RESEARCH PAPER

PHYLOGENETIC ANALYSIS OF *COLLETOTRICHUM FALCATUM* ISOLATES CAUSING RED ROT IN SUGARCANE

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Abstract

The fungal pathogen Colletotrichum falcatum, causing red rot, exhibits enormous variation. Representative isolates of C. falcatum collected from major red rot endemic regions in the country were characterized by cultural, pathological and molecular methods using the 5.8s-ITS region. Phylogenetic analysis of ITS sequences confirmed the divergent and unique nature of C. falcatum from other Colletotrichum spp. and separated the Indian isolates under three distinct molecular groups as Group I, II, and III. Although the dark coloured, non-sporulating least virulent types similar to isolates of other countries were distinguished in Group III, diversity among Group I and II was not much supported by cultural or pathogenicity patterns. However, overall correlation could be made with respect to phenotypic and genotypic characters based on their predominance in each group. This is the first attempt to group a large collection of C. falcatum isolates using molecular approach and correlate with other traits.

***Key words**: Sugarcane, Colletotrichum falcatum, 5.8s-ITS, phenotype, genotype*

Introduction

Red rot caused by *Colletotrichum falcatum* Went (Teleomorph: *Glomerella tucumanensis* (Speg) Arx and Muller) is one of the oldest known diseases of sugarcane, occurring in many cane growing countries. The disease was responsible for the decline and failure of many noble canes, which prompted sugarcane breeding for red rot resistance. Severe epidemics occurred in different parts of the country during the last century, which led to the elimination of several elite sugarcane varieties (Viswanathan 2010) and various approaches are being attempted to manage the disease under farmers' field conditions. Among all the approaches, breeding for red rot resistance plays a significant role in maintaining good crop stand in the field and hence no variety is being released without red rot resistance. However, we encounter breakdown of resistance very frequently due to the emergence of newer pathotypes. A systematic screening programme in sugarcane includes all the newer virulent isolates to assess red rot resistance. Major pathotypes used in screening programmes were distinguished at morphological, cultural, serological and pathogenicity level (Viswanathan et al. 2003). Earlier, attempts were made to characterize the isolates using RAPD profiles (Mohanraj et al. 2002; Suman et al. 2005). Recently we have characterized major pathotypes of *C. falcatum* based on the 5.8 – Internal spacer region of rDNA and vegetative compatibility grouping (Malathi et al. 2010).

However, no correlation could be obtained with all these methods as the studies were conducted in different periods of time with routine and limited number of isolates. The available information clearly revealed that some molecular tools are needed to understand evolutionary relationship of the pathogenic isolates and to identify important factors for pathogenicity in *C. falcatum*. For this purpose we used various conserved genes to understand their evolutionary role and relation to other characters *viz*., origin, cultivars and period of isolation following the breakdown of resistance. In this context, a preliminary attempt has been made to group the isolates representing various regions over a decade using 5.8s-ITS. Based on this result, grouping correlation has been made with other parameters.

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Materials and Methods

C. falcatum isolates and phenotypic characters

About 80 isolates representing various agroclimatic regions of India (Punjab - 2, Haryana - 16, Uttar Pradesh - 4, Bihar - 4, Orissa - 1, Andhra Pradesh - 11, Gujarat -16, Tamil nadu - 24, Kerala - 2) collected in different periods of time from various cultivars and maintained as part of red rot type culture collections at the Plant Pathology lab of the Institute were employed for this study. The isolates were grown on oat meal agar (OMA) for studying their cultural characters. Phenotypic characters including cultural parameters and pathogenicity were recorded as carried out for limited isolates (Malathi et al. 2010). On solid medium the pathotypes were grown for 8 days at 28-30°C and observations were recorded on mycelial growth, its colour and texture, acervuli initiation, and sporulation (conidiation). For assessing pathogenic virulence, pathogenicity reaction was tested by means of plug method of inoculation in the field on canes of the susceptible cultivar CoC 671 with 4 canes each. Sixty days after inoculation, the inoculated canes were split open longitudinally and disease development was scored based on a 0-9 scale (Srinivasan and Bhat 1961).

Molecular analysis using 5.8s rDNA - ITS gene sequences

Fungal DNA was extracted and purified from fresh mycelial mat grown on potato dextrose broth following a cetyltrimethylammonium bromide (CTAB) protocol after grinding with liquid Nitrogen (Saghai-Maroof et al. 1984). DNA was diluted to a concentration of 10ng/µl and the 5.8S rDNA-ITS region was amplified using *Colletotrichum* specific ITS primers, col1 and col2 (Martinez-Culebras *et al*. 2003). The amplified products were resolved on 1.2% (w/v) agarose gel and the resolved products were eluted and sequenced. The corrected sequences as per chromogram yielded 402bp length and were aligned using the Multiple-sequence alignment program CLUSTAL W (Thompson et al. 1994).

