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Assessment of genetic diversity in *Colletotrichum falcatum* Went accessions based on RAPD and ISSR markers

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ABSTRACT

Sugarcane is susceptible to red rot disease caused by phytopathogenic fungus *Colletotrichum falcatum* Went which ultimately affect the economy of farmers as well as sugar based industry. One of the various ways to control this devastating disease is to develop disease resistance sugarcane cultivar and this requires the complete understanding of genetic makeup of pathogen. Although South Gujarat is well known sugarcane cultivating area, less published data can be found about PCR-based genetic diversity in prevalent *C. falcatum* accessions. So, present investigation aims at finding molecular variation among the ten accessions of *C. falcatum* using RAPD and ISSR molecular markers. A total of 35 RAPD and 39 ISSR primers were screened across 10 *C. falcatum* accessions, of which 15 RAPD and 21 ISSR primers have showed consistent amplification. Statistics related to genetic variation were estimated using NTSYS-PC by means of Dice's coefficient. The results revealed 80.6% and 68.07% polymorphism and similarity coefficient ranged from 0.43 to 0.91 and 0.73 to 0.93 in RPAD and ISSR analysis respectively. The dendrogram generated using RAPD, ISSR and combined RAPD-ISSR grouped accessions into different clusters which reveal considerable level molecular variation among the *C. falcatum* accessions. It is also evident from PCA plots that accessions are rather dispersed with tested marker systems indicating good genetic base. So, in nut shell, we found considerable genetic variation and relatedness within *C. falcatum* accessions collected from different areas of south Gujarat, India using RAPD and ISSR markers.

1. Introduction

Sugarcane (*Saccharum officinarum* L.) belonging to family *Poaceae* is one of the most important agro-industrial crops under cultivation in tropical and subtropical regions in the world. In India sugarcane is grown in 21 states and it's a second highest crop after cotton under cultivation. In Gujarat, total 4.5 Lakhs families grow sugarcane and that provides total of 5.5 Lakhs employments. Particularly South Gujarat is entire sugarcane growing belt with eleven operational sugar factories, which is highest compared to other region of state and Bardoli sugar factory is one of the Asia's biggest sugar mill. As an industrial crop, sugarcane is used for production of sugar, bioethanol, jaiggery, molasses, cattle feed and syrups. Under field condition sugarcane is susceptible to many bacterial, viral and fungal diseases but among them fungal infections is most critical as it can affect all parts of plant. There are two main fungal diseases in sugarcane, wilt and red rot, among them red rot is oldest and severe disease of sugarcane caused by fungus *Colletotrichum falcatum* Went (telomorph, *Glomerella tucumanensis*). In India, first time this disease was reported by Barber (1901) in Andhra Pradesh [1].

Red rot pathogen infects various parts of the cane plant but it is typically considered as a stalk and a seed borne disease [2]. Infection on leaves may not affect the overall yield, while stem infection with fungus is very crucial as it directly reduces the sugar content. At early stage of the cane crop it is difficult to recognize the presence of disease in the field whereas at the later stage of sugar formation characteristic symptoms of the disease develops on the stem as reddening of the internodal tissues with intermittent red and white patches and leads to 29% reduction in sugar cane weight and 31% loss in sugar recovery [3]. Though the plant pathogen interactions studies provided an overall understanding of the genetic analysis of pathogenicity, yet the exact mechanism for development of disease is not clear. Further cultural and morphological characterization of *C. falcatum* isolates and use of differential host reaction to detect pathogenic variability is time

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consuming [4,5]. Since, the pathogenicity is variable with environmental conditions sometimes leading to disease misinterpretation. As the genetic make-up of pathogen *C. falcatum* changes continuously, prompt characterization and its biological control is crucial to control the disease. Continuous evolution of newer races is also one of the key factors that make several elite varieties to become susceptible [6]. High variability and adaptability of the *C. falcatum* endangers the farming of different elite sugarcane cultivars. Obviously use of disease resistant variety is the important approach to prevent the disease, but due to frequent genetic mutations the pathogenicity often overcomes the resistance introduced and cultivars surrender to the pathogen soon [7,8]. The natural selection of new, more virulent accession and collapse of host resistance is majorly due to gradual diminishing of resistance sources. [9]. Understanding of genetic variation in and diversity pattern of *C. falcatum* prevalent in specific area is important mean to device disease management strategy if any epidemics arise. So variety of molecular approaches have been developed and used as morphological criteria are not accurate to discriminate between *Colletotrichum* species. Recently, various molecular markers have been used in categorization of different *Colletotrichum Spps* [4,10,11]. Random Amplified Polymorphic DNA (RAPD) marker amplifies random DNA fragment with decamer primers using polymerase chain reaction (PCR) [12]. RAPD technique was the most widely used molecular method owing to its inexpensiveness, technical simplicity and it does not require prior sequence information and can efficiently distinguish taxa below the species level [13]. Inter-simple sequence repeats (ISSR) is also PCR-based molecular tool that amplifies genomic regions between SSR sequences repeat. ISSR markers are robust, reliable, quick, efficient and reproducible. This method was first reported as a technique for the study of molecular variation in plants and animals and later on used to find DNA markers in fungi [14,15]. The RAPD and ISSR markers have became the markers of choice to study the genetic variations due to their technical simplicity, inexpensiveness and repeatable amplification of DNA sequence using single primer. There is a lack of information on the genetic diversity of the *C. falcatum* in South Gujarat. So, the present research work was carried out using RAPD and ISSR markers to find molecular variation among *C. falcatum* accessions collected from red rot infected sugarcane cultivars from south Gujarat.

