

RESEARCH ARTICLE

**COLLETOTRICHUM FALCATUM CAUSING RED ROT IN SUGARCANE:
GENOMIC AND PROTEOMIC APPROACHES TO CHARACTERIZE THE
PATHOGENIC VARIATION****P. Malathi, K. Kaverinathan, M. Scindiya, E. Elamathi, A. Ramesh Sundar
and R. Viswanathan*****Abstract**

Red rot caused by *Colletotrichum falcatum*, is the major constraint in sugarcane production in India and varietal resistance plays an important role in managing the disease. However, breakdown of resistance in sugarcane varieties to new pathogen variants which co-evolve concurrently is a serious concern and warrants a clear understanding on pathogenic variation in response to virulence. Hence, a detailed study has been carried out through genomic and proteomic approaches to characterize representative group of *C. falcatum* pathotypes and isolates varying phylogenetically based on 5.8S-ITS sequence analysis. At genomic level the isolates were characterized using conserved gene sequences viz., actin, calmodulin and GPDH and molecular markers viz., RAPD and ISSR. Molecular analyses with conserved gene sequences highly implicated the existence of one major group of virulent isolates and a minor group of least virulent isolates which was confirmed by definite nucleotide variation. Further studies with molecular markers viz., RAPD and ISSR also confirmed separate grouping of least virulent isolates and different groups among the virulent isolates. Among the markers, ISSR was more efficient in grouping the isolates based on virulence, since it clearly differentiated the least virulent isolates like conserved sequences and some of its primers were able to differentiate the virulent isolates with specific markers. For proteomic analysis, a protocol has been standardized to develop *C. falcatum* proteome profiles of phylogenetically differentiated virulent and least virulent isolates and demonstrated unique and differential expression of spots related to pathogen virulence. Some of the unique proteins viz., serine protease, DJ-1/PfpI family protein and glutathione S-transferase identified from *C. falcatum* were closer to well characterized pathogenicity related genes in *Colletotrichum* spp and other fungi. Outcome of the study formed a basis for characterizing virulence related genes in *C. falcatum*.

Keywords: *Colletotrichum falcatum*, conserved genes, molecular markers, proteome profile

Introduction

Sugarcane is an important field crop grown in almost all the states in India and seed transmissible red rot caused by the fungal pathogen *Colletotrichum falcatum* Went (Teleomorph: *Glomerella tucumanensis* (Speg.) Arx and Muller) is one of the most destructive diseases of sugarcane in the country. Severe red rot epidemics occurred in different parts of the country during the last century led to the

elimination of several elite sugarcane varieties and the emerging new variants of the pathogen were responsible for the breakdown of released resistant varieties (Viswanathan 2010). Major pathotypes used in screening programme were distinguished at morphological, cultural, pathogenicity, serological, genetic and molecular level to certain extent (Malathi et al. 2010; Mohanraj et al. 2002; Suman et al. 2005; Viswanathan et al. 2003). Further, our phylogenetic study with 75 *C. falcatum* isolates based on 5.8S-ITS revealed that

P. Malathi, K. Kaverinathan, M. Scindiya, E. Elamathi, A. Ramesh Sundar and R. Viswanathan*

Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore – 641 007, Tamil Nadu, India

*Corresponding author: rasaviswanathan@yahoo.co.in

Submitted: 26 February 2020; Accepted: 29 March 2020

there were two major groups comprising of 95% isolates with virulent nature and one minor group with 5% isolates of least virulent nature (Malathi et al. 2011). Gene sequences for conserved proteins are used extensively in phylogenetic studies along with ITS data to reveal the genetic variation in understanding and to explore evolutionary relationship in the fungal taxonomy (Guerber et al., 2003; Hirata et al. 2007; Whitelaw-Weckert 2007).

Apart from ITS gene sequences, molecular markers could be used to characterize the isolates for phylogenetic grouping. There are different kinds of markers viz., RFLP, AFLP, RAPD, SSR, ISSR etc. and each one has its advantages and disadvantages. Since random amplified polymorphic DNA (RAPD) has been used in earlier studies for characterizing *C. falcatum* isolates, RAPD was compared with recent widely used inter simple sequence repeats (ISSR) markers.

Moreover it is understood from the earlier studies that the *C. falcatum* isolates vary in the production of various pathogenicity determinants viz., enzymes, toxin and melanin (Malathi and Viswanathan, 2012; Kaverinathan et al. 2017) and responsible for expression of various pathogenicity related genes during its pathogenesis (Scindiya et al. 2017). Hence, further research is needed to characterize the genes/ proteins related to pathogenicity factors of *C. falcatum*. In recent years, 2-dimensional gel electrophoresis (2-DE) has been widely used to study the proteome of phytopathogenic fungi and this approach is useful in obtaining the proteomic maps of disease causing fungi which will be highly useful to compare and identify unique and differentially expressed proteins between isolates varying in virulence. Results from fungal proteomic studies showed that it is a potential tool for identifying pathogenicity factors, therapeutic targets and for basic research (Fernandez-Acero et al., 2007). Reports indicated that 2-DE

has been effectively utilized for comparative analysis of mycelial protein and secretomes in basidiomycete fungi (González-Fernández et al. 2014; Alfaro et al. 2014).

With this background, the present study on genomic approach was carried out to appraise the feasibility of utilizing nucleotide sequences of conserved genes viz., actin, calmodulin and glyceraldehyde 3-phosphate dehydrogenase (GPDH) along with 5.8S-ITS to confirm their molecular grouping in a representative group of *C. falcatum* isolates. Besides, molecular markers viz., RAPD and ISSR were used to confirm the existence of least virulent isolates in the group and possibility of identifying suitable marker linked to any specific trait related to virulence, origin etc. Furthermore, phylogenetically differentiated virulent and least virulent isolates were selected and utilized in proteomic approach for identification of proteins related to *C. falcatum* pathogenicity. In this context, preliminary attempt has been made to standardize the protocol to develop proteome profiles of genetically distinct isolates to characterize unique/ differentially expressed proteins in relation to pathogen virulence.