Results

5.8s-ITS based grouping

The amplified 5.8s-ITS region of *C. falcatum* was found to be of 402 nucleotides which include partial regions of ITS1 (127bp) and ITS2 (96bp) with complete sequence of 5.8s rDNA (179bp) region. The same nucleotide positions aligned with other *Colletrotrichum* spp. yielded 411 nucleotide positions. Though the primers could not yield complete ITS 1 and 2 regions, they were able to reveal all the variable regions of *Colletotrichum* spp. Also this primer set has the ability of amplifying all the *C. falcatum* isolates uniformly and constantly by distinguishing defective isolates. In the present study, out of 80 isolates, sequences of five isolates from subtropical region were found to be partial and hence they were not included for all the studies. Phylogenetic analysis of ITS sequences identified the genetic divergence of 75 confirmed isolates under three distinct molecular groups with 40, 32, and 3 isolates for Group I, II, and III respectively. Comparative analysis of sequence data with other *Colletotrichum* spp. clearly demonstrated the most divergent group of *C. falcatum* from other species with a high bootstrap value of 100 and moderate support for intra-specific variation (Figure not shown). However, it identified three molecular groups within the Indian population and the divergence of *C. falcaum* isolates received equal moderate support of 42 for Group I and II which could be due to 1 bp difference between these groups and the isolates within the group were identical. The most divergent Group III, genetically more identical to isolates of other countries *viz*., Bangladesh, Mexico, Hong-Kong and USA, was differentiated by 6bp with a high bootstrap value of 82 (Table 2).

Table 2. Sequences variability of 5.8s rDNA-ITS region between Group I, II and III

Group	Variable regions		
	ITS160-80bp	ITS190-110bp	ITS2310-330bp
I	GGCCCCTC**C**CGGGGCCGA	GGATCAC**C**CAACTCTATT	CTCAAGCC**CG**GC
II	GGCCCCTC**T**CGGGGCCGA	GGATCAC**C**CAACTCTATT	CTCAAGCC**CG**GC
III	GG**T**CCC**G**C**T**CGGGGCCGA	GGATCAC**G**CAACTCTATT	CTCAAGCC**TC**GC

Variation in phenotypic characters

Variation in cultural characters was recorded in terms of their growth rate, colour and texture of mycelium, time of acervuli initiation and intensity of sporulation. Of the 75 isolates confirmed by molecular data, majority were found to be fast growing (73.7%), light coloured (63.1%), thin (75.0%), sporulating (58.8%) and virulent (57.5%). In all these categories, it was found that the proportions of tropical isolates were significantly higher than the subtropical ones (Fig.1). Among the different cultural characters, growth rate of the isolates was found to have good correlation with pathogenic virulence. It also revealed that the subtropical region is dominated by less virulent types (61.5%) and the tropical regions by highly virulent types (66.7%). Besides, it was found that the five defective isolates from subtropics were extremely slow growing and non-pathogenic on sugarcane.

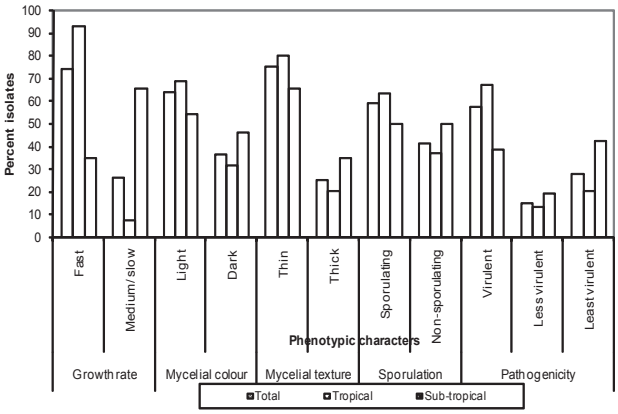

Fig. 1. Variation in phenotypic characters of *C. falcatum* isolates

Molecular grouping in relation to phenotypic characters

Analysis of molecular grouping in relation to origin, cultural and pathogenic characters indicated that Group I had mixed population of tropical (25) and subtropical (15) isolates and Group II had majority of tropical isolates (26) and less number of subtropical isolates (6) (Fig. 2). Also, it indicated that the mean pathogenicity is significantly lower in Group I (2.5 to 5.0) than Group II (4.0 to 7.0) and it was only one in Group III. Based on overall correlation of phenotypic characters with molecular grouping an illustration has been developed with the representative isolates (Fig. 3).

