTATTGAATG, Phyto_qPCRR) and reverse (3' CTACTTCGCAACAGCAAAGC) were used to generate the amplicon of ~106 bp size. The 25 µL of real-time PCR mix contained 12.5 µL of master mix, 1.0 µL each of forward and reverse primers (10 pM), 1.0 µL of genomic DNA and 9.5 µL of water. Reaction was performed with the following conditions, 95°C for 10 min followed by 95°C for 10 s, 60°C for 20 s, 60°C

Inventors
Thava Prakasa Pandian, R., Bhat, A. I., Biju, C. N. and Sasi, S.
ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

for 20 s and 65-95°C for 1-5 s. The assay was found to be highly sensitive and could detect the target DNA upto 10^{-8} (1.5 fg).

Application:

The PCR and real-time PCR based protocol developed would provide rapid and accurate detection of *Phytophthora* spp. in spices-plantation crops based cropping systems which would further help the growers for timely adoption of management measures.

LAMP and Real-Time LAMP based genus specific diagnostic assay for the detection of *Phytophthora* spp.

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Objective:

To develop a genus-specific diagnostic assay for *Phytophthora* spp. through loop-mediated isothermal amplification-based protocol

Brief description:

LAMP reaction was standardized with selected *P. capsici* isolate. The 25 µL of LAMP reaction was set up which included 1 µL template DNA (150 ng), 2x thermopol buffer (New England Bio Labs, USA), 1.4 mM dNTPs, 6 mM $MgSO_4$, 0.8 M betaine, 0.2 µM external primers, 2 µM inner primers, 1 µM loop primers and 8 U of Bst polymerase (New England Bio Labs, USA). The reaction was performed at 65°C for 60 min followed by incubation at 80°C for 5 min to inactivate the Bst polymerase. LAMP products were analyzed through agarose gel electrophoresis, which exhibited a characteristic ladder-like pattern while, no amplification was observed in the negative control.

Optimization of the real-time LAMP assay components was carried out in 0.2 mL strips (Optigene, UK) using *P. capsici* DNA. The real-time LAMP reaction mixture contained 1 µL template, 15 µL isothermal master mix (Optigene, UK) and primers as mentioned in the LAMP assay. The reaction was performed at 65°C for 60 min in real-time LAMP equipment (Genie II, Optigene, UK). The primers used were, LF 5' CCTGTTCAGCCGAAGCCA and LB 3' TGCTGTTGCGAAGTAGGGTGG; F3 5' GCTGCGGCGTTTAAAGGA and B3 3' AGTGCACACAAAGTTCCCAA;FIP5' ACGCCACAGCAGGAAAA GCATTGA GTGTTCGATTCGCGGTA and BIP 3' GGCTTGGCTTTT GAATCGGCTTTGGATCGACCCTCGACAG. Real-time LAMP detected the target region in *P. capsici* DNA within 6 min and a single peak of annealing curve was observed at Tm 89°C. Real-time LAMP reaction detected the target region in all *Phytophthora* spp. used but not in *Pythium vexans* and water control. This assay was specific for target regions and the time taken for the detection varied from 6-12 min in different samples. The specificity of real-time LAMP product was confirmed through annealing curve which showed a single peak at 89°C for *Phytophthora* sp. (isolate code: 98-93) and *P. palmivora* (isolate code: 12-31) and 88°C for others.

Application:

The LAMP-based protocol developed would provide rapid and accurate detection of *Phytophthora* spp. in spices-plantation crops based cropping systems.

Inventors

Thava Prakasa Pandian, R., Bhat, A. I., Biju, C. N. and Sasi, S.

ICAR-Indian Institute of Spices Research, Marikunnu PO,Kozhikode, Kerala - 673012

Fusarium

In India, crops are grown under varied agro-climatic conditions and *Fusarium* spp., occur regularly causing considerable crop losses and produce several mycotoxins. The genetic variability of the members of genus *Fusarium* increases the difficulties encountered in developing resistant host genotypes, as well as in effectively deploying available tolerant cultivars. *Fusarium oxysporum* is a species complex comprising ubiquitous soil-borne plant pathogens with more than 150 host specific forms. *Fusarium* wilt incidence in tomato, chilli and safflower has been recorded from various parts of India. The pathogen invades the root epidermis and extends into the vascular tissue. It colonizes the xylem vessels producing mycelium and conidia. The characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging. Since *F. oxysporum* f. sp. *lycopersici* (Fol) is an asexual fungus, genetic exchange occurs *via* somatic fusion and hetreokaryon formation between vegetative compatible strains. Tomato is one of the world's most widely cultivated vegetable crops for consumption as fresh fruits and various types of processed products. *Fusarium* wilt caused by the soil-borne fungus, *F. oxysporum* f. sp. *lycopersici*, is one of the most devastating diseases of tomato. The disease is characterized by yellowed leaves and wilted plants with minimal or no crop yield. There may be a 30-40% yield loss due to the disease and this may go upto 80% under favorable weather conditions.

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Identification of vegetative compatibility groups (VCGs) in *Fusarium oxysporum* f. sp. *ciceris* causing chick pea wilt

Objective:

To determine existence of vegetative compatibility groups (VCGs) among the Indian populations of *Fusarium oxysporum* f. sp. *ciceris*

Brief description:

Fusarium wilt caused by *F. oxysporum* f. sp. *ciceris* is one of the most important biotic stresses of chickpea (*Cicer arietinum* L.), with the potential to cause 100% yield loss.

Nitrate non-utilizing (nit) mutants were selected by growing isolates of Foc on quarter-strength potato dextrose agar instead of minimal media (MM) for 3–4 days. 2 mm blocks of fungal mycelium was transferred to 9 mm petri plates containing MM with 2.5% potassium chlorate ($KClO_3$) and incubated at 28±1°C for 2–3 weeks. The colony growth in the medium was restricted for the first week, and after that fast growing sectors appeared. These fast growing sectors from initially restricted colonies were then transferred to a MM that contained sodium nitrate as a nitrogen source. Mutant cells that could not utilize nitrate emerged as fast growing sectors on chlorate, and sectors having thin and expanding areal mycelium on MM were considered to be nit mutants.

Vegetative compatibility was determined by observing complementation reaction between two nit mutants. Two-mm blocks of complementary nit mutants were transferred from MM to a fresh 9 cm petri dish in daisy configuration with a nit M mutant at the center and nit1 and nit3 mutants from different isolates at the outer circle 4 cm apart, and incubated at 28±1°C for 10–15 days. Three types of scorings, namely, strong reaction (++), moderate or weak reaction (+) and no reaction (-), were made . If strong and moderate reactions occurred at the intersection of the colonies, then the isolates were of the same VCG; if it remained thin, then the isolates were of different VCGs.

Application:

The protocol developed can be used to identify vegetative compatibility groups (VCGs) among the Indian populations of *F. oxysporum* f. sp. *ciceris* causing chickpea wilt .

Related publication:

Saabale, P. R. and S. C. Dubey (2014) Pathogenicity and vegetative compatibility grouping among Indian populations of *F. oxysporum* f. sp. *ciceris* causing chickpea wilt. *Phytoparasitica*, 42, (4): 465–473.

Inventors
Saabale, P. R. and Dubey, S. C.
ICAR- Indian Agricultural Research Institute, Pusa, New Delhi - 110012.

A protocol to identify genetic diversity in *Fusarium oxysporum* f. sp. *ciceris*

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Objective:

To determine genetic diversity in *F. oxysporum* f. sp. *ciceris* causing chick pea wilt

Brief description:

Virulence of *Foc* isolates were analysed on 10 chickpea differential cultivars, namely, C 104, JG 74, CPS 1, BG 212, WR 315, Chaffa, JG 62, KWR 108, GPF 2 and DCP 92-3 under greenhouse conditions. Ten seeds of each cultivar (surface-sterilized in 2% sodium hypochlorite for 2 min and rinsed thrice in sterile water) were sown in surface-sterilized plastic pots (15 cm diameter) filled with 2 kg of sterilized soil (1% formalin for 15 days) and inoculated with 15-day old inoculum of the pathogen grown on sorghum grains (10 g kg^{-1} soil) five days before sowing. The wilt incidence was recorded at 15 days interval up to maturity of the crop plants.