2. Materials and methods

2.1. Fungal accessions

Ten accessions of *C. falcatum* were isolated from red rot infected stems of nine sugarcane cultivars used in present study [16]. Accessions one to nine were collected different region of South Gujarat and Cf8436 was obtained from Sugarcane Breeding Institute, Coimbatore (Table 1). Pure cultures of each accession were made by single spore isolation and were maintained on oatmeal agar slants at 4 ± 1 °C until used for DNA isolation.

2.2. Genomic DNA extraction

Total fungal DNA was isolated from the mycelia grown for 5–7 days at room temperature on complete media broth (CMB) (yeast extract at 6 g/l, Casien Acid Hydrolysate at 6 g/l and Sucrose at 10 g/l) using modified method of Raeder and Broda [17]. The mycelium harvested using filter paper (Whatman no. 1) was dried overnight in a centrifugal evaporator. Using liquid nitrogen the mycelium was grounded into a fine powder in a pestle and mortar. Dried powder of each isolate (70 mg) was suspended in 750 µl of DNA extraction buffer (700 mM NaCl, 50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 2% cetyl trimethyl ammonium bromide, 1% Polyvinyl pyrolidone (PEP) and 1% β-mercaptoethanol) and incubated for 45 min with intermittent shaking at 62 °C. After incubation, suspension was subjected to centrifugation at 10,000 rpm for 15 min and supernatant was collected into a fresh tube. Later, the supernatant was extracted twice with chloroform: isoamyl alcohol (24:1) and later two volumes of absolute ethanol were added to the aqueous extract and the mixture was placed on ice for 35 min. The contents were centrifuged at 12,000 rpm for 10 min to collect the precipitated DNA. The DNA pellet was washed with 70% ethanol, briefly air-dried and resuspended suspended in 200 µl of 1X Tris-EDTA (TE). The RNA contamination from the genomic DNA was removed by adding 5 µl of RNase (10 µg/ml) to each tube, DNA was redisolved by taping the pellet and incubated for 1 h at 37 °C. Further, genomic DNA was purified by phenol: chloroform: isoamyl alcohol (25:24:1) treatment and precipitation with 3 M ammonium acetate and absolute alcohol and finally dissolved in TE buffer. The quantity and quality of isolated DNA was analysed on spectrophotometer and agarose gel (0.8%) electrophoresis, respectively. Purified DNA samples were stored at 4 °C for further RAPD and ISSR analysis.

2.3. PCR amplification and agarose gel electrophoresis of PCR products

PCR was performed in a programmable gradient thermocycler (Eppendorf) using 200 µl PCR tubes (Himedia). In total 35 RAPD primers (Operon technologies, USA) and 39 ISSR primers were screened for genetic analysis of *C. falcatum* accessions. PCR for RAPD and ISSR was carried out in of 25 µl volume containing buffer MgCl2, primer, deoxynucleotides mixtures and Taq polymerase (Bangalore GeNei, India, GeNei TM), and genomic DNA. The optimal annealing temperature was found to vary according to the G + C content of the di- or tri-nucleotide repeats of containing ISSR primers. PCR products were separated on 1.5% agarose gels and visualized in Gel Doc system (BioRad). A 100-base pair ladder (Bangalore GeNei Pvt Ltd, Bangalore, India) was used as a standard DNA marker. Analysis was replicated twice to confirm reproducibility of results.

Table 1
Details of *Colletotrichum falcatum* accessions collected from different regions of South Gujarat for genetic diversity analysis.

Sr No	Isolate	Place of collection	Host Cultivar	Latitude (°N), Longitude (°E)
1	cfNAV	Navsari, Gujarat	Co 671	20.9467° N, 72.9520° E
2	cfVES	Vesma, Gujarat	CoS 707	21.0314° N, 72.9752° E
3	cfPAR	Pardi, Gujarat	Co 671	20.5230° N, 72.9594° E
4	cfTIM	Timbarva, Gujarat	Co 86002	21.1864° N, 73.1364° E
5	cfMAR	Maroli, Gujarat	CoN 10071	21.0243° N, 72.8899° E
6	cfGAN	Gandevi, Gujarat	Co 86032	20.8077° N, 72.9992° E
7	cfKAM	Kamrej, Gujarat	Co 94004	21.2676° N, 72.9609° E
8	cfCHA	Chalthan, Gujarat	Co 94008	21.1544° N, 72.9623° E
9	cfMAD	Madhi, Gujarat	Co 86249	21.1170° N, 73.1076° E
10	Cf8436	Coimbatore, Tamil Nadu	Co 8436	11.0045° N, 76.9616° E

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