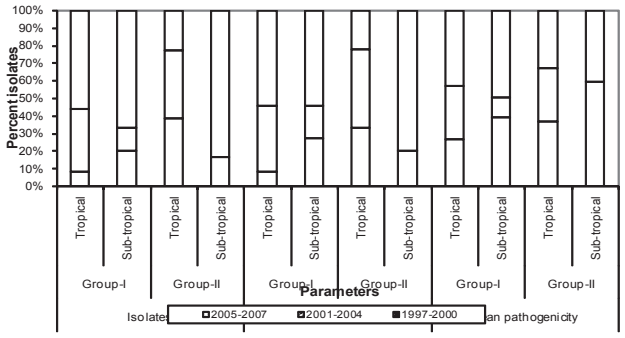

Fig. 2. Molecular grouping in relation to origin and period of isolation of *C. falcatum* isolates

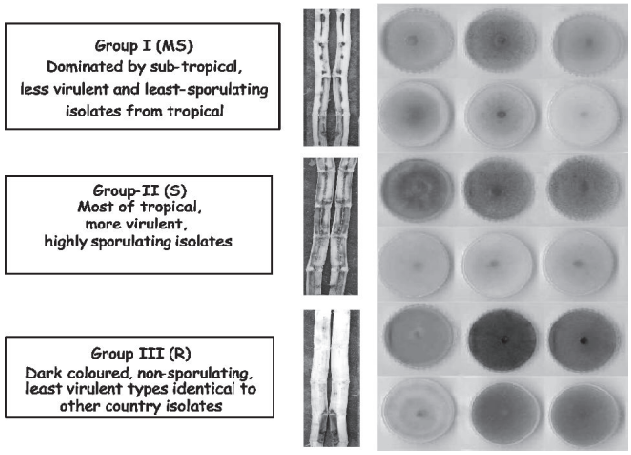

Fig.3. Phenotype and genotype correlation with representative isolates of three molecular groups by ITS.

Evolutionary relationship among the isolates

Although virulence, represented as average disease reaction produced, of Group I isolates was less (4.5) than that of Group II (6.0), breakdown of resistance is more frequent from Group I as it is clear from the number of cultivars infected by the isolates of Group I and II. Of 46 cultivars used for isolating the pathogen, about 28 were infected by isolates of Group I and only 18 by the isolates of Group II (Fig. 2). Addition of newer virulent tropical isolates in Group I indicated divergence of newer pathotypes from this group in breaking down the resistance of newly released cultivars.

Discussion

Pathogen variability in *C. falcatum* was first studied by Edgerton and Moreland (1920). In early 20th century, variability *in C. falcatum* was described culturally as light and dark isolates in correlation with pathogenicity (Abbott 1935) and then they were identified with alphabets (Rafay and Singh 1957) and numbers (Gupta

et al. 1980) without any designated nomenclature. Later, efforts were made to identify the pathotypes based on differential host interaction in different periods of time in tropical and subtropical regions (Padmanaban *et al*. 1996) and the isolates were also named as tropical and subtropical based on origin. However, the results became inconsistent due to the influence of weather factors (Raid and Lentini 2002). Since this pathogen represents a species that encompasses a wide range of pathogenic variability on set of host differentials, currently the major isolates used in screening programme are named as CF01, CF02, CF03, etc. During the last decade, there were many approaches *viz*., cultural, pathogenicity-based differentials, serological, genetically based on vegetative compatibility grouping and molecular tools using RAPD and 5.8s-ITS sequences with limited number of major isolates used in screening programme to characterize the pathotypes. As it required large number of isolates for correct understanding and confirmation, present study was undertaken with large number of isolates.

In this investigation, diversity of *C. falcatum* from other species supports the earlier reports of Martinz-Culebras et al. (2003) with col1 and 2 primers and Whitelaw-Weckert et al. (2007) with ITS 1 and 4 for 5.8s-ITS region. Comparison of *C. falcatum* sequences with other species as out group identified the unique nucleotide pattern of *C. falcatum* from other species. Previous studies also strongly recommend the use of rDNA sequence information for inferring phylogenetics and measuring genealogical relationship in other *Colletotrichum* (Sreenivasaprasad et al. 1996a; Johnston and Jones 1997; Freeman et al. 2000; Hsiang and Goodwin 2001) and analysis of ITS1 region provided more information for phylogenetic analysis than the ITS2 region (Sreenivasaprasad *et al*. 1994; Abang et al. 2002; Martinz-Culebras *et al*. 2003; Whitelaw-Weckert et al. 2007). This confirms the present results of more variable ITS1 region in *C. falcatum* than ITS2.