Materials and Methods

C. falcatum isolates

Sixteen *C. falcatum* isolates representing three groups of ITS based phylogenetic analysis (Malathi et al. 2011) were selected for validation with conserved gene sequences and molecular markers, finally selected two extremely varied isolates in response to virulence and studied differential expression of proteins. For this purpose, 10 designated pathotypes viz., CF01- Cf1148(H1), CF02 - Cf7717(H2), CF03 - Cf64(H3), CF04 - Cf419(AP1), CF05 - Cf997(AP2), CF06 - Cf671(TN1), CF07- Cf64(H5), CF08 - Cf64(H6), CF09 - Cf767(H7) and CF10 - Cf89V74(AP11) selected under differential interaction studies in

both tropical and sub-tropical conditions were employed. Besides some of the isolates viz., Cf92061(TN3), Cf90063(TN5) and Cf64 always showing virulent reaction and some of the isolates viz., Cf94101, Cf86032 and Cf92020 belonging to least virulent group for a long period were also included for comparison.

PCR amplification and sequencing for conserved genes

Genomic DNA from fungal cultures were extracted based on CTAB method and subjected to PCR amplification with 5.8s rDNA-ITS region and other conserved genes viz., actin, calmodulin, and Glyceraldehyde 3-phosphate dehydrogenase (GPDH) gene sequences. The amplified products were purified and sequenced to study nucleotide variation among virulent and least virulent isolates. The primers used in the study were referred from Carbone and Kohn (1999) for actin and calmodulin, from Guerber et al. (2003) for GPDH and from Martinez-Culebras et al. (2003) for *Colletotrichum* specific ITS primers, col1 and col2.

Analysis with molecular markers

Random Amplification of Polymorphic DNA (RAPD): Eight decamer oligonucleotide primers were used for the random amplification of red rot DNA with 6 RAPD primers (OPA 01, 03, 09, 15, 16 and 18) referred from earlier reports (Suman et al., 2005). PCR amplification was performed in a final volume of 20 µl with 1µl of template DNA (25 ngµl⁻¹), 2 µl of 10X PCR buffer, 1.6 µl of dNTPs (2.5 mM of each of the dNTPs), 1µl of MgCl₂, 1µl of primer (10 µM), 1 unit of Taq DNA polymerase and 13.07 µl of Milli Q water. The DNA was amplified in a programmable thermal cycler with an initial denaturation step of 5 min at 94°C followed by 40 cycles of 1 min denaturation at 94°C, 2 min annealing (at 42°C for OPA03 and OPA15, 46°C for OPA01 and 48°C for

OPA09, OPA16 and OPA18), 2 min at 72°C for extension. A final extension step was at 72°C for 5 min to ensure complete extension of the amplified products.

Inter Simple Sequence Repeats (ISSR).

Eight ISSR primers (AF80820, AF80821, AF80822, AF80823, AF80824, AF80825, G5 and G6) previously reported for *Colletotrichum* (Ratanacherdchai et al., 2010) were used for the analysis of ISSR polymorphism among the red rot isolates. PCR amplification was performed in a final volume of 20 µl with 1 µl of template DNA (25 ng/µl), 2 µl of 10X PCR buffer, 1.6 µl of dNTPs (2.5 mM of each of the dNTPs), 1 µl of MgCl₂, 1 µl of primer (10 µM), 1 unit of Taq DNA polymerase and 13.07 µl of Milli Q water. PCR conditions were 2 min initial denaturation at 94°C followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing (at 49°C for primer AF80820, 51°C for AF80823, 55°C for AF8082 and AF80822, 57°C for AF80824 and 59°C for AF80825, G4 and G5) and 1.5 min at 72°C for extension. A final extension step was carried out at 72°C for 6 min.

Amplified PCR products were separated by electrophoresis on 1.5% agarose gel and the DNA bands were visualized under an ultraviolet transilluminator (AlphaImager, Alphainnotech, USA). Comparison of banding profile for each primer was carried out on the basis of presence (1) or absence (0) of the RAPD and ISSR products of the same size. Scoring has been done from the bands obtained from ISSR and RAPD analyses and phylogenetic tree has been constructed by using DARWIN 5-SINGLE software.

Standardization on development of proteome profile of *C. falcatum*

The representative isolates of virulent group (Cf671) and least virulent group (Cf94101) were used to standardize proteomic approach. For

protein extraction, the isolates were grown on complete medium broth (CMB) for 72 h and the developing young mycelia before melanization was vacuum dried and used for protein extraction. Different protein extraction methods viz., acetone precipitation, TCA precipitation and acetone-TCA methods were attempted for protein extraction from fungal mycelia. In all the protocols, the mycelia were pulverized to a fine powder in a mortar and pestle with liquid nitrogen to minimize proteolysis and other modes of protein degradation. The quantitative protein yield in fungal mycelia was assessed in all the three methods of protein extraction. Acetone precipitation and TCA precipitation methods individually yielded much lower quantity of proteins. TCA/acetone precipitation method of protein extraction helped to remove non-protein contaminants and yielded higher concentration. After extracting the protein, the protein present in the sample was quantified by Bradford's method at an absorbance of 280 nm (Bradford 1976).