Single-spore cultures of *Foc* isolates were multiplied in potato dextrose broth at 25±1°C on a shaking incubator for seven days. The mycelium was harvested by filtration and kept at -80°C prior to grinding in liquid nitrogen to a fine powder. DNA was extracted by the CTAB method . The mycelium (1 g) was triturated with a pre-cooled mortar and pestle and mixed with pre-warmed (65°C) 2% CTAB DNA extraction buffer. The tubes were incubated in a water bath at 65ºC with gentle shaking for 10 min then, after cooling at room temperature, an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) was added and mixed gently to denature proteins. Centrifugation followed at 12,000 rpm at room temperature for 20 min. The aqueous phase was transferred to a new sterile tube and an equalvolume of chloroform:isoamyl alcohol (24:1 v/v) was added, mixed gently and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a new sterile tube and the last step was repeated once again to obtain a pure DNA preparation. The aqueous phase was transferred to a new tube and DNA was precipitated with 0.6 vol of ice cold isopropanol and 0.1 vol of 3M sodium acetate, kept at -20°C for 3-4 h and centrifugedat 10,000 rpm for 10 min at 4°C. The supernatant was poured off, the DNA pellet was washed twice with 70% ethanol, dried either at room temperature or at 37°C, then resuspended in 200 µl TE buffer

Inventors
Durai, M., Dubey S.C. and Tripathi, A.
ICAR- Indian Agricultural Research Institute, Pusa, New Delhi - 110012.

and stored at -20°C.

Amplification with ITS1, 5.8S rDNA and ITS2 region of *Foc* isolates was achieved with a set of universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR condition was as follows: 1X buffer, 50 ng template DNA, 1 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 6 pmol of each of primer (ITS1 and ITS4) in a reaction mix of 25 µl. Amplification was done in Master cycler gradient thermal cycler with the following reaction conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1 min and extension at 72°C for 2 min with an elongation of 72°C for 5 min. Amplified products were resolved by electrophoresis on agarose gel (1.4%) in 1X TAE buffer stained with ethidium bromide. The amplified PCR products were digested with the restriction enzymes .Twenty µl of reaction solution was prepared by combining 10 µl PCR products, 1X reaction buffer, 2 units enzyme and sterile distilled water. One µl of 6X dye was added to the reaction solutions to stop the reaction. After digestion, 10 µl of each reaction mix was electrophoresed through 2% agarose gel in 1X TAE buffer at 70 V for 40 min and detected. The amplified fragments of ca. 540 bp were eluted, purified and custom sequenced.

Application:

The set of differential indicators standardized can be used for differentiating the changed populations of *Foc* isolates into various races and which will help to determine the distribution pattern of *Foc* races in India. Determination of virulence and genetic variability of *Foc* isolates will help to develop area specific resistant varieties of chickpea.

Related publication:

Durai, M., Dubey, S.C. and Tripathi, A. (2012) Analysis of virulence and ITS region based genetic variability among the Indian populations of *F. oxysporum* f. sp. *ciceris* causing chick pea wilt. *Journal of Plant Pathology*, 94 (3): 651-662.

Development of a SCAR marker for specific detection of *Fusarium oxysporum* f. sp. *carthami*

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Objectives:

To develop a molecular marker for specific detection of *Fusarium oxysporum* f. sp. *carthami* in safflower

Brief description:

Rapid and reliable detection of the pathogen is essential for undertaking appropriate and timely disease management measures. Traditional methods are now being increasingly replaced by molecular detection techniques, which are much faster and more specific. A SCAR marker of 162 bp is developed from ITS sequences and used for identification of *F. oxysporum* f. sp. *carthami* isolates. The SCAR marker shows specific amplification product of 162 bp of *F. oxysporum* f. sp. *carthami* isolates, which is not amplified in other *Fusarium* species. The designed SCAR primer pair can be used consistently to detect *F. oxysporum* f. sp. *carthami* isolates, isolated from the diseased samples.

Application:

F. oxysporum f. sp. *carthami* isolates can be specifically identified from other *Fusarium* species.

Inventors
Prasad, R. D. and V. Dinesh Kumar
ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad - 500030

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Identification of SSR markers linked to *Fusarium* wilt resistance

Objectives:

Introgression of *Fusarium* wilt resistance from wild species into cultivated safflower and marker assisted selection for wilt resistance

Brief description:

Fusarium wilt susceptible safflower (*Carthamus tinctorius* L.), Nira was crossed to wilt resistant wild species, *C. oxyacantha, C. palaestinus, C. lanatus, C. creticus, C. turkestanicus* and *C. galaucus.* Screened F1-F7 generations against *Fusarium* wilt in wilt sick plot/pots. The plants with any intensity of wilt symptom were designated as susceptible and the one without any wilt symptoms was designated as resistant. Single dominant gene control of wilt resistance was found. Of the 320 SSR markers used, 45 confirmed true hybriditity of interspecific hybrids. Bulk segregant analysis employed in F2 mapping populations identified 34 species-specific SSRs linked to *Fusarium* wilt resistance. Using these markers, marker-assisted selection for wilt resistance was practiced successfully in F2-F7 generations of all interspecific crosses. Wilt resistant individuals in each generation could be selected at seedling stage using markers prior to commencement of wilt in the field. Total 116 wilt resistant interspecific inbred lines were derived through markerassisted selection (MAS).

Application:

The SSR markers linked to wilt resistance are useful for selection of *Fusarium* wilt resistant individuals at seedling stage itself and for pyramiding of wilt resistant genes.

Related publications:

Anjani, K., Pallavi, M., Pradsa, R. D., Dinesh Kumar, V. and Madanmohan, B. (2010). Relevance of marker-assisted breeding for *Fusarium* wilt resistance in safflower (*Carthamus tinctorius* L.). National Symposium on “Genomics and Crop Improvement Relevance and Reservations”, February, 25-27, 2010, Hyderabad.

Anjani, K., Bhavna, P., Debadutta Mishra and Prasad, R. D. (2016). Exploitation of wild species, *Carthamus oxyacantha* Bieb. for disease resistance and yield improvement in safflower (*C. tinctorius* L.). Oral presentation during National Seminar on “Breeding of field crops for biotic and abiotic stress in relation to climate change” from 28-29 March, 2016 at College of Agriculture, VNMKV, Parbhani.

Inventors
K. Anjani and R. D. Prasad
ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad - 500030

Chitin synthase, a candidate gene for the molecular characterization of *Fusarium* isolates

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Objective:

Molecular characterization of *Fusarium* isolates infecting tomato and chilli using chitin synthase gene

Brief description:

Wilt of tomato and chilli caused by *F. oxysporum* and *F. solani*, respectively has the potential to severely affect tomato and chilli production and can cause complete yield loss under greenhouse and field conditions. Chitin synthase gene (*CHS*), which is responsible for virulence in *Fusarium*, was amplified with specific primers CHS79-F (5'-TGGGGCAAGGATGCITGGAAG AAG-3') and CHS354-R (5'-TGGAAGAACCATCTGTGAGACTTG-3'). PCR reactions were carried out in a 25 µL volume consisting of 1x PCR buffer (Fermentas Inc., USA), 0.2 mM of each dNTPs (Fermentas Inc., USA), 2 mM $MgCl_2$ (Fermentas Inc, USA), 0.5 µL of each primers (Eurofins), 1 U of Taq DNA polymerase (Genei, Bangalore), 2.5 µL of template DNA and sterile distilled water was added to make up the final volume. The thermal cycling conditions used were: initial denaturation at 95˚C for 10 min followed by 35 cycles of 95˚C for 15 s, 55˚C for 20 s and 72˚C for 60 s with a 5 min extension at 72˚C on the final cycle. The isolates of *F. solani* and *F. oxysporum* were found positive and yielded 300 bp fragment of *CHS* gene.

