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Rapid identification of endophytic fungi of sugarcane (*Saccharum* spp. hybrid) using PCR-RFLP of rDNA

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Abstract

Aim : Restriction fragment length polymorphism analysis of PCR-amplified internal transcribed spacer (ITS) regions of rDNA is a cheap and convenient molecular tool used in fungal taxonomy. This tool has been used for rapid identification of the endophytic fungi of sugarcane belonging to different genera for their further potential utilization.

Methodology : Eight isolates belonging to three endophytic fungi viz. *Colletotrichum falcatum*, *Fusarium moniliforme* and *Trichoderma viride* isolated from sugarcane plant were used for differentiation using PCR-RFLP pattern of rDNA. For this, the ITS1-5.8S-ITS2 region of rDNA was amplified with the help of universal primers and the PCR products were digested with various restriction enzymes.

Results : The size variation of amplified products ranged from 637 bp (*C. falcatum* isolates), 592-614 bp (*F. moniliforme* isolates) to 631-698 bp (*T. viride* isolates). Five restriction enzymes viz. *Alu I*, *EcoR I*, *Bam H I*, *Mse I* and *Sma I* used for PCR-RFLP of ITS fragments yielded a total of 54 bands that were arranged in three RFLP patterns.

Interpretation : Banding pattern obtained with the combination of *Alu I* and *Mse I* enzymes presented the best profile to identify the endophytic fungi and allowed distinction at molecular level. This reflected the potential of PCR-ITS-RFLP using a combination of restriction endonucleases as a rapid and convenient differentiation tool to distinguish these endophytic fungi.

Rapid identification of fungi

PCR-RFLP (Restriction fragment length polymorphism) based identification of endophytic fungi of sugarcane for their potential utilization

Amplification of ITS1-5.8S-ITS2 region of rDNA of 8 isolates belonging to 3 endophytic fungi viz. *Colletotrichum falcatum*, *Fusarium moniliforme* and *Trichoderma viride*

Restriction digestion of PCR amplicons with five restriction endonucleases viz. *Alu I*, *EcoR I*, *Bam H I*, *Mse I* and *Sma I*

A total of 54 PCR-RFLP bands obtained were arranged in three RFLP patterns viz. I, II and III

Combination of *Alu I* and *Mse I* enzymes presented the best profile to identify the endophytic fungi

PCR-ITS-RFLP using a combination of restriction endonucleases is a potential tool for rapid identification of fungal species

Introduction

Endophytes commonly refer to a group of fungi that exist asymptomatically inside the living plant tissues for complete or part of their life cycle without causing any visible symptoms of their presence (Sánchez Márquez *et al.*, 2008). They inhabit in various tissues, seeds, roots, stems and leaves (Johri, 2006). Their occurrence on various host plants suggests that fungal endophytes are ubiquitous in plant species (Hyde and Soyong, 2008; Sánchez Márquez *et al.*, 2008; Zhang *et al.*, 2006). Sugarcane (*Saccharum* spp.) is an important cash crop of India, primarily used for the production of sugar as well as biofuel alcohol. The association of microbial communities with sugarcane and their influence on bio-productivity is a concern of investigation. Various endophytic fungi belonging to different genera have been isolated from sugarcane cropping system (Fávaro *et al.*, 2012; Stuart *et al.*, 2010). Of these, *Colletotrichum falcatum*, *Fusarium moniliforme* and *Trichoderma viride* are the three important endophytic fungi of sugarcane. Their isolates have been identified based on cultural and morphological features. It is important to further identify these endophytic fungi for their potential utilization in growth promotion and disease resistance activities using molecular techniques.