The protein extracts using above three protocols were subjected to SDS-PAGE and finally selected TCA/Acetone precipitation method. The extracted protein was focussed on 18 cm (pH 4-7) IPG strip, electrophoresed on 12% SDS-PAGE and stained with CBB. This standardized protocol coincides with the procedure followed for the cane proteome profile (Amalraj et al., 2010) except the sample preparation. In this method, the mycelial proteins from both the isolates were precipitated by TCA-acetone (10% w/v) method containing 0.07% DTT and the obtained pellet was washed with acetone and lyophilized. Then the proteins were solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v IPG buffer pH 4-7 and 0.3% w/v DTT), quantified using BSA as standard

and the quality was checked in 12% SDS-PAGE. Then 315 µl of sample representing ~600 µg of protein was loaded in strip holder and passively rehydrated overnight onto 18 cm IPG strips, pI 4-7 (GE Healthcare). After rehydration, the strips were focused on EttanIPGphor II platform (GE Healthcare) until attaining a total of 45 kVh. The focused strips were reduced and alkylated by equilibrating twice (with 1% DDT and 2.5% iodoacetamide) for 20 min each in equilibration buffer containing 6M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 50 mM Tris-HCl buffer. Then the strips were subjected to 2nd dimension in 12% SDS-PAGE gels with Tris-glycine buffer in EttanDALTsix Electrophoresis Unit (GE Healthcare). After the run was completed the gels were treated with fixing solution (40% methanol, 10% acetic acid and 50 % distilled water) for 30 min and stained with colloidal CBB solution (Kang et al. 2002) (0.02% (w/v) CBB G-250, 5% (w/v) aluminium sulfate hydrate, 10% (v/v) ethanol (96%) and 2 % (v/v) orthophosphoric acid (85 %) overnight. The stained 2D gels were digitalized in Image Scanner III (GE Healthcare) incompatible image formats (300dpi, 16bit, .tiff and .mel), the images were imported, warped and matched in ImageMaster2D Platinum version 7 software (GE Healthcare). Differentially abundant protein spots with statistical significance and protein spots exclusively present only in virulent isolate Cf671 were selected, picked manually and sent for MS analysis. Gel pieces were Coomassie destained and trypsin (Promega) digested. The digested peptides were subjected to MALDI-TOF using an AB Sciex 5800 TOF/TOF System, MALDI TOF TOF (Framingham, MA, USA). The obtained charge-mass ratio was analysed using MATRIX SCIENCE – MASCOT search software to identify the respective protein.

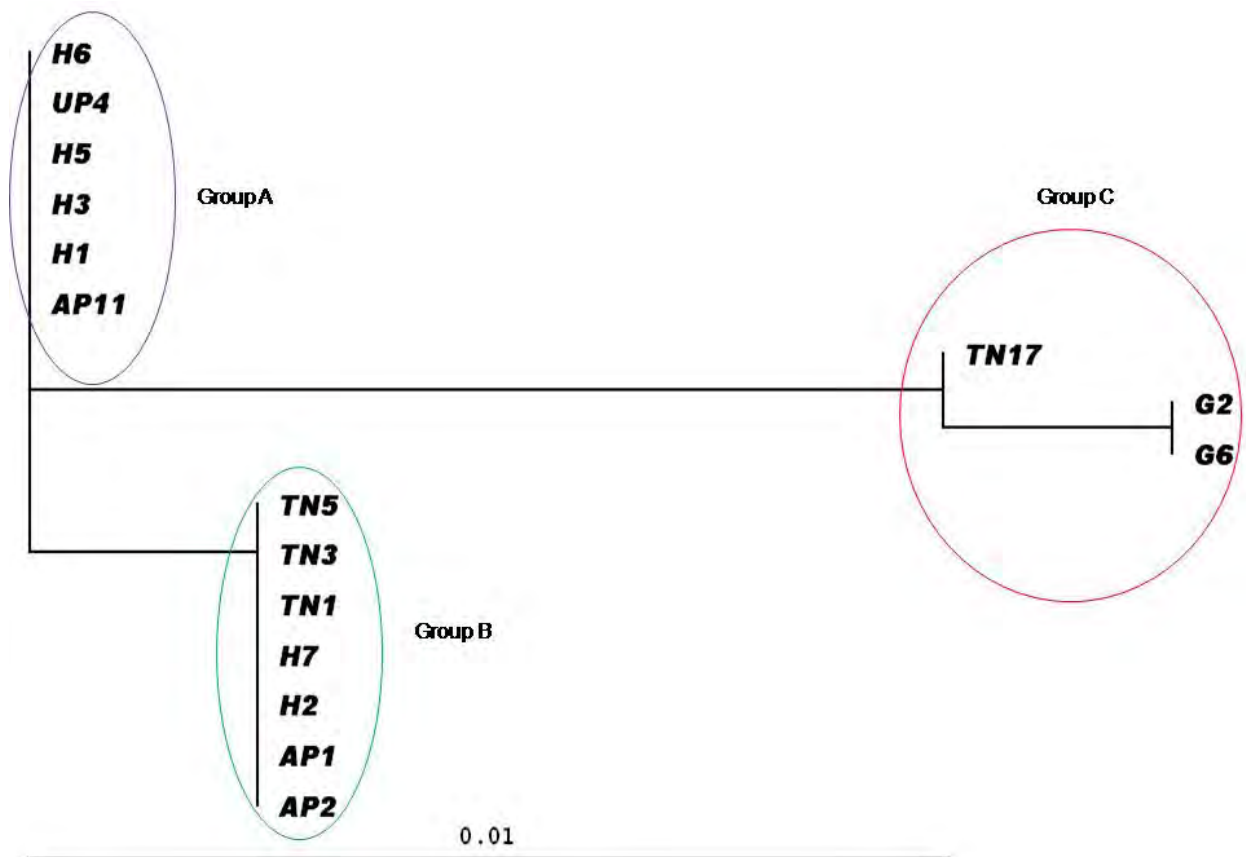


Fig. 1. Phylogenetic tree derived based on 5.8S ITS sequences of 16 *Colletotrichum falcatum* isolates

Results and Discussion

Molecular analysis based on conserved gene sequences

Phylogenetic analysis on 5.8S-ITS region of 16 *C. falcatum* isolates yielded three molecular groups (Fig. 1) as reported earlier (Malathi et al., 2011). Among the three groups, the first two groups were differentiated by 1bp and the third group was differentiated by 6bp as per our earlier report. Analysis on nucleotide sequence showed that there were 402 nucleotides including partial regions of ITS1 (127 bp) and ITS2 (96 bp) with complete sequence of 5.8S rDNA (179 bp) region. Though the primers could not yield complete ITS 1 & 2 regions, it was able to reveal all the variable regions of *C. falcatum* isolates. Previous

studies also strongly recommend the use of rDNA sequence information for inferring phylogenetics and measuring genealogical relationship in *Colletotrichum* and analysis of ITS1 region provided more information for phylogenetic analysis than the ITS2 region (Martinez-Culebras et al., 2003; Whitelaw-Weckert et al., 2007). This confirms the present result of more variable ITS1 region in *C. falcatum* than ITS2.