Application:

CHS gene can be used as the candidate gene for the identification of *Fusarium* isolates infecting tomato and chilli.

Inventors

Loganathan, M., Venkataravanappa, V., Nagendran, K., Pandey, K. K., Saha, S. and Rai, A. B.

ICAR-Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh - 221305

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Identification of races of *Fusarium oxysporum* f.sp. *lycopersici*

Objective:

To identify the race of *Fusarium oxysporum* f. sp. *lycopersici* infecting tomato in India

Brief description:

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is one of the most economically important and widespread disease of the cultivated tomato. The virulence profile of *F. oxysporum* f. sp. *lycopersici* isolates affecting tomatoes has been grouped into three races according to their ability to infect a set of differential cultivars carrying distinct resistance loci. Two sets of specific primers based on *polygalacturonase* gene PGLENDO-F (CCAGACTGCGCATACCGATT) and PGLENDO-R (AAGTGTTGGTAGGATAGTTG) that can amplify race 1 & 2 and race 2 & 3 were used. PCR reactions were carried out in a 25 µL volume consisting of 1x PCR buffer (Fermentas Inc., USA), 0.2 mM of each dNTPs (Fermentas Inc. Maryland, USA), 2 mM $MgCl_2$ (Fermentas Inc. Maryland, USA), 0.5 µL of each primers (Eurofins), 1 U of Taq DNA polymerase (Genei, Banagalore), 2.5 µL of template DNA and final volume made up with sterile distilled water. The thermal cycling conditions used were: initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation, annealing and elongation steps respectively at 94°C for 60 s, 61°C for 60 s and 72°C for 2 min and a final extension step of 72°C for 10 min. *Polygalacturonase* gene was amplified in the isolates of FOL with specific primers PGLENDO-F/R with an amplicon size of 1.5 kb.

Specific primers developed based on *polygalacturonase* gene can be used for the race identification in *F. oxysporum* f. sp. *lycopersici* isolates. Molecular identification of races in *F. oxysporum* f. sp. *lycopersici* isolates using *polygalacturonase* gene-based specific primers will help in early detection of races and also to identify the establishment of new pathogenic races of the fungus.

Application:

The markers can be used for the race identification in the *F. oxysporum* f. sp. *lycopersici* isolates.

Inventors
Nagendran, K., Loganathan, M., Pandey, K. K., Saha, S., Venkataravanappa, V. and Rai, A. B.
ICAR-Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh - 221305

Ralstonia

Ralstonia solanacearum (Smith) Yabuuchi *et al.* is a major soil-borne bacterial pathogen which is limiting the production of many crop plants around the world. This organism is the causal agent of brown rot of potato, bacterial wilt of tomato, ginger, tobacco, eggplant and some ornamentals and Moko disease of banana. The pathogen is a widely distributed pathogen found in tropical, subtropical and some temperate regions of the world. The species as a whole has a very broad host range and infects hundreds of species in many plant families. The majority of hosts are dicots with the major exception being bananas and plantains. High temperatures and high soil moisture generally favours *R. solanacearum*, the exception being certain race 3 strains that are pathogenic on potato and are able to grow well at lower temperatures. The pathogen is found in many different soil types and over a wide range of soil pH. The organism survives in infected plant material, vegetative propagative organs, wild host plants and soil. Alternate hosts, especially latently infected weed species, are thought to play a major role in the overwintering ability of the organism in temperate regions. Sources of inoculum for agricultural fields and methods of spread include irrigation and surface water, aquatic weeds, infested soil and field weeds, contaminated planting material, latently infected vegetative propagative material and contaminated farm tools and equipment. Once established in a field, plant-to-plant spread may occur when bacteria move from roots of infected plants to roots of healthy plants. *R. solanacearum* enters the plant through wounds in the roots from cultivating equipment, nematodes, insects and through cracks where secondary roots emerge. The bacteria reach the large xylem elements and are spread into the plant, where they multiply. Once established in the xylem vessels, the bacteria are able to enter the intercellular spaces of the parenchyma cells in the cortex and pith in various areas of the plant. Here, *R. solanacearum* is able to dissolve the cell walls and create slimy pockets of bacteria and cell debris. Production of highly polymerized polysaccharides increases the viscosity of the xylem, which results in plugging.

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Detection of *Ralstonia solanacearum* in different substrates with Bio-PCR

Objective:

To detect *Ralstonia solanacearum* from asymptomatic plants, irrigation water and soil

Brief description:

Bacterial wilt of tomato caused by *R. solanacearum* is a serious disease, which causes losses upto 60 % depending on environmental conditions, soil property and cultivars. Bio-PCR reaction was performed in a final volume of 20 µL amplification reaction mixture containing buffer 2.0 µL (5x), primer F (20 pmol/ µL) 0.4 µL, primer R (20 pmol/ µL) 0.4 µL, $MgCl_2$ (25 mM) 1.0 µL, dNTPs (10 mM) 0.4 µL, Taq polymerase 0.25 µL. Amplification conditions included denaturation step at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 51°C for 30 s and 72°C for 30 s and then one cycle of 72°C or 10 min in thermal cycler gradient PCR (BIORAD; model: C1000TM). Amplified PCR products were separated by electrophoresis on 1.2% agarose gel (80V) for 1 h and visualized under UV light (300 nm) after staining with ethidium bromide under gel documentation system. The genomic DNA of *R. solanacearum* isolates race 1 biovar 3 & 4 was amplified at 323 bp.

Nucleotide sequences of virulence, hypersensitive response and pathogenicity (*hrp*) gene were used to design a pair of primer (Hrp_rs 2F: 5′-AGAGGTCGACGCGATACAGT-3′ and Hrp_rs 2R: 5′-CATGAGCAAGGACGAAGTCA-3′) for amplification of bacterial genome. The primer was found specific for detecting viable and virulent strains of *R. solanacearum* and useful for the diagnosis of *R. solanacearum* in tomato seedlings and monitoring of pathogen in irrigation water and soil.

Application:

Detection of *R. solanacearum* from asymptomatic plants, irrigation water and soil.

Related publication:

Dinesh, S., Yadav, D. K., Shweta, S. and Garima, C. (2014). Detection of *Ralstonia solanacearum* from asymptomatic tomato plants, irrigation water and soil through non-specific enrichment medium with *hrp* gene based bio-PCR. *Current Microbiology* 69: 127-134.

Inventors
Dinesh Singh, Shweta Sinha, Yadav, D. K. and Garima Chaudhary
ICAR-Indian Agricultural Research Institute, Pusa, New Delhi - 110012

Loop-mediated isothermal amplification assay for detection of *Ralstonia solanacearum* in tomato

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Objective:

To develop a LAMP assay for the detection of *Ralstonia solanacearum* infecting tomato plants

Brief description:

R. solanacearum, causing a vascular wilt disease in tomato, has been ranked as the second most important bacterial pathogen. It is one of the most destructive pathogen identified and induces rapid and serious wilting symptoms in affected plants. A rapid and sensitive detection of *R. solanacearum* using a loop mediated isothermal amplification (LAMP) was developed. Isothermal Master Mix (12.5 µL) and consisted of 5 primers, at the final concentration 0.2 µmol L1 each of F3 and B3, 2 µmol L1 each of FIP and BIP, l mol L1 dNTPs, l mol L1 betaine, 6 mmol L1 $MgSO_4$, 2.5 µL 10x BstDNA polymerase buffer, 8 U Bst DNA polymerase and 5 µL template. All reactions for evaluation of the test were performed in a thermal cycler instrument using negative control and positive control. The reaction time was optimized for 15, 30, 60, 90 and 120 min at 65°C, while the reaction temperature was optimized by incubating 60, 61, 62, 63, 64, 65 and 66°C for 60 min. The final standardized LAMP reactions were run at 60°C for 60 min. The concentrations of betaine and Mg^{2+} ion were optimized using a series of concentrations from 1 mol L1 to 4 mol L1 and from 1 mmol L1 to 7 mmol L1. The reaction was terminated at 80°C for 10 min using dry bath cycler. The LAMP products were run on 2% agarose gels and stained with ethidium bromide. The possibility of LAMP detection of *R. solanacearum* using HNB was examined by dissolving in distilled water at 1.5 mM to prepare stock solution.