Although ITS sequence analysis identified three groups among *C. falcatum* isolates, the nucleotide variation was least, and considering the origin, the genotypes were found to have monophyletic evolutionary relationship among them. Similar results on least intra-specific variation in worldwide isolates of *C. acutatum* causing lupin anthracnose were confirmed by ITS, *tub2* and his4 gene sequences (Talhinhas *et al*. 2002). Looking into the correlation among cultural, pathogenic and molecular characters, the Group III in the phylogenetic tree was well distinguished by thick, dark coloured, non-sporulating, least virulent isolates, while Group II was dominated by homogenous population of tropical genotypes with high virulence and the Group I had heterogeneous population from both tropics and sub-tropics with moderate genetic support. It is similar to the findings of previous reports on *C. acutatum* grouping. In *C. acutatum,* grey almond isolates were differentiated from pink colonies by PCR profiles using repeat primers and sequences of ITS 2 rDNA (Freeman *et al*. 2000, 2001) and by intron sequence of GPDH (Guerber *et al*. 2003). Similarly, weakly virulent fast–growing *C. gloeosporioides* isolates causing Yam anthracnose in Nigeria were well distinguished from moderate and high virulent isolates having various phenotypes by 5.8s-ITS sequences (Abang et al. 2002). However, Valerio et al. (2005) reported no association between virulence phenotypes and molecular profiles of *C. graminicola* isolates causing sorghum anthracnose in Brazil.

Results on phenotypic characters indicated that there is a positive association between fast growing and highly sporulating isolates with virulence and virulent isolates were mainly from tropical conditions. Padmanaban *et al*. (1996) also indicated that pathogen isolates originating from tropical India were more virulent than the sub-tropical ones in a study with tropical and sub-tropical isolates on sugarcane differentials. Hence we could confirm our phenotypic patterns with the origin and to a certain extent with the molecular grouping. Thus we hypothesize that the genotypes related to Group I might have originated from subtropical areas and the genotypes of Group II from tropical areas. Similar findings were reported for *C. acutatum* isolates from sub-tropical Australia (Whitelaw-Weckert et al. 2007). They opined that the sub-tropical Australian isolates have genetic similarity even with non-grape isolates of that area with lack of host specificity and the tropical isolates were separated under two subgroups. Influence of origin as tropical or subtropical conditions even on species specificity of *Colletotrichum* was reported with fruit rot causing *Colletotrichum* spp. in Brazil (Pers et al. 2002). Hence the origin of isolates may play a vital role in deciding the genetic diversity.

Prevalence of most of the recent isolates in Group I could be attributed to development of new variants from that group by adaptation in tropical areas and development

of newer races in breaking down the newly released cultivars. Hence newly emerging virulent isolates of Group I have to be used along with the highly virulent tropical types for screening of sugarcane clones for red rot resistance. Earlier we demonstrated adaptation of well defined incompatible subtropical pathotypes Cf1148 and Cf7717 by cross inoculation (Malathi et al. 2006). Though these isolates have similar cultural behaviour, variation was proved at serological (Viswanathan et al. 2000), RAPD (Mohanraj et al. 2002; Suman et al. 2005), phenotypic (Viswanathan *et al*. 2003), vegetative compatibility grouping and using 5.8S-ITS with CITS1 and CITS2 primers (Malathi et al. 2010). In the present investigation also these two isolates were distinguished in Group I and II by the 5.8s-ITS sequences. Least genetic variation among the isolates could be attributed to isolates of commercial sugarcane genotypes bred from the common ancestor *Saccharum officinarum* with high sugar gene pool. Evolution of new variants from Group I might also be due to extreme winter and summer in subtropics which is shown by development of more number of genetically altered isolates having different cultural, pathological and genetic behaviour (five isolates could not be confirmed) and simultaneously high frequency of virulent types knocking down the newer cultivars. In subtropical plains of India, high relative humidity and temperatures prevail during the monsoon period in the months of July-August making sugarcane genotypes more vulnerable to *C. falcatum* attack resulting in complete devastation of the crop (Suman *et al*. 2005).

Results of the study revealed that though molecular grouping could confirm phenotypic/ pathogenic pattern of Group III and distinguished I and II by ITS analysis, it is more complicated to determine the isolates phenotypically / pathogenicity unless it is supported by genetic confirmation. Hence, it is suggested that any new variants should be confirmed for genetic variation before they are being included in disease screening programme. Based on the above information it is very clear that the use of many conserved genes in addition to ITS region will be highly useful to understand the phylogenetics/ evolutionary relationship of *C. falcatum* isolates at genomic level. Further a proteomic approach is being standardized to identify differentially expressed proteins in relation to pathgenicity.

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