Individual phylogenetic analyses of other three genes viz., actin, calmodulin and GPDH highly implicated the existence of Group III as in ITS analysis with high boot strap support, while movement of gene sequences between Group I and II were different (Results not shown). PCR amplification of actin gene with ACT-512F and ACT-783R yielded 253 bp product of *C.*

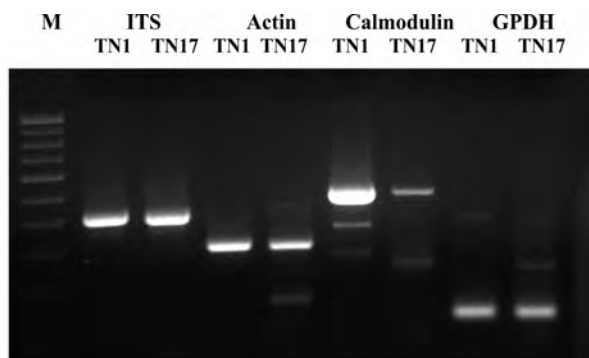


Fig. 2a. PCR amplification of 5.8S-ITS, Actin, Calmodulin and GPDH in *Colletotrichum falcatum* isolates varying in virulence

falcatum. Like ITS, the nucleotide difference was 1bp between Group I & II and Group III was distinguished by 5 bp and confirmed the least virulent isolates in Group C. However, movement of isolates between first two groups were different. Similarly, the calmodulin sequence of 485 bp also differed among *C. falcatum* isolates, wherein only 1bp differentiated the Group I from Group II and the Group III has 4 variable nucleotides from others. Further sequence analysis of GPDH intron of 152 bp showed only two groups by separating least virulent group with 1 bp difference from

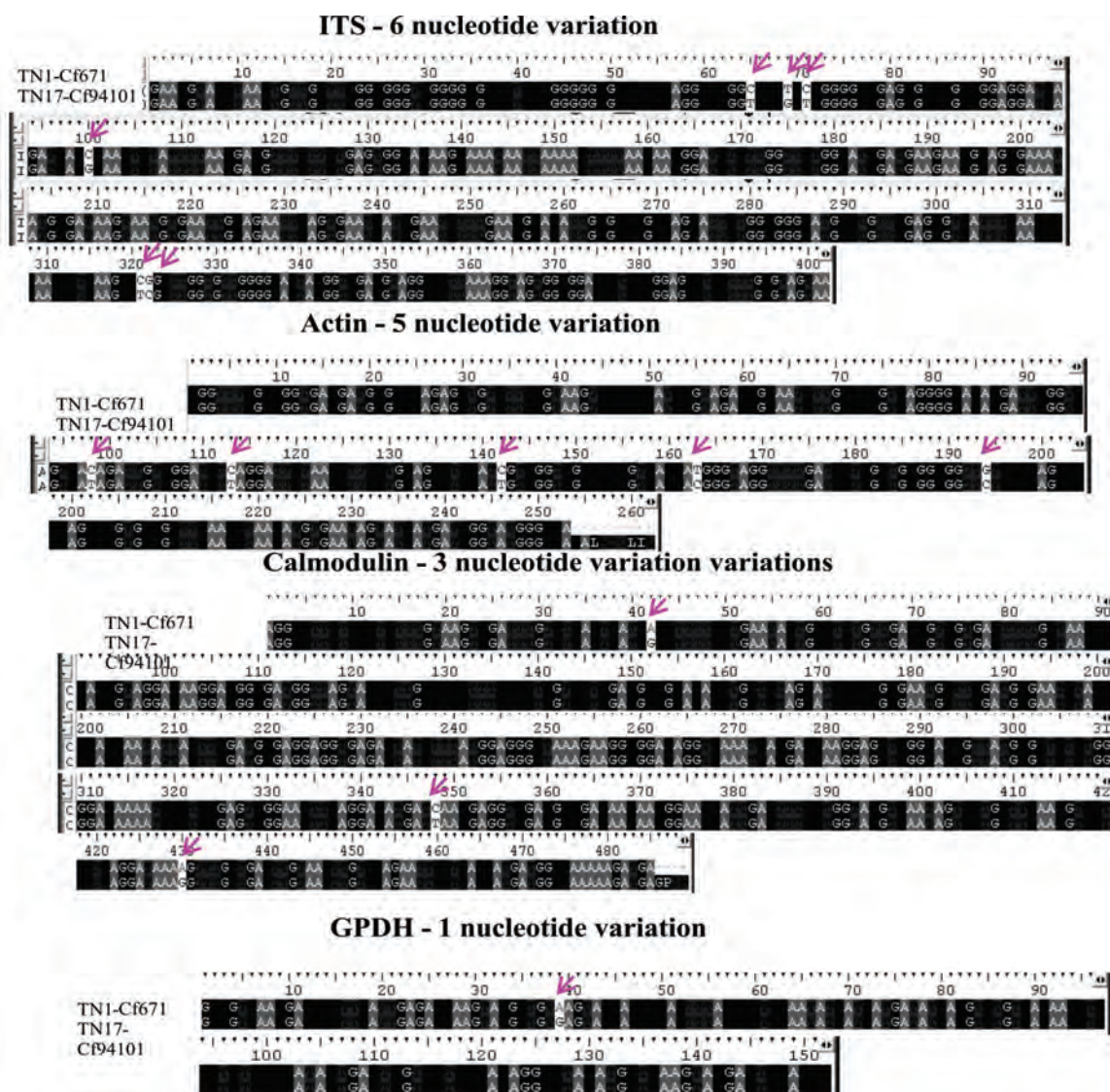


Fig. 2b. Variation in conserved gene sequences of highly virulent and least virulent isolates of *Colletotrichum falcatum*