Application:

Detection of *Ralstonia solanacearum* in tomato plants.

Inventors

Dinesh Singh, Garima Chaudhary and Dhananjay Kumar Yadav

ICAR-Indian Agricultural Research Institute, Pusa, New Delhi - 10012

35

Development of PCR-based methods to detect *Ralstonia solanacearum* in soil, plant and weeds

Objective:

To detect *Ralstonia solanacearum* in soil, plant and weeds

Brief description:

PCR-based sensitive detection was standardized and validated using one forward primer and two reverse primers designed from ITS region among Indian isolates of *Ralstonia solanacearum*. Conventional PCR: The 10 µL of reaction mixture contained 200 µM dNTPs, 1x buffer, 1.5 mM $MgCl_2$, 5% DMSO, 20 µg BSA, 1 U Taq DNA polymerase, 1 µM specific primers (RS-ITS FP- AGTCGTAACAAGGTAGCCGTATCG, RS-ITS RP-GCCAAGGCATCCACCACATGC) and 10-20 ng of the DNA/bacterial ooze/bacterial growth. The following PCR conditions were set: initial denaturation at 94^{O}C for 2 min, followed by 34 cycles of denaturation at 94^{O}C for 20 s, annealing at 68^{O}C for 20 s and extension at 72^{O}C for 20 s and the final extension at 72^{O}C for 10 min. PCR product generated was resolved on 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL). BIO-PCR: one mL soil suspension (10 g/100 mL) was inoculated into nine mL broth and incubated at RT for two days. Five µL of this was taken as template for PCR.

Application:

Rapid, economic and reliable method of *R. solanacearum* detection from soil, plant and weeds.

Inventors
Ramesh, R., Anthony, J., Jaxon, T. C. D., Gaitonde, S. and Achari, G.
ICAR-Central Coastal Agricultural Research Institute, Old Goa - 403402

PCR-based rapid detection of *Ralstonia solanacearum* from infected plants

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Objective:

To detect *Ralstonia solanacearum* directly from the infected plants using PCR-based approach

Brief description:

Ralstonia solanacearum (Smith) causes sudden wilting in plants and difficult to detect at the initial level as similar symptom may also occur with many fungal organisms like *Fusarium* spp. and *Verticillium* spp.

Samples were cut into small pieces aseptically and were placed in 1 mL sterile nuclease and protease free water. Then those were kept for incubation at room temperature for 5 min or until the water became turbid followed by a final incubation at 96°C for 6 min in thermal cycler. Finally, the suspension was directly used for PCR amplification using previously reported Rsol-fliC primers (Forward: 5'GAA CGC CAA CGGTGC GAA CT 3'; Reverse: 5' GGC GGC CTT CAG GGA GGTC 3') designed to amplify a ~400 bp fragment of *fliC* gene specific for *R. solanacearum.* The PCR has 1x PCR buffer, 3 mM $MgCl_2$, 6% DMSO, 50 µM of each dNTPs, 10 pmol of each primer, 1 U of Taq DNA polymerase and 2 µL of bacterial ooze suspension. A known and identified bacterial strain of *R. solanacearum* was used as positive control and sterile nuclease free water as negative control. The PCR cycling conditions are: initial denaturation at 96°C for 9 min, followed by 34 cycles of 95°C for 30 s, 64°C for 1 min, 72°C for 2 min and final extension of 72°C for 10 min. The primer pairs for *fliC* gene (Rsol-fliC), can amplify 400 bp region of *fliC* gene. The method requires only 3-5 hours against the conventional methods which generally require minimum three days to detect the pathogen. PCR based method can be used go detect *Ralstonia solanacearum* from bacterial ooze from infected tissues. The PCR-based method developed is very simple, robust and inexpensive.

Application:

Rapid detection of *Ralstonia solanacearum* from diseased plants.

Inventors
Ram Dutta, Amrita Banerjee, Gajanan T. Behere, Jina Devi, K. Satish Chandra and Ngachan. S. V.
ICAR Research Complex for North Eastern Hill Region, Umaiam, Meghalaya - 793103

Related publications:

Ram Dutta, Amrita Banerjee, Gajanan T. Behere, Jina Devi, K., Satish Chandra and Ngachan, S. V. (2015). PCR detection of *Ralstonia solanacearum*. A new approach for rapid detection of bacterium from infected plants. *Indian Journal of Agricultural Sciences* 85(8): 1007-11.

Development of loop-mediated isothermal amplification (LAMP) PCR for detection of *Ralstonia solanacearum* in brinjal

37

Objective:

To detect bacterial wilt from field samples

Brief description:

The final loop-mediated isothermal amplification (LAMP) reaction mixture consisted of 2.4 µM of FIP and BIP, 0.2 µM of F3 and B3 and 0.4 µM of loop primers, 800 µM dNTPs, 1x assay buffer, 4 mM $MgSO_4$, 20 µg BSA, 1 M Betaine and 2 µL template. The reaction mixture was heated for 10 min at 94^{O}C before adding 8 U Bst 2.0 DNA polymerase. The reaction tubes were incubated for 1 h at 65^{O}C and thereafter at 80^{O}C for 10 min for terminating the reaction. LAMP products were subjected to electrophoresis at 75 V for 2 h on a 1.5% agarose gel. Suitability of several dyes for visual observation of positive LAMP reaction for detection of *R. solanacearum* under field conditions was standardized.

Application:

Detection of *R. solanacearum* and diagnosis of bacterial wilt at field level and the minimum threshold of detection from soil was 70 cfu.

Inventors
Ramesh, R., Achari, G., and D'Souza, M.
ICAR-Central Coastal Agricultural Research Institute, Old Goa - 403402

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Development of field diagnostics for *Ralstonia solanacearum* race 4

Objective:

To detect *R. solanacearum* in soil, water and seed rhizomes of ginger

Brief description:

A set of six primers *viz.,* RsGyrB F3, RsGyrB B3, RsGyrB FIP, RsGyrB BIP, RsGyrB LOOPF and RsGyrB LOOPB were designed from the *gyrB* gene region and on *in silico* validation, no hairpin formation or self dimers was observed. The primers were validated by LAMP which resulted in characteristic ladder-like amplification only for race 4 strains of *R. solanacearum* and not for race 1 strains from brinjal, tomato and potato or race 3 strains from potato.

Real-time LAMP: The reactions were performed in 25 µL volume containing 15 µL of ampligene real-time LAMP mastermix, 2.5 µL of primer mix (0.5 µL of F3, B3.FIP and BIP and 0.25µL of LOOPF and LOOPB 0.25 µL) and 50 ng template DNA. The reactions were carried out at 65^{O}C for 45 min with an annealing curve analysis from 98^{O}C-80^{O}C at 0.05°C/s.

Soil: One gram of soil was dispensed in 10 mL of sterile distilled water and mixed in a vortex at maximum speed for 5 min. The tubes were then kept still for about 30 min. After this one mL of the supernatant was centrifuged at 8000 rpm for 1 min at room temperature and resuspended in 10 µL of water. From this one µL was used as the template for LAMP.

Rhizome: The core of the infected rhizome was removed aseptically (0.1 g) and homogenized in 1 mL of 50 mM phosphate buffer (pH 7.0), resulting suspension (100 µL) was heated at 97^{O}C for 4 min and cooled rapidly on ice. To this sample, 900 µL of sterile water was added. One µL of this sample was used as template for LAMP.

