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Molecular characterization of Trichoderma isolates by ISSR Marker

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Abstract: *Biocontrol agent Trichoderma has attained importance for substitute of chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters with antagonistic ability. Twelve isolates belonging to Trichoderma harzianum and Trichoderma viride were assessed for their antagonistic effect on Fusarium oxysporum F.sp. lycopearsicie and Xanthomonas campestris. Pv.vesicatoria. Trichoderma harzianum isolates were more aggressive than T. viride isolates. The dataset generated through morphological characters and ISSR markers showed a comparable output grouping the isolates of T. viride in one cluster and all T. harzianum isolates in another cluster. It is obvious from the present study that genetic diversity analysis had a positive correlation with the antagonistic ability of Trichoderma isolates. Thus an integrated approach of morphological and molecular markers can be employed to identify a superior strain of Trichoderma for its commercial exploitation.*

Key Words: *Trichoderma, antagonism, morphological and molecular characters, ISSR.*

I. Introduction

Plant disease epidemics have created an ecologically unbalanced system in modern agriculture. Deterrence of such epidemics for the most part achieved through use of chemical fungicides which have greater repercussion on environment and human health. Also progressive con-frontation in a midst of pathogen resistance to accessible chemical plant protectants has engrossed the need of alternative methods of disease control. Fungi of the genus Trichoderma are important biocontrol agents of several soil borne phytopathogens (Benitez et al., 2004).

The Trichoderma species serves as a potential alter-native to chemical control measure and growing pathogen resistance crop cultivars. Trichoderma is easily identified in culture media, which produces large number of characteristics small, green or white conidia, from phialides present on the profusely or meagerly branched conidiophores. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters of the species. Molecular analysis of several strains revealed that classification based on morphological data has been, erroneous to great extent resulting in re-classification of several isolates and species (Samuels, 1996).Trichoderma use different mechanism for control of phytopathogens which includes mycoparasitism, out compete pathogenic fungi for nutrients, secretion for antibiotics and fungal cell wall degrading enzymes (Harman et al., 2004; Renio et al., 2008). Furthermore it is difficult to predict the degree of synergism and the behaviour of a biocontrol agent in a natural pathosystem. Thus the present study was to characterize the cryptic species of Trichoderma associated with biological control and to establish the relation between bioefficacy and morphological and molecular characters existed if any.

Currently molecular techniques like DNA sequencing (Apple and Gordon, 1996), Random Amplification of Polymorphic DNA (RAPD) analysis (Woo et.al, 1996), Restriction Fragment Length Polymorphism (RFLP) analysis (Meyer et.al, 1992), internal transcribed sequences (ITS) of the ribosomal DNA (rDNA) analysis (rDNA-ITS1) and universally primed polymerase chain reaction (UP-PCR) have been used to characterize isolates of Trichoderma (Cumagun et.al, 1996). Inter simple sequence repeats (ISSR) have been used as another effective method to characterize genetic variability. Since the evolutionary rate within ISSR is considerably higher than other types of DNA, the likelihood of finding poly-morphism is greater compared to RAPD (Charlesworth et al., 1994).

II. Material And Methods

Isolation and identification of Trichoderma

Soil samples were collected from the different tomato fields in 10 districts of western Maharashtra. A total number of twelve isolates of Trichoderma species was isolated and identified on potato dextrose agar (PDA). The identification of Trichoderma isolates were confirmed both by colony characters and microscopic characters as given by Rifai (1969).

Confrontation assays in vitro

In vitro confrontations were studied by performing dual culture technique described by Dennis and Webster (1971) and inhibition zone technique was used to test the antagonistic ability of Trichoderma isolates against the Fusarium oxysporum f.sp. lycopersici and Xanthomonas campestris pv. vesicatoria. The host bacterium grown on NA, fungus and Trichoderma were grown on potato dextrose agar (PDA) for a week at room temperature (28±2°C). Growth parameters in all dual cultures and inhibition zone technique were read after 7 days. The plates containing only the target pathogenic organisms without Trichoderma were taken as control to evaluate the percent growth inhibition.

Morphological variation

Growth characteristics of Trichoderma isolates obtained from different locations were studied on PDA medium. Morphological characteristics such as colony growth, colour, reverse side colour, mycelia form, mycelia colour, conidiation and chlamydospores for each isolate were noted. The observations were recorded up to seven days at 24 hrs interval.