Identification of fungi is traditionally done based on morphological characters (such as of conidial anatomy and appressoria shape), pathogenicity tests and physiological and biochemical approaches (Zhang *et al.*, 2006). However, special care is required for differentiating closely related or morphologically identical fungal genera at species and sub-species level, because the morphological characteristics of some fungi may vary depending on the culture media and conditions as these factors substantially affect their vegetative and sexual compatibility (Hyde and Soyong, 2008; Zhang *et al.*, 2006). Nowadays, the most precise tools for differentiating two closely related species/genera are molecular biology-based tools, such as AP-PCR (arbitrarily primed- polymerase chain reaction) or RAPD (Randomly amplified polymorphic DNA), mt DNA RFLPs (mitochondrial DNA restriction fragment length polymorphisms), and rDNA (ribosomal DNA) restriction analysis (Morelock *et al.*, 2002; Promputtha *et al.*, 2005). These approaches have greatly facilitated detection of array of pathogens like viruses, bacteria, phytoplasmas and fungi (Martínez-Culebras *et al.*, 2002; Moritz *et al.*, 2000). Among the molecular methods, those which are based on the nucleotide variability of ITS (internal transcribed spacer) region, have been shown to be useful for the taxonomic characterization of many fungal species because the ITS regions of nuclear rDNA consisting of two non-coding variable regions of ITS1 flanked by the 18S and the 5.8S subunits and ITS2 flanked by the 5.8S and the 28S subunits are often highly variable between isolates of the same species of fungus and, are therefore, the best tools for the phylogenetic analysis and differentiation of closely related fungal taxa (Salazar *et al.*, 1999).

RFLP (Restriction fragment length polymorphism) analysis of the PCR-amplified internal transcribed spacer (ITS) regions of rDNA has further facilitated microbial taxonomy e.g. identification of strains of *Penicillium* subgenus *Bivertillium* (Dupont *et al.*, 2006), differentiation of *P. expansum* among fungal species (Garcia *et al.*, 2006), grouping of *A. flavus* isolates infecting maize (Mohankumar *et al.*, 2010) or bacterial diversity analysis of a municipal sewage treatment plant (Devi and Ramya, 2015). In the present study we developed and evaluated a PCR-RFLP assay of rDNA for rapid and easy identification and differentiation of endophytic fungi of sugarcane without any immediate need of sequencing.

Materials and Methods

Preparation of total genomic DNA : A total of 8 isolates of three endophytic fungi belonging to *Colletotrichum falcatum* (isolates Cf 07, Cf 08 and Cf 09), *Fusarium moniliforme* (isolates Fm 01, Fm 02 and Fm 03) and *Trichoderma viride* (isolates Tv 01 and Tv 02) were selected for their molecular identification using PCR-RFLP. Isolation of these isolates was done from leaf and stem of sugarcane on potato dextrose agar medium (PDA composition: potato 250 g, dextrose 20 g and agar 20 g per liter of water). The isolates were maintained in the laboratory at 20 °C. About 1 gm of fungal mycelia was harvested with the help of a sterilized stainless steel spatula from actively growing cultures in PDA plates and ground in a pestle and mortar by adding adequate liquid nitrogen. Total genomic DNA was extracted using a slightly modified CTAB extraction protocol (Srivastava and Gupta, 2001). Isolated DNA was purified using RNase and pellets were dissolved in 1 X TE buffer. The purified DNA was visualized in 0.8% agarose gel stained with ethidium bromide to check its purity and to estimate its quantity. Uniform concentration of 5 ng / µl was standardized for the polymerase chain reaction. DNA was kept at 4 °C until further use and then stored at -20 °C in deep freezer.

Amplification of ITS region : The universal oligonucleotide primers ITS-4 (5'- TCC TCC GCT TAT TGA TAT GC -3') and ITS-5 (5'- GGAAGT AAAAGT CGT AAC AAG G -3') were used (White *et al.*, 1990) to amplify a nuclear rDNA gene fragment containing ITS1, ITS2 and the 5.8S rRNA gene by PCR using gDNA as template. The PCR amplification reactions were performed in a total volume of 50 µl. Each reaction mixture contained 10 to 100 ng of template DNA, 2 mM MgCl₂, 20 pmol of each primer, 200 mM of each dNTP and 2 U of DNA polymerase (Bangalore Genei, Bangalore) in a PCR buffer containing 75 mM Tris HCl (pH 9.0), 50 mM KCl, 20 mM (NH₄)₂SO₄ and 0.001% BSA. The polymerase chain reaction was carried out in a PTC 200 Thermal Cycler (BioRad, U.S.A.) programmed to perform an initial denaturation step of 95 °C for 5 min, followed by 35 cycles consisting of 30 sec at 95 °C for denaturation, 30 s at 55 °C for annealing and 1 min at 72 °C for extension with 10 min elongation at 72 °C followed by cooling at 4 °C for 15 min. PCR products were resolved on a 2%