virulent group. Based on all these results, the nucleotide sequences of four conserved genes of virulent pathotype TN1 and least virulent isolate TN17 were amplified with respective primers (Fig. 2a) and the sequence variation has been shown (Fig. 2b). Evidences were available on the use of GPDH gene sequences in phylogenetics of ascomycetous and basidiomycetous fungi (Kruezing et al. 1996) and at intra-species level for *C. acutatum* grouping (Guerber et al. 2003). Templeton et al. (1992) characterized this gene with coding and non-coding regions in *Glomerella cingulata*. The other two loci actin and calmodulin were exceedingly used in delineating speciation of *Magnaporthe* sp (Cough and Kohn 2002; Hirata et al. 2007). 5.8S-ITS and β -tubulin gene sequences have been widely used to study the phylogenetic relationships in correlation with pathogenicity and other groupings of various *Colletotrichum* spp. causing anthracnose in various crops (Whitelaw-Weckert 2007).

Molecular grouping based on genetic markers

All the 10 pathotypes and 6 isolates were subjected to RAPD and ISSR analysis and grouped them accordingly. Both the markers confirmed the existence of the minor group (Fig. 3a & 3b) and grouped other isolates and pathotypes into two groups with some contradictions among them.

However they confirmed the similarity of tropical isolates/ pathotypes particularly from Tamil Nadu (TN1, TN3 and TN5) and Andhra Pradesh (AP1 and AP2) in one group along with H2 and H7 from Haryana. While H1 was in another group along with most of the sub-tropical isolates and incompatibility of H1 and H2 was shown by keeping them in separate groups. It supports the results of Mohanraj et al. (2002) who concluded that subtropical pathotypes H2 and H1 were widely distinct while the tropical pathotype TN1 and the isolate TN3 exhibited closest genetic relationship among the isolates studied. This was also further confirmed by vegetative compatibility grouping and ITS gene based sequences (Malathi et al. 2010). Suman et al. (2005) examined for genetic relationships using RAPD markers among six *C. falcatum* pathotypes prevalent in the subtropical India and reported existence of two groups among sub-tropical pathotypes with H1 and H2 in two different clusters. Of the two analyses in the present study, ISSR primers clearly confirmed the existence of ITS based Group III isolates in a separate group and grouped the isolates/ pathotypes based on their origin and virulence nature to some extent (Fig. 4).

Results of our present study revealed that ISSR primers are highly reliable in understanding the

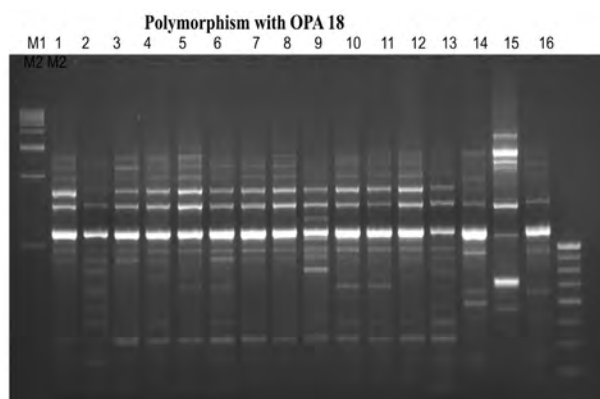


Fig. 3a. Profile of 16 *Colletotrichum falcatum* isolates generated by RAPD primer OPA 18

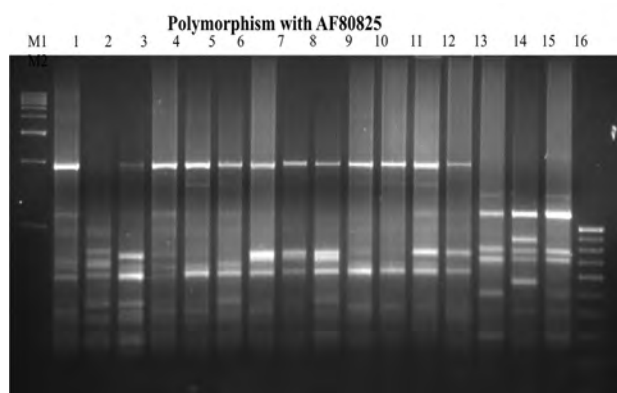


Fig. 3b. Profile of 16 *Colletotrichum falcatum* isolates generated by ISSR primer AF80825