The method can be used to detect *R. solanacearum* in soil, water and seed rhizomes of ginger. Soil supernatant is used as template for easy and on-farm diagnosis. The time taken for detection is only 3-4 h.

Application:

The detection limit/sensitivity of this method was 10^3 cfu/g of soil or rhizomes. It can be easily adopted in the field which will benefit farmers to choose pathogen-free soil as well as disease-free seed material for planting.

Inventors
Suseela Bhai, R., Prameela, T. P., Kumar, A. and Anandaraj, M.
ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

Antimicrobial activity of mango ginger extract against *Ralstonia solanacearum*

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Objective:

To evaluate the antimicrobial activity of mango ginger extract against *R. solanacearum*

Brief description:

The dried mango ginger slices were powdered and 50 g of rhizome powder was sequentially extracted with n-hexane, chloroform and methanol in the order of their increasing polarity at room temperature. After each extraction the solvents were filtered and concentrated using a rotary evaporator. The yield of extracts was noted. The extracts were screened for anti-microbial activity by agar well diffusion method. The solvent extracts were tested against *R. solanacearum* at different concentrations ranging from 5, 10, 25, 50, 75 mg/ml and 100 mg/ml. Essential oil was extracted from 500g of dried and crushed mango ginger rhizomes by hydro distillation using Clevenger type apparatus. The isolated essential oils were tested at 1%, 5% and 10% (v/v) against *R. solanacearum.* The virulent strains of *R. solanacearum* were cultured on Casamino acid-Peptone-Glucose (CPG) agar medium supplemented with 0.005 % (wt/vol) 2,3,5-triphenyl tetrazolium chloride (TZC) medium at 28 °C (Kelman, 1954) and the colonies formed were multiplied in CPG (Casein Peptone Glucose) broth for 16 h. The density of the bacteria which were in actively growing phase was adjusted to a concentration of 10^8 CFU/ mL. The broth was centrifuged and the bacteria were pelleted. The media was discarded and the pellet was mixed with sterile water. The bacterial culture, 100 µL at OD-0.1 (10^8 CFU/mL) was spread on the surface of CPG agar plates.

The sequential extraction with hexane, chloroform and methanol yielded 4.2, 0.9 and 2.8g of extracts respectively. The preliminary screening of all the three extracts resulted in varying but nearly same level of antibacterial activity against *R. solanacearum* as indicated by a clear zone of inhibition around the agar well supplemented with extract.

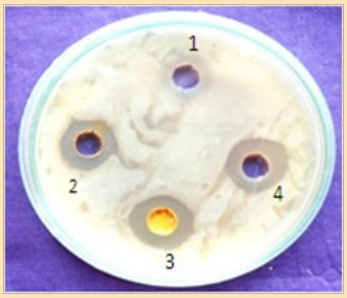
The sequential extraction with hexane, chloroform and methanol yielded 4.2, 0.9 and 2.8 g of extracts respectively. The preliminary screening of all the three extracts resulted in varying but nearly same level of antibacterial activity against *R. solanacearum* as indicated by a clear zone of inhibition around the agar well supplemented with extract.The essential oils exhibited antibacterial activity at all the three concentrations *viz*., 1 %, 5 % and 10 % (vol/vol) against R. solanacearum. The GC-MS analysis identi-

Inventors
Prasath, D., Karthika, R., Leela, N, K., Bhai, R.S. and Anandaraj, M.
ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

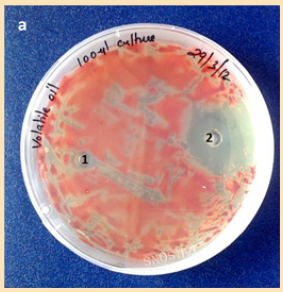
fied the chemical constituents of the essential oils.

Application:

Our results represent new contributions on the antibacterial properties of mango ginger rhizome extracts against *R. solanacearum*. The results are very encouraging and the identification of the novel antibacterial compounds could be useful in the control of bacterial wilt infection in ginger. The incorporation plant derived bactericides from the plants with limited known economic values like mango ginger into integrated disease management approach will ensure a better management of the bacterial wilt disease affecting ginger.



Petriplate showing zone of inhibition of 100 µl of hexane, chloroform and methanol extracts on *R. solanacearum* 1) Control (DMSO) 2) Hexane extracts 3) Chloroform extract 4) Methanol extract



Effect of essential oil of mango ginger on *R. solanacearum* (1. Control; 2. Essential oil)

Related publication:

Karthika R, Prasath D, Leela NK, Bhai RS and Anandaraj M. 2017. Evaluation of the antibacterial activity of mango ginger rhizome extracts against bacterial wilt pathogen *Ralstonia solanacearum*. *Journal of Spices and Aromatic Crops*, 26 (2):95-100.

Candidate genes for resistance to bacterial wilt in ginger

40

Objective :

To identify candidate genes through transcriptome and gene expression analysis for resistance to bacterial wilt in ginger

Brief description:

To identify genes involved in resistance to ginger bacterial wilt caused by *Ralstonia solanacearum*, the transcriptome from bacterial infected and healthy leaf tissues collected from wilt-sensitive ginger (*Zingiber officinale*) and wilt-resistant mango ginger (*Curcuma amada*) using paired-end (PE) Illumina sequencing technology. Comparative transcriptome analysis identified genes belonging to different pathways of plant defense against biotic stresses that are differentially expressed in either ginger or mango ginger. Nine candidate reference genes selected from transcriptome database were evaluated comprehensively across rhizome and leaf tissue samples using quantitative real time PCR analysis. The primers specific to the sequences were designed using Primer-quest tool (Integrated DNA Technologies, Coralville, IA). The potential of ERF, HMGR, HMGS, ABC, WRKY8, β-1, 3-glucanase, Callose synthase, heat shock protein and MLO genes were studied in 45-60 days old ginger and mango ginger plants inoculated with *R. solanacearum.*

The pattern of the accumulation of the transcripts of the selected defence-response genes differed with respect to each other after pathogen exposure. These changes in the transcript levels may underlie the outcome of the host-pathogen interaction in mango ginger and ginger. All the nine genes under study were induced in both pathogens under inoculated and uninoculated conditions. The present results suggest that defence related genes are induced rapidly in response to pathogen invasion might help the mango ginger to limit infection by *R. solanacearum.*

Application:

The intrinsic roles of the candidate genes differentially expressed may provide a new viewpoint towards the understanding the defence response of ginger to *R. solanacearum.*

Related publication:

Prasath D, R Karthika, NT Habeeba, EJ Suraby, OB Rosana, A Shaji, SJ Eapen, U Deshpande and M Anandaraj (2014) Comparison of the Transcriptomes of Ginger (*Zingiber officinale* Rosc.) and Mango Ginger (*Curcuma amada* Roxb.) in Response to the Bacterial Wilt Infection. *PLoS ONE* 9(6): e99731. doi:10.1371/journal.pone.0099731.

Inventors
Prasath, D., Karthika, R. and Anandaraj, M.
ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

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A serological method to detect virulent strains of *Ralstonia solanaceraum*

Objective:

To detect virulent strains of *Ralstonia solanacearum* present in soil and plant samples

Brief description:

A serological method such as DAC-ELISA was developed for detection of virulent strains of *R. solanacearum*. A simple strategy used for production of polyclonal antiserum for the detection of virulent strains of *R. solanacearum.* The whole bacterial cells of *R. solanacearum* fixed with glutaraldehyde and the purified extra cellular polysaccharides are used as antigens for immunization to produce a high titre polyclonal antiserum. The type of antigen used for immunization significantly influenced the specificity of polyclonal antibodies produced. The rabbit immunized using glutaraldehyde-killed bacterial cells (Ag1) produced polyclonal antibodies with higher specificity than that immunized using the extracellular polysaccharides (Ag2). Both the antiserum produced successfully detected original bacterial culture of different concentrations as well as natural infection of *R. solanacearum* in infected tomato, brinjal and chilli crops and infested soil at 1:4000 dilutions. The OD values in soil samples were less when compared to plant samples. Thus this is a semi-quantitative method and is proportional to the pathogen concentration. The ELISA developed here has shown to be very sensitive, detecting as few as 10^1 cells/ml of *R. solanacearum* with a titre of 1:4000 dilutions.