Table 1 : Restriction profile of ITS regions of ribosomal DNA of fungal strain with different endonucleases and size estimates (base pairs) for fragment patterns

	Amplicon	Fragment length in base pair using restriction enzyme					Restriction profile type
		Eco RI	Sma I	Alu I	Mse I	Bam HI	
Cf07	637 bp	327 310	526 111	637	210 202 180 36 9	637	I
Cf08	637 bp	327 310	526 111	637	209 202 181 9	637	
Cf09	637 bp	327 310	526 111	637	211 202 179 36 9	637	
Fm01	592 bp	304 288	592	422 139 31	592	592	II
Fm02	614 bp	304 288	362 252	475 139	614	614	
Fm03	592 bp	304 288	592	422 139 31	592	592	
Tv01	631 bp	335 296	631	631	631	631	III
Tv02	698 bp	358 340	698	698	698	569 129	

agarose gel containing 1 mg ml⁻¹ ethidium bromide in tris-acetate buffer (0.04 M tris-acetate, 0.001 M EDTA, pH 8.0) at 80 V for 45 min in BioRad SubCell GT electrophoresis unit. The amplified DNA fragments were visualized by UV transillumination and analyzed using Alphamager™ Gel Documentation System (Protein Simple, USA).

Cleaning-up and restriction of PCR products : The PCR products were eluted from the gel and purified using the Ultrafree DA Gel Extraction Kit (Millipore, USA) following the manufacturer's instructions. Purified PCR products of each isolate were digested with the restriction enzymes *Alu I*, *EcoRI*, *Bam HI*, *Mse I* and *Sma I* (Bangalore Genei, Bangalore) in a total volume of 20 µl containing approximately 200 ng amplified DNA, 10 U of enzyme in final concentration of 1X digestion buffer. Incubation was done at 37 °C for 16 hrs in the thermal cycler. Restricted fragments were separated in 10% Acrylamide-bisacrylamide gel with 0.5 X TBE buffer in vertical electrophoresis unit of Bio Rad. Electrophoresed gels were silver stained and amplified bands were visualized and documented in Alphamager™ Gel documentation system (Protein Simple, USA). Size of fragments was estimated by doing comparison with

a standard 100 bp DNA ladder (Bangalore Genei, Bangalore).

Results and Discussion

PCR- RFLP identification : The ITS4 and ITS5 primer pair described by White *et al.* (1990) was used in this study to amplify the ribosomal DNA region from eight isolates of three endophytic fungi of sugarcane viz. *C. falcatum*, *F. moniliforme* and *T. viride*. The amplified PCR products belonged to two non-coding regions of ITS1 and ITS2, and the 5.8S rRNA gene (*ITS1-5.8S-ITS2*). There was no intraspecific difference in size of amplified PCR products in *C. falcatum* isolates and *F. moniliforme* isolates Fm 01 and Fm 03, but it differed between the genera and among rest of the isolates. The size variation ranged from 637 bp (*C. falcatum* isolates), 592-614 bp (*F. moniliforme* isolates) to 631-698 bp (*T. viride* isolates) as presented in Fig. 1. Such size variations are not unexpected in PCR using ITS region primers. Gomes *et al.* (2002) found extensive length polymorphism in ITS region (from 560 to 750 bp) in 26 isolates of 8 ectomycorrhizal fungi genera. Highly variable lengths of ITS1/ITS4 amplified fragments were observed in different fungal genera ranging from as small as 380 bp to up to 880 bp (Korabecna (2007) and 539 to 626 bp (Diguta *et al.*, 2011).

Restriction digestion of ITS amplified fragments was done for further detection of the variability among these endophytic fungal isolates. Five restriction enzymes consisting of 4-bp and 6-bp cutter enzymes viz. *Alu I*, *EcoR I*, *Bam H I*, *Mse I* and *Sma I* were used, which yielded a total of 54 bands. For some enzymes, length of restricted fragment was same, whereas, some enzymes did not cut all the genera. Size similarity of restricted fragments may occur due to sequence similarity of ITS region being cut. Nikolcheva *et al.* (2003) also found conserved restriction sites for *Msp I* and *Rsa I* while using different restriction enzymes for terminal restriction fragment length polymorphism (T-RFLP) analysis of aquatic hyphomycete community. In such cases, selection of appropriate restriction enzymes becomes crucial