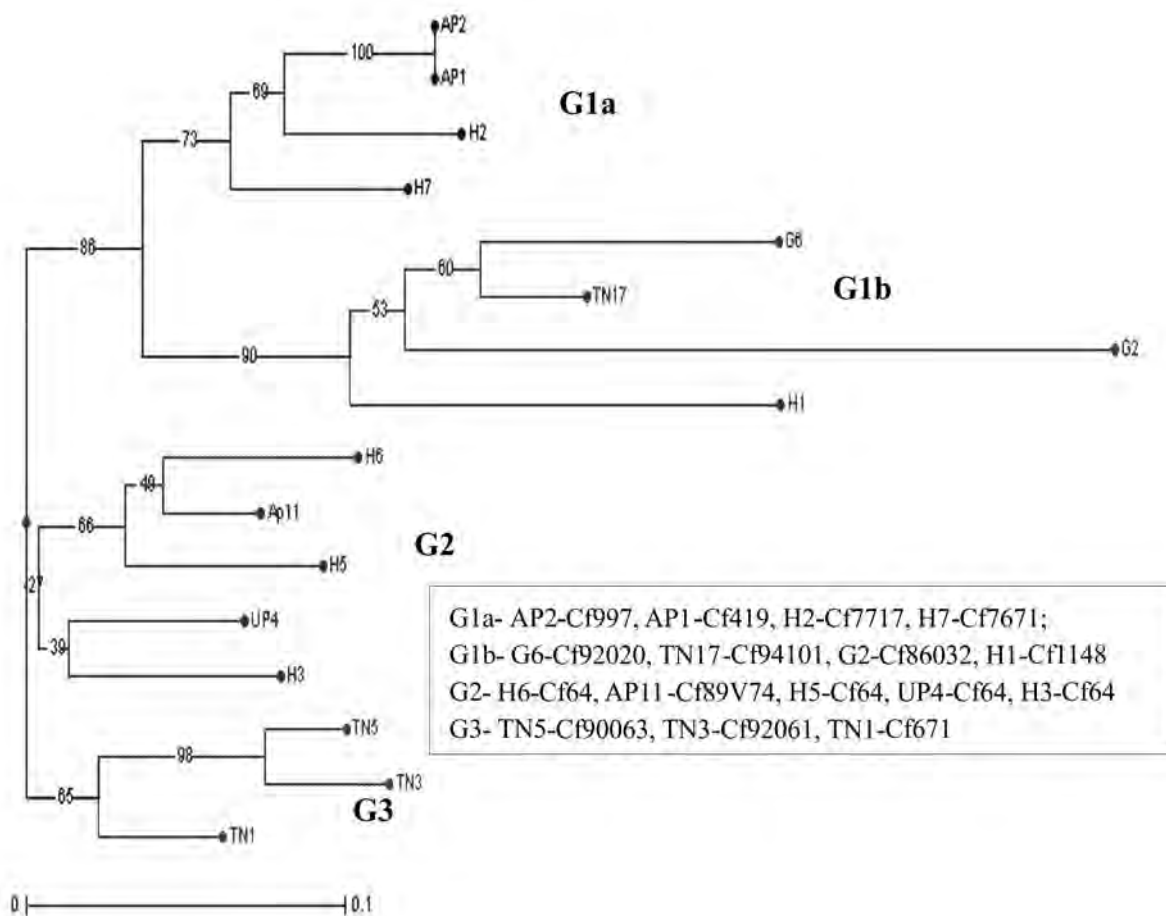


Fig. 4. Phylogenetic tree derived from 8 ISSR finger prints of 16 *Colletotrichum falcatum* isolates

evolutionary relationship and also the virulence pattern of *C. falcatum* isolates, although RAPD could confirm some of the results. Reports available indicated that ISSR markers were widely used to characterize the fungal populations and the potential of ISSR primers in understanding the phylogenetic relationship of fungi was shown by various workers. Studies of Ratanacherdchai et al. (2010) with ISSR analysis in chilli anthracnose pathogen indicated that there are two distinct groups of *C. gloeosporioides* and *C. capsici*. Furthermore, genetic diversity was correlated with geographic distribution, while no clear relationship between genetic diversity and pathogenic variability was established. Likewise, pathogenic and molecular variability have been studied with regional specific

isolates using molecular markers (RAPD, URP and ISSR) and ITS-RFLP analyses. However, they could not obtain any tangible results (Mishra and Behera 2009; Kumar et al. 2010).

Development of *C. falcatum* proteome profiles to differentiate *C. falcatum* isolates

In the present study, the 2-DE profiles showed a total of 449 spots in CF06 and 427 spots in Cf94101, which indicates a critical difference for number of spots between them (Fig. 5). The two profiles were analysed using Image Master 2D Platinum 7 software and 294 matching spots were identified. The remaining unmatched spots probably considered as unique spots for the respective strains. Randomly eight spots were analysed by MALDI-TOF and subsequent mass