Application:

The serological method can be used to detect virulent strains of *R. solanacearum* present in soil as well as in plant samples and is sensitive in detecting the bacterium as few as 10^1 cells/mL with a titre of 1:4000 dilutions.

Inventors
Krishna Reddy, M., Gopalakrishnan, C. and Manasa
ICAR-Indian Institute of Horticultural Research, Bengaluru, Karnataka - 560089

Total RNA isolation protocol from the leaf and rhizome tissues of ginger and mango ginger

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Objective:
To standardize total RNA isolation technique from the leaf and rhizome tissues of ginger and mango ginger.

Brief description:
The protocol by Deepa *et al.* (2014) for RNA isolation from *C. longa* has been slightly modified to isolate RNA from the rhizome tissues of ginger and mango ginger.

To 100 mg of sample tissues added 2 mL of pre-warmed extraction buffer (100 mMTris-HCl, 25 mM EDTA (pH-8), 2 % SDS, 1 % β-mercaptoethanol and 2 % PVP) and ground using mortar and pestle, and transferred to a sterile epperdorf tube. The homogenate was centrifuged at 13,000 g for 15 min at 4°C and the supernatant was transferred to a fresh tube. To the supernatant added an equal volume of acid phenol: chloroform (24:1), mixed gently but thoroughly. The tubes were then centrifuged at 13,000 g for 10 min at 4 °C. To 700 µL of the supernatant added 0.3 volume of 5 M sodium acetate (pH 4) and 0.7 volume of acid phenol: chloroform (24:1) and mixed gently. The tubes were centrifuged at 13,000 g for 10 min at 4 °C. To 700 µL of the supernatant added 0.1 volume of 3 M sodium acetate (pH 4) and an equal volume of ice cold isopropanol and mixed gently. The mixture was transferred to an RNA spin column and centrifuged at 10,000 g for 1 min at 4 °C and the flow through was discarded. To the column added 350 µL of RW1 wash buffer (Qiagen, USA). The column was then centrifuged at 10,000 g for 1 min at 4°C. The flow through was discarded. To the column added 80 µL of DNase buffer mix (Invitrogen, USA) and incubated for 30 min at 37 °C. After incubation the column was washed with 300 µL of RW1 wash buffer at 10,000 g for 1 min at 4 °C and flow through was discarded. The column was then washed with 500 µL of RPE wash buffer (Qiagen, USA) at 10,000 rpm for 1 min at 4 °C. The step was repeated again. The column was given an empty spin at 8000 g for 2 min at 4 °C. The column was then transferred to clean eppendorf tube and eluted with 30 µL of DEPC treated water and stored at -20 °C.

Application
The protocol yielded the highest amount of RNA that could be used for the comparative analysis of the candidate genes of mango ginger and ginger elicited in response to the bacterial wilt infection.

Inventors
Prasath, D., Karthika, R. and Anandaraj, M.
ICAR-Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

Related publication:

Karthika R, Prasath D, Anandaraj M. 2014. Investigation of the pattern of gene expression in ginger (*Z. officinale* Rosc) and mango ginger (*C. amada* Roxb) following inoculation with *Ralstonia solanacearum*. In abstracts: International symposium on plantation crops (PLACROSYM XXI) ISBN: 978-81-86872-48-2. p.222, December 10-12, Kozhikode, Kerala.

Protocol to develop RNAi construct for developing resistance against potato bacterial wilt

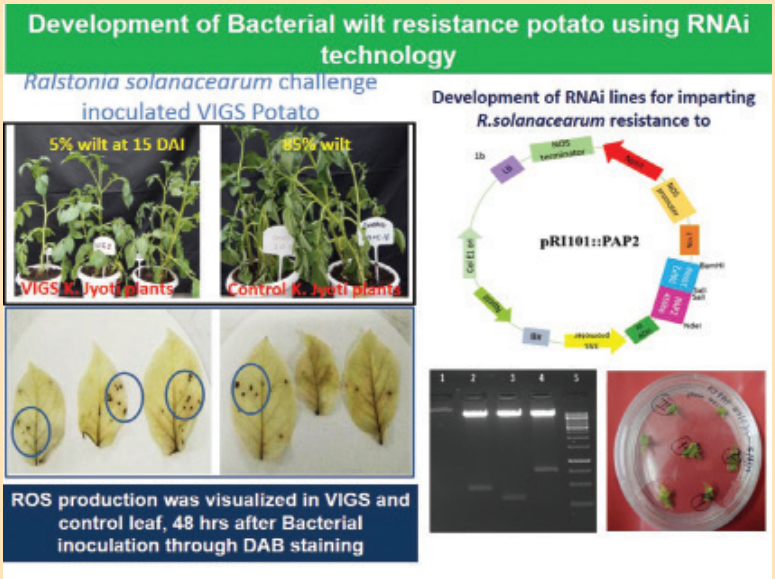
43

Objective:

To develop bacterial wilt resistant potato varieties

Brief description:

Since none of the potato genotypes including wild species has resistance genes, the identification and discovery of resistance gene/s for successful breeding programme is a challenging task. This necessitated development of a strategy to understand the candidate genes required for susceptibility (plant disease susceptibility factors). Virus-induced gene silencing (VIGS) is a powerful tool for analyzing gene function and this technology was used to screen the genes related to disease susceptibility using potato variety Kufri Jyoti. The *R. solanacearum* responsive genes *i.e. PAP2* gene were randomly cloned into the pTRV2 vector and transformed into *Agrobacterium tumefaciens* and then inoculated into Kufri Jyoti to create VIGS plants. Screened VIGS plants that barely showed wilting symptoms after inoculation with the pathogen *R. solanacearum*. Resistant plants showed



Inventors

Sundaresha, S., Vinay Sagar, Singh, B. P., Sanjeev Sharma and Shubhangi Sharma

ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh - 171001

accelerated cell death and rapid accumulation of reactive oxygen species (ROS). To authenticate the silencing and resistant nature of the VIGS plants, qRT-PCR was performed that showed reduced expression of PAPA2 and increased expression of *FGS2* (*Flagellin Sensing receptor gene*) to achieve effective resistance against *R. solanacearum.* A RNAi construct was developed for developing resistant varieties in potato against bacterial wilt.

Application:

Development of bacterial wilt resistant varieties of potato.

A protocol for rapid regeneration from apical meristems of taro [*Colocasia esculenta*]

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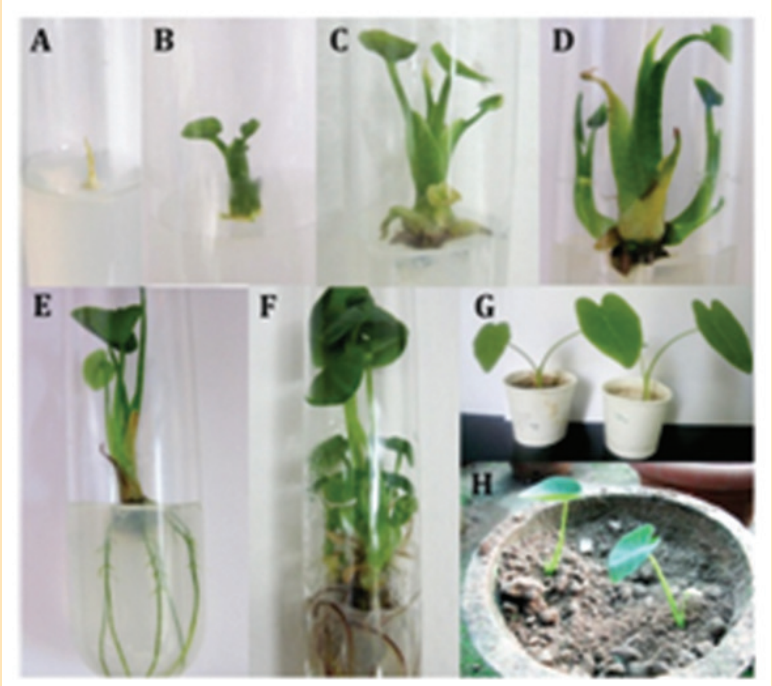
Objective:

To mass multiply disease free taro plants

Brief description:

Taro [*Colocasia esculenta (L.) Schott.*] is a traditional root crop of the tropics grown for its edible corms and leaves. *In vitro* techniques offer an alternative, reliable and rapid method for the production of high quality, disease-free planting material. Rapid multiplication of many high yielding cultivars of taro and cv. Muktakeshi is a leaf blight-resistant variety on a commercial scale is essential to supply disease free, good quality planting material to farmers.