Fig. 1 : Agarose gel electrophoresis of PCR amplicon of different fungal isolates using rDNA gene sequence as primers ITS 4 and ITS 5. (Lane 1 to 9: M – 100bp DNA ladder of Bangalore Genei; 1, 2, 3 = Cf07, Cf08, Cf09; 4, 5, 6 = Fm01, Fm02, Fm03 and 7, 8 = Tv01, Tv02 respectively)

Fig. 2 : Silver stained PAGE of ribosomal DNA restriction profile of different fungal isolates after digestion with the restriction endonucleases a. RFLP profile with *Alu I*; b. RFLP profile with *Eco R I*; (Lane 1 to 9: M – 100bp DNA ladder of Bangalore Genei; 1, 2, 3 = Cf07, Cf08, Cf09; 4, 5, 6 = Fm01, Fm02, Fm03 and 7, 8 = Tv01, Tv02 respectively)

which can be done by hit and try approach. Nikolcheva *et al.* (2003) further found that the restriction enzyme *Dde I* gave the most variable RFLP pattern in the pure aquatic hyphomycete controls followed by the enzymes *Cfo I*, *Hae III*, *Hinf I*. In our study also, different restriction enzymes showed different cutting patterns with different endophytic fungi. e.g. *Alu I* cut only the three *Fusarium* isolates and *Bam H I* did not cut any isolate except *Trichoderma* isolate Tv 02. Similarly *Sma I* did not cut two *Fusarium* isolates- Fm 01 and Fm 03, and *Trichoderma* isolates Tv 01 and Tv 02. For restriction enzyme *Mse I*, there was no cutting site in the genus *Fusarium* and *Trichoderma* (isolate Tv 02). Mergulhão *et al.* (2008) could also not find any restriction sites for the enzyme *Alu I*, however, they observed that the restriction patterns of *Hinf I* and *Mbo I* in combination with other enzymes were able to differentiate four AMF species. In the present study also, the combination of restriction enzymes showed polymorphism in size of restricted fragments, based on which three RFLP patterns viz. RFLP patterns I, II and III were discerned which could differentiate these endophytic fungi (Table 1). The representative RFLP patterns with restriction enzymes *Alu I* and *Eco R I* are depicted in Fig. 2 a, b.

The RFLP pattern I was generated by the three *Colletotrichum* isolates. The amplified 5.8S-ITS rDNA region of these isolates had one restriction site each for *Eco R I* and *Sma I* and four sites for *Mse I*. The restriction enzyme *Alu I* and *Bam H I* yielded only a single fragment of same size as the amplified region. The RFLP pattern II was generated by the three isolates of *Fusarium*, where, amplified ITS region had one restriction site for *Eco R I* in all the three isolates. Digestion with *Alu I* revealed two restriction sites in isolates Fm 01 and Fm 03 and one restriction site in Fm 02. For restriction enzymes *Bam H I* and *Mse I*, there was no cutting site in *Fusarium*. The RFLP pattern III was represented by the two isolates of *Trichoderma*, where *Eco R I* had one restriction site in amplified products. Restriction enzymes *Sma I*, *Alu I* and *Mse I* did not cut the amplified fragment. The restriction enzyme *Bam H I* cut only Tv 02 isolate.

Analysis of RFLP pattern of ITS1-5.8S-ITS2 PCR amplicons with restriction endonucleases : Amplification of 5.8S rRNA gene, the conserved coding region along with two non-coding and variable flanking regions ITS1 and ITS2, generally show low intraspecific variability but high interspecific polymorphism and are thus suitable for identification of different fungi (Salazar *et al.*, 1999). ITS length variations (between 500 to 550 bp) and, presence of SNPs (single nucleotide polymorphisms) and INDELS (insertions /deletions) were observed in ITS1 and ITS2 regions of ribosomal DNA, encompassing the 5.8S rRNA gene nuclear ribosomal DNA of ten species of *Pestalotiopsis* (Gehlot *et al.*, 2012). In the present study, the use of PCR primers ITS4 / ITS5 resulted in amplification of single fragment in all isolates of endophytic fungi but its size varied among the isolates from 592 to 698 bp (637 bp in *C. falcatum*, 592 - 614 bp in *F. moniliforme* and