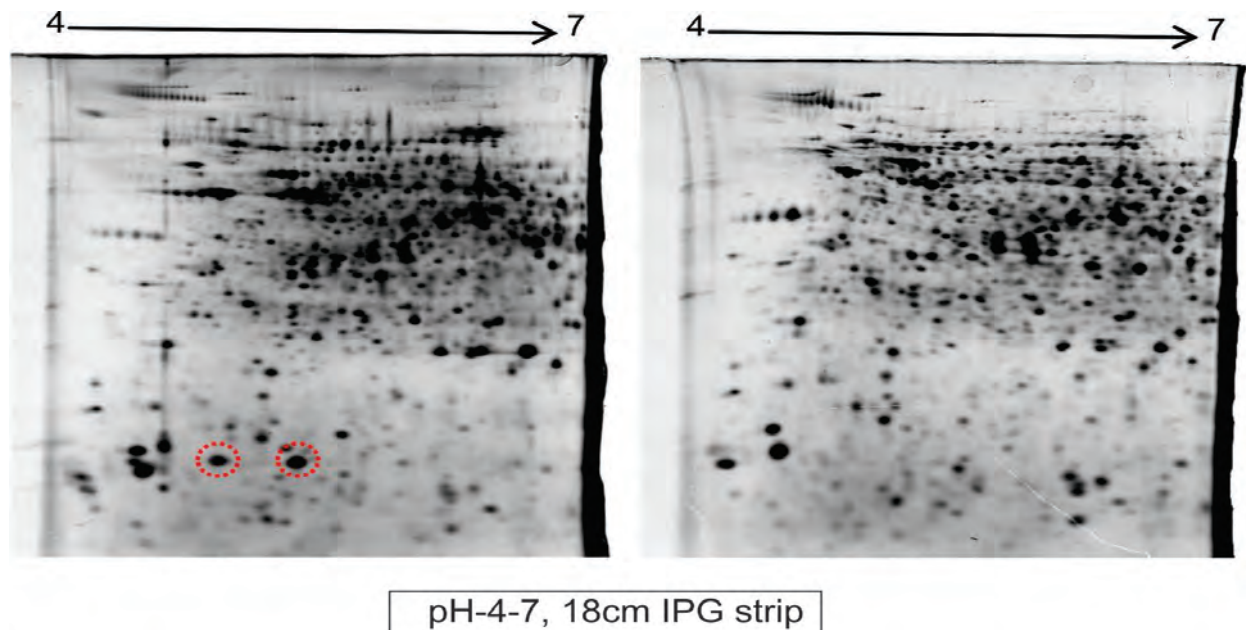


Fig. 5. Proteome profiles of virulent (left) and least virulent (right) isolates

data search in MASCOT identified the spots as galactokinase, serine protease, protease subunit alpha, phospho pyruvate hydratase, Bys1 family protein mRNA, hypothetical protein partial mRNA, DJ-1/PfpI family protein partial mRNA and glutathione S-transferase domain-containing protein partial mRNA. Among these proteins, serine protease (Muszewska et al. 2017), DJ-1/PfpI family protein (Chu et al. 2015;) and glutathione S-transferase (Calmes et al. 2015) act as virulent factors for fungal pathogens, while protease subunit alpha acts as ubiquitin dependent catabolic protein involved in lysis of host defense proteins (Pan et al. 2013) and Bys1 family protein (Ashwin et al. 2017) was reported to be involved in sporulation.

Proteome profiles developed in this study for two *C. falcatum* pathotype/ isolate clearly demonstrated the greater possibility of this approach for studying pathogen variability and further applicability of pathogenicity related genes identification. In *Botrytis cinerea*, the mycelial 2-DE protein profile revealed the existence of qualitative and quantitative differences between

the analyzed strains based on virulence and most of the identified proteins were malate dehydrogenase (MDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fernández-Acero et al. 2007). Likewise, many reports were available on proteins involved in pathogenicity process of various fungi. Recently, Gonzalez Fernandez et al. (2014) identified malate dehydrogenase or peptidyl-prolylcis-trans isomerase from the mycelium, and endopolygalacturonase, aspartic protease orcerato-platanin protein from the secretome and have been reported as virulence factors, which are involved in host-tissue invasion, pathogenicity or fungal development of the necrotrophic fungus *Botrytis cinerea*. Similarly, comparative secretome analysis of *C. falcatum* by a Ashwin et al. (2017) identified a pathogen-associated molecular pattern (PAMP) and its functional characterization showed a major cerato-platanin domain (EPL1 Δ N1-92) which is a defense inducing and hypersensitive response (HR) agent in sugarcane and tobacco, respectively.

Results from the study based on conserved gene sequences clearly indicated that the existing

pathotypes/ isolates of *C. falcatum* have least genetic variation and exhibits monophyletic origin except for confirming the least virulent isolates. However, selection of phylogenetically differentiated virulent and least virulent isolates for proteomic study will be highly helpful in identification and characterization of pathogenicity/ virulence related genes involved in *C. falcatum* pathogenesis. Hence, the pathogenic variation could be attributed to the adoption of existing isolates to the newer cultivars resulting in co-evolution of new variants of the pathogen, which has been proved in our earlier study with cross inoculation of incompatible pathotypes (Malathi et al. 2006). Recent study very clearly elaborated on evolution of a new pathotype CF12 of *C. falcatum* from the tropical region in India from the virulent pathotype CF06. The new pathotype has acquired further virulence to knockdown the recently introduced varieties for cultivation by the acquired virulence (Viswanathan 2017).

Conclusion

Results revealed that the ISSR based markers probably differentiate the isolates based on virulence and it needs further detailed study with large number of isolates using selected markers from this study. The present study forms the basis to study further on confirming new isolates using conserved gene sequences, identifying virulence related markers based on ISSR profiles from a large group of isolates and proteomic based analysis of phylogenetically differentiated isolates to characterize differentially expressed gene/ proteins in relation to virulence.

Acknowledgement

The authors are grateful to the Director, ICAR-Sugarcane Breeding Institute for providing facilities and constant encouragement.

References

- Alfaro M, Oguiza JA, Ramírez L, Pisabarro AG (2014) Comparative analysis of secretomes in basidiomycete fungi. *Journal of Proteomics* 102: 28-43.
- Amalraj RS, Selvaraj N, Veluswamy GK, Ramanujan RP, Muthurajan R, Palaniyandi M, Agrawal GK, Rakwal R, Viswanathan R (2010) Sugarcane proteomics: Establishment of a protein extraction method for 2-DE in stalk tissues and initiation of sugarcane proteome reference map. *Electrophoresis* 31: 1959–1974.
- Ashwin NMR, Barnabas L, Sundar AR, Malathi P, Viswanathan R, Masi A, Agrawal GK, Rakwal R (2017) Comparative secretome analysis of *Colletotrichum falcatum* identifies a cerato-platanin protein (EPL1) as a potential pathogen-associated molecular pattern (PAMP) inducing systemic resistance in sugarcane. *Journal of Proteomics* 169: 2-20.
- Bradford M (1976) A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.
- Calmes B, Morel-Rouhier M, Bataillé-Simoneau N, Gelhaye E, Guillemette T, Simoneau P (2015) Characterization of glutathione transferases involved in the pathogenicity of *Alternaria brassicicola*. *BMC Microbiology* 15(1): 123.
- Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91: 553-556.
- Chu J, Li WF, Cheng W, Lu M, Zhou KH, Zhu HQ, Li FG, Zhou CZ (2015) Comparative analyses of secreted