Apical meristems (~1.0 cm) were excised from leaf blight resistant taro cultivar cv. Muktakeshi. Multiple shoots were initiated on Murashige and Skoog (MS) medium supplemented with 5.0 mg L^{-1} 6-benzyladenine and 1.0 mg L^{-1} α-naphthalene acetic acid. Four week old shoots were rooted by transferring to MS basal



Inventors

Nath, V. S., Sankar, M. S., Hegde, V. M., Jeeva, M. L., Misra, R. S. and Veena, S.S.

ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala - 695017

medium without plant growth regulators. After two weeks, well developed plantlets were hardened in plastic cups in potting mixture (vermiculite+sand, 1:1, v/v). Acclimatized plants were transferred to 30 cm plastic pots containing top soil and vermi-compost (3:1, v/v) where they grew well. The protocol developed for the initiation of multiple shoots and roots from apical meristem explants of taro cv. Muktakeshi is very simple and highly efficient. The protocol can be applied for commercial mass production and distribution good quality planting material of taro cultivars to farmers.

Application:

Commercial mass production and distribution of high yielding cultivars of taro and leaf blight resistant taro cultivar Muktakeshi to farmers.

Related publication:

Nath, V. S., Sankar, M. S., Hegde, V. M., Jeeva, M. L., Misra, R. S., Veena, S. S. (2012). A simple and efficient protocol for rapid regeneration and propagation of taro (*Colocasia esculenta* (L.) Schott.) from apical meristems. *International Journal of Plant Developmental Biology* 6(1): 64-66.

Protocol for isolation of apoplastic microorganisms from ginger

45

Objective:

To isolate apoplastic microorganisms from ginger

Brief description:

Fresh and healthy ginger plants were washed thoroughly with tap water for apoplastic fluid extraction. The leaves and pseudostems were cut individually into bits of 7-10 cm length. Surface sterilized the cut pieces by immersing in 70% ethanol in a sterile polyethylene bottle for 10 minutes and transferred to 5% H_2O_2 for 5 min. Then washed four times in sterile distilled water. Extracted the apoplastic fluid from surface sterilized leaf and pseudostems by vacuum infiltration and centrifugation. For this, 5-8 leaf / pseudostem bits were immersed in sterile distilled water in a glass beaker and place in vacuum desiccators. Applied vacuum at 40 kPa pressure for 15 minutes for leaves and 30 minutes for pseudostems. Released and re-applied vacuum at 5 minutes intervals. After vacuum infiltration, transferred the leaves and pseudostem bits into sterile filter paper and dried inside a laminar air flow cabinet. After drying, rolled vertically 3-4 leaves and placed inside a centrifuge tube with conical bottom in such a way that the petiole was facing towards the bottom of the tube. Carried out centrifugation at 3000 g for 10 minutes at 4°C. Do the vacuum infiltration and centrifugation for pseudostem also as described above. After centrifugation, removed the leaves/pseudostems from the tubes and took the remaining fluid in the bottom as the apoplastic fluid. Plated the extracted apoplastic fluid either directly (depending on the quantity extracted) or serially diluted up to 10^{-6} and plated 0.1 ml of this on selective media *viz*. Tryptic soy agar (TSA) for bacteria, Rose Bengal agar (RBA) for fungi and Actinomycetes Isolation Agar (AIA) for actinomycetes. Incubated TSA and RBA plates at 28°C for five days at day- night intervals and AIA plates at 28°C for 15 to 20 days. Selected bacteria based on their morphological difference, purified and maintained in 40% glycerol at -80°C.

Application:

The protocol can be used to isolate apoplastic microorganisms from ginger

Inventors

Suseela Bhai R., Prameela T. P., Kumar A., and Anandaraj M.

ICAR- Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

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Protocol for isolation of endophytic fungi from black pepper (*Piper nigrum* L.)

Objective:

To isolate endophytic fungi from black pepper from black pepper

Brief description:

Collected leaf, root and stem samples of black pepper were washed individually in running tap water and cut into sections under sterile conditions. Sterilized the sections using 0.5% sodium hypochlorite for 2 min, and then with 70% ethanol for 2 min and rinsed in sterile distilled water followed by drying on sterile filter paper. Discarded the exposed surface of each tissue and place the remaining sterile tissues individually in malt extract agar medium (MEA) containing antibiotic solution. Prepared the antibiotic solution as follows: stock - 0.02 g of tetracycline, penicillin and streptomycin in 10 mL sterile distilled water and filter sterilize; from this add 1 mL to one litre of media. Incubated the plates for 30 days at room temperature under dark. Surface sterilization, was ensured by sterilizing every tissue segments by pressing on to the surface of MEA medium. The final wash solution on MEA was pour plated and incubated for 30 days at room temperature under dark with periodic observations at every 24 h. Took the tissue samples with no growth in sterility check plates and sub-cultured them on to PDA and store at 15°C. Black pepper seeds both young and ripened were used for endophytic fungal isolation. Washed the seeds thoroughly in running tap water. Cut the washed and dried seeds into pieces, removed carefully the pericarp from the endocarp with sterile scalpel and surface sterilized by dipping in 0.5% sodium hypochlorite for 3 min, and then treated with 70% ethanol for 2 min and rinsed in sterile distilled water thrice, dried on sterile filter paper and incubated on MEA medium. Observed the plates periodically for fungal growth, purified the isolates and stored at 15°C.

Application:

The protocol can be used to isolate endophytic fungi from black pepper

Related publication:

Sreeja, K., Anandaraj, M. and Suseela Bhai, R. (2016) *In vitro* evaluation of fungal endophytes of black pepper against *Phytophthora capsici* and *Radopholus similis*. *Journal of Spices and Aromatic Crops* 25(2): 113-122

Inventors
Anandaraj M., Sreeja K., and Suseela Bhai R.,
ICAR- Indian Institute of Spices Research, Marikunnu PO, Kozhikode, Kerala - 673012

Detection of polyketides producing *Bacillus* spp. by multiplex polymerase chain reaction (PCR)

47

Objectives:

Screening and detection of polyketides antibiotic genes producing *Bacillus* spp. from genomic DNA and soil

Brief description:

Polyketides are natural metabolites that comprise the basic chemical structure of various antibiotics, parasiticides, immuno modulators, anticancer and antifungal agents. A multiplex polymerase chain reaction (PCR) protocol was developed to detect polyketides producing *Bacillus* spp. A PCR protocol was used to amplify from obtained template in a reaction mixture of 5x taq buffers, 1.5 U Taq polymerase (Promega), 10 mM dNTPs, 25 mM $MgCl_2$, 1 µl of each forward and reverse primers (10 µM), 1 µL of DNA (100 ng) and sterile H_2O in final volume of 25 µL. The thermo cycling conditions were initial denaturation 95^{O}C for 5 min, followed by 32 amplification cycles of 95^{O}C for 30 s, 52^{O}C for 0.45 s and 72^{O}C for 1 min and final extension 72^{O}C for 8 min using a BIO-RAD C1000 thermo cycler. The PCR products were resolved by using a 1.5% agarose gel stained with ethidium bromide and photographed using the gel documentation system. To determine the detection threshold of multiplex PCR primer sets of polyketides genes were done by diluting upto 10^{-3} of genomic DNA (100 ng) of *B. amyloliquefaciens* ,1.0 µL of aliquots were used as DNA template. Specificity of these primer sets was tested by using DNA template of *B. subtilis, B. licheniformis, B. pumulis, B. cereus, Pseudomonas fluorescens, Ralstonia solanacearum,* and *Xanthomonas campestris* pv. *campestris* along with *Bacillus amyloliquefaciens* DSBA-11.