631 - 698 bp in *Trichoderma viridae*). The analysis of the restriction profile (RFLP pattern) of these ITS fragments resulted in identification of two restriction endonucleases *Alu I* and *Mse I* that could reliably be used for rapid and easy identification of the three endophytic fungi in sugarcane. The PCR-RFLP using *Alu I* and *Mse I*, revealed 2 to 5 fragments per restriction enzyme in each fungal isolate, with three distinct restriction patterns overall (Table 1). According to restriction profile, there were one to two restriction sites for *Alu I* in *F. moniliforme* that cleaved the PCR products of isolates Fm 01 and Fm 03 into 3 fragments of 422 bp, 139 bp, and 31 bp while two products of 475 bp and 139 bp were obtained in isolate Fm 02 (Fig. 2a). There were 4 restriction sites in *Colletotrichum* isolates for *Mse I* that cleaved the PCR products into 5 fragments of 210, 202, 180, 36 and 9 bp each. On the other hand, there was no restriction site for *Alu I* and *Mse I* in the PCR products of *Trichoderma* isolates. Digestion of the PCR products with endonucleases *Eco R I* showed one cutting site in the ITS region of all the endophytic fungi resulting in two bands of nearly 327 bp and 310 bp in *Colletotrichum*, 304 bp and 288 bp in *Fusarium* and 358 bp, 340 bp, and 296 bp in *Trichoderma* (Fig. 2b). For the endonuclease *Bam H I*, there was one cleavage site in *Trichoderma* strain Tv 02 resulting in two bands (569 bp and 129 bp), however no cutting sites in remaining endophytic fungi were observed. The digestion fragments ranging from 31 to 422 bp in *Alu I* and 9 to 211 bp in *Mse I* were differentiated between the three endophytes (Fig. 2a and 2b). No restriction sites were found in *C. falcatum* and *T. viride* digested with *Alu I* (Table 1). Consequently, no variation between the *C. falcatum* and *T. viride* was detected with this enzyme. On the other hand, the combination of the restriction patterns of enzymes *Alu I*, *Mse I*, *EcoR I* and *Sma I* allowed the molecular distinction of all species, mainly with the profiles obtained with endonuclease *Mse I*.

The detailed comparison of the restriction profile of the ITS fragments with *Alu I* and *Mse I* proved that the size profiling of the PCR amplified bands after restriction using endonucleases *Alu I* and *Mse I*, is extremely useful as one of the rapid tools for identification and monitoring of endophytic fungal species. Using a combination of the restriction patterns of two or more enzymes to allow the molecular distinction is not uncommon. Lee *et al.* (2003) used combinations of three restriction enzymes, *Hinf I*, *Alu I* and *AsuC21* for RFLP of partial small subunit of ribosomal DNA of AMF (Arbuscular mycorrhizal fungi) and plant pathogenic species of *Fusarium*, and found that distinct polymorphism was achieved only with *AsuC21*. A similar type of observation was made by Mergulhao *et al.* (2008), while doing PCR-RFLP of ITS region of AMF. They observed that the restriction enzymes *Hinf I* and *Mbo I* were able to differentiate four AMF species, whereas, no restriction sites were obtained for the restriction enzyme *Alu I*. Similarly, species of *Phaeoacremonium* were identified by restriction digestion pattern of ITS1F/ITS4 amplified PCR-amplicons using restriction enzymes *BssK I*, *EcoO109 I*, and *Hha I* (Aroca and Raposo, 2007). Viaud *et al.* (2000) also

substantiated the fact that the PCR-RFLP of ITS region could be used as an efficient molecular tool to distinguish a large number of fungal samples without going for sequence analysis.

Interpretation of the restriction pattern obtained is generally rapid, reliable and cheaper as compared to morphological traits and no need to go for the conventional sequencing of PCR products; hence the process can be adopted on a large scale in a short time. The present study also proved it to be a rapid and convenient tool for differentiation of endophytic fungi of sugarcane using PCR-RFLP with only three steps to perform: (1) to amplify the ITS fragment with the universal ITS primers (2) to digest PCR products with suitable restriction enzymes and (3) to run the digested product on vertical PAGE to identify the samples based on their restriction profiles. The method excluded the influence of artificial factors and thus improved the validity of identification greatly and hence has a great potential for utilization as a tool to differentiate the major endophytic fungi in sugarcane with high phylogenetic affinity.

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