- proteins from the phytopathogenic fungus *Verticillium dahliae* in response to nitrogen starvation. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* 1854(5):437-448.
- Cough BC, Kohn LM (2002) A multilocus gene genology concordant with host preference indicates segregation of new species *Magnaporthe oryzae* from *M. grisea*. *Mycologia* 94(4): 683-693.
- Fernández-Acero FJ, Jorge I, Calvo E, Vallejo I, Carbú M, Camafeita E, Garrido C, López JA, Jorrin J, Cantoral JM (2007) Proteomic analysis of phytopathogenic fungus *Botrytis cinerea* as a potential tool for identifying pathogenicity factors, therapeutic targets and for basic research. *Archives of Microbiology* 187(3): 207-15.
- González-Fernández RK, Aloria RK, Valero-Galván J, Redondo I, Arizmendi JM, Jorrin-Novo JV (2014) Proteomic analysis of mycelium and secretome of different *Botrytis cinerea* wild-type strains. *Journal of Proteomics* 97:195-221.
- Guerber JC, Liu B, Correll C, Johnston PR (2003) Characterization of diversity in *Colletotrichum acutumsensulato* by sequence analysis of two introns, mtDNA and intron RFLPs and mating compatibility. *Mycologia* 3: 872-895.
- Hirata K, Kusaba M, Chusm I, Osu J, Nakayashiki H, Mayama S, Tosa Y (2007) Speciation in *Pyricularia* inferred from multilocus phylogenetic analysis. *Mycological Research* 111: 799-808.
- Kang D, Gho YS, Suh M, Kang C (2002) Highly sensitive and fast protein detection with coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Bulletin of Korean Chemical Society* 23(11): 1511.
- Kaverinathan K, Scindiya M, Malathi P, Viswanathan R, Sundar AR (2017) Role of melanin in *Colletotrichum falcatum* pathogenesis causing sugarcane red rot. *Sugar Tech* 19(6): 584-591.
- Kreuzinger N, Podeu R, Gruber F, Gobl Kubicek CP (1996) Identification of some ectomycorrhizal basidiomycetes by PCR amplification of their *gpd* (Glyceraldehyde-3-Phosphate Dehydrogenase) genes. *Applied and Environmental Microbiology* 62: 3432-3438.
- Kumar N, Jhang T, Sharma TR (2010) Molecular and pathological characterization of *Colletotrichum falcatum* infecting subtropical Indian sugarcane. *Journal of Phytopathology* 159(4): 260-267.
- Malathi P, Viswanathan R, Jothi R (2006) Specific adaptation of *Colletotrichum falcatum* pathotypes to sugarcane cultivars. *Sugar Tech* 8(1): 54-58.
- Malathi P, Viswanathan R, Ramesh Sundar A, Prakasam N, Padmanaban P, Jothi R, Renuka Devi SR, Poongothai M (2010) Variability among *Colletotrichum falcatum* pathotypes used for screening red rot resistance in sugarcane. *Sugar Cane International* 28: 47-52.
- Malathi P, Viswanathan R, Ramesh Sundar A, Padmanaban P, Prakasam N, Jothi R (2011) Phylogenetic analysis of *Colletotrichum falcatum* isolates causing red rot in sugarcane. *Journal of Sugarcane Research* 1: 69-74.
- Malathi P, Viswanathan R (2012) Identification of pathogenicity determinants in *Colletotrichum falcatum* using wild and mutant cultures. *Sugar Tech* 14(4): 383-390.
- Martinez-Culebras PV, Querol A, Surez-Fernandez MB, Garcia-Lopez MD, Barrio E (2003)

- Phylogenetic relationships among *Colletotrichum* pathogens of strawberry and design of PCR primers for their identification. *Journal of Phytopathology* 151: 135-143.
- Mishra MK, Behera B (2009) Pathogenic and molecular variability of *Colletotrichum falcatum* Went. isolates from sugarcane with red rot disease symptoms. *Journal of Crop Science and Biotechnology* 12(1) : 31-35.
- Mohanraj D, Kumaresan S, Sreenivasan TV (2002) Molecular characterization of isolates of the sugarcane red rot pathogen *Colletotrichum falcatum* Went. *Indian Phytopathology* 55(2): 147-151.
- Muszevska A, Stepniewska-Dziubinska MM, Steczkiewicz K, Pawlowska J, Dziedzic A, Ginalska K (2017) Fungal lifestyle reflected in serine protease repertoire. *Scientific Reports* 7: 9147.
- Pan X, Zhu B, Luo Y, Fu D (2013) Unraveling the protein network of tomato fruit in response to necrotrophic phytopathogenic *Rhizopus nigricans*. *PloS one* 8(10): 1371.
- Ratanacherdchai K, Wang HK, Lin FC, Soyong K (2010) ISSR for comparison of cross-inoculation potential of *Colletotrichum capsici* causing chilli anthracnose. *African Journal of Microbiological Research* 4(1): 076-083.
- Scindiya M, Malathi P, Kaverinathan K, Viswanathan R, Sundar AR (2017) Molecular characterization of pathogenicity gene homologs in *Colletotrichum falcatum* causing red rot in Sugarcane. *Sugar Technology* 19(6): 563-572.
- Suman A, Sunita Lal, Shasany AK, Asha Gaur, Singh P (2005) Molecular assessment of diversity among pathotypes of *Colletotrichum falcatum* prevalent in subtropical Indian sugarcane. *World Journal of Microbiology and Biotechnology* 21: 1135-1140.
- Templeton MD, Rikkerink EH, Solon SL, Crowhurst RN (1992) Cloning and molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase –encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. *Gene* 122: 225-230.
- Viswanathan R (2010) *Plant Disease: Red Rot of Sugarcane*. Anmol Publishers, New Delhi India. p306.
- Viswanathan R (2017) Pathogen virulence in sugarcane red rot pathogen versus varieties in cultivation: Classical case of loss in virulence in the pathotype CF06 (Cf671). *Sugar Technology* 19: 293-299.
- Viswanathan R, Malathi P, Padmanaban P (2003) Variation in sugarcane red rot pathogen *Colletotrichum falcatum* Went. In: *Frontiers of Fungal Diversity in India* (Eds Rao GP, Manoharachari C, Bhat DJ, Rajak RC, Lakhanpal TN). International Book Distributing Co Lucknow. 639-667.
- Whitelaw-Weckert MA, Curtin SJ, Haung R, Steel CC, Blanchard CL, Roffey PE (2007) Phylogenetic relationships and pathogenicity of *Colletotrichum acutatum* isolates from grape in subtropical regions. Australia. *Plant Pathology* 56: 448-563.