Application:

Detection of polyketides antibiotic genes producing *Bacillus* spp. direct from soil samples.

Inventors
Dinesh Singh, Dhananjay Kumar Yadav and Garima Chaudhary
ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala - 695017

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Real-time PCR protocol to detect and quantify *Radopholus similis* in soil

Objective:

To detect and quantify of *Radopholus similis* infestation in soil

Brief description:

A real-time PCR-based method was developed for the detection and quantification of *Radopholus similis* in soil. Primers for the assay were designed from ITS region of *R. similis*. In order to quantify *R. similis* in soil, a standard curve was constructed using 1:2 dilution of genomic DNA of *R. similis*. Using this standard curve, the population was quantified in each sample containing fixed number of nematodes. The assay was further used for detecting the presence of *R. similis* in soil using soil DNA isolated from *R. similis* infected (positive) and sterile (negative) soil samples and only the positive sample showed amplification.

Application:

Highly sensitive protocol to detect and quantify *R. similis* in black pepper soil.

Inventors
Santhosh J. Eapen, Krishna, P. B., Bhat, A. I. and Rosana, O. B.
ICAR- Indian Institute of Spices Research, Marikunnu PO,Kozhikode, Kerala - 673012.

Application of DArT markers for generating high density genetic linkage map in rubber (*Hevea brasiliensis*)

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Objectives:

To develop high density genetic linkage map in rubber using SNP and silico DArT markers.

Quantitative trait loci (QTL) mapping for disease resistance against abnormal leaf fall disease in rubber caused by *Phytophthora* spp.

Brief description:

An interspecific cross between *Hevea brasiliensis* clone RRII 105 (high yielder with low level of resistance to *Phytophthora* spp.) and *H. benthamiana* (low yielder with high level of tolerance to *Phytophthora* spp.) was made and a progeny population of 86 individuals were used for the study. Around 24004 markers obtained from DArT sequencing were used for construction of genetic linkage map using DArT PL's OCD MAPPING program. Linkage maps were produced for each parent and consensus map between the two parental maps established using common bridge markers. Phenotyping for disease resistance was carried out using zoospore suspension from a virulent isolate of *Phytophthora* through *in vitro* challenge inoculation of the progeny population. *In vitro* leaf disc assay quantified disease reaction and categorized individuals based on size of lesion produced. The genotypic and phenotypic data for disease resistance were merged together to identify marker trait relationship for identification of QTLs.

Applications:

High density genetic linkage map construction using both dominant (silico DArT) and codominant (SNP) markers. Genome wide association mapping for agronomic traits of interest and QTL analysis using genetic map.

Related publications:

Bindu Roy, C., Bikku, B., Anuja, S., Jayasree, M. and Saha, T. (2016). Transgressive segregation for resistance to *Phytophthora* pathogen in an interspecific hybrid population of rubber (*Hevea brasiliensis*). In: 6th International Conference on "Plant, Pathogens and People" organized by Indian Phytopathological Society, IARI, New Delhi.

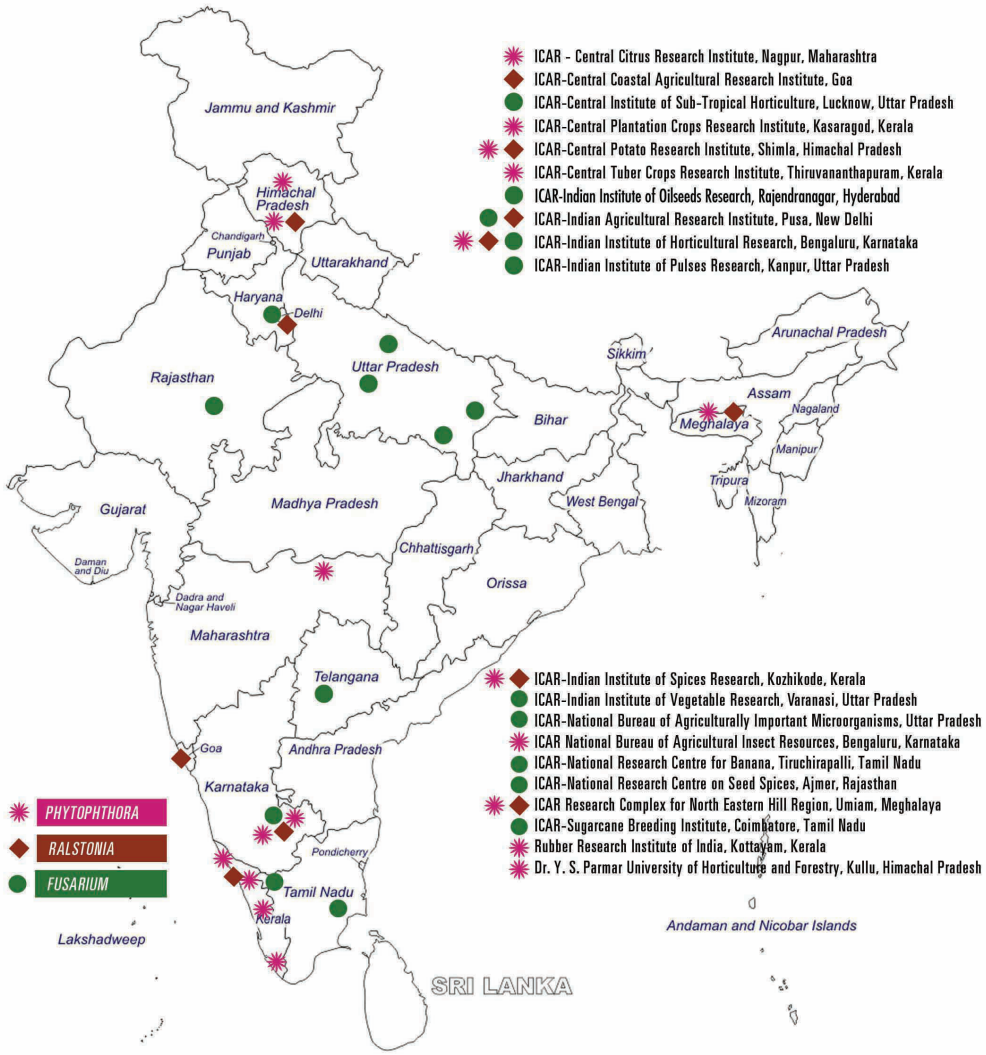
Bindu Roy, C., Bikku, B., Andrzej Kilian, Jayasree, M. and Saha, T. (2016). Construction of a high density genetic linkage map using SNP and silico DArT markers and QTL mapping for disease resistance in rubber tree (*Hevea brasiliensis*). In: 22nd Biennial Symposium on Plantation Crops (PLACROSYM 22), ICAR- CPCRI, Kasaragod, 15-17 December, 2016.

Inventors

Bindu Roy, C., Saha, T. and Andrzej Kilian

Rubber Research Institute of India, Kottayam, Kerala - 686009

LIST OF PHYTOFURA CENTRES



PhytoFuRa Nodal Centre

ICAR-Indian Institute of Spices Research
Post bag No. 1701, Marikunnu P. O.,
Kozhikode - 673 012, Kerala, India.
Phone: 0495-2731794/2731410,
Fax: 0495-2731794,
e-mail: mail@spices.res.in,
Web site: www.spices.res.in