Molecular characterization

The total genomic DNA was extracted from each isolate of Trichoderma based on cetrimide tetradecyl trimethyl ammonium bromide (CTAB) mini extraction method of Crowhurst et al. (1995) with modification

Inter simple sequence repeats (ISSR) analysis

The procedure described by Bornet et al. (2002) with minor modi-fications was used for carrying out the polymerase chain reaction (PCR) reactions for ISSR analysis. Nineteen primers were tested for amplification at different annealing temperatures of genomic DNA of the isolates. Out of these, Ninteen primers consisting of anchored ISSR gave satisfactory amplification and band resolution. The PCR amplification was carried out with 25 ηg of genomic DNA, 2.5 mM MgCl2, 1 U Taq DNA polymerase, 1 X PCR buffer without MgCl2, 1 µM ISSR primer and 0.2 mM dNTP mix. The volume was made up to 25 µl. PCR reactions were carried out in a Perkin Elmer Gene-Amp 9600 thermocycler under the conditions involving denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, primer annealing at temperature specific to each primer for 1 min and primer extension at 72°C for 2 min; final extension step at 72°C for 7 min.

The PCR products of ISSR reactions were resolved on 1.4% agarose gel in 1 X TBE buffer pre-stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 90 volts for 1.5 h followed by 70 volts for 2 h and visualized under UV light on a UV- Transillu-minator. The gel was photographed using a Gel documentation system.

Data analysis

The amplification products ISSR were scored for the presence "1" and absence "0" and missing data as "9". The genetic associations between isolates were evaluated by calculating the Jaccard"s similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated. The computations were performed using the program NTSYS – PC. version 2.02h (Rohlf, 1997). The Jaccard"s similarity matrix was subjected to principal component analysis. This coordination method makes use of multidimensional solution of the observed relationships. PCA resolves complex relationships into a function of fewer and simpler factors. In this technique, the data matrix is derived from the distances (or similarities) between the operational taxonomic units.

III. Result

Confrontation assay in vitro

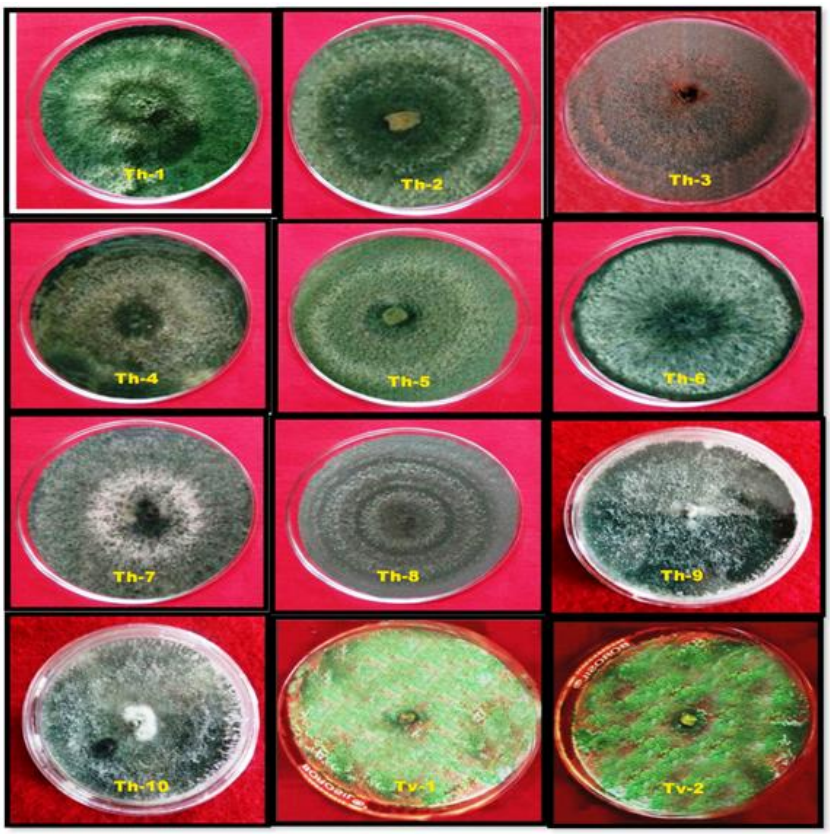
The isolates of Trichoderma spp. viz., Th1, Th2, Th3, Th4, Th5, Th6, Th7, Th8, Th9, Th10 belonging to T. harzianum and Tv1, Tv2 of T. viride were evaluated for their antagonistic ability against the Fusarium oxysporum f.sp. lycopersici based on percent inhibition mycelial growth, inhibition zone and mean mycelial growth (in cm^2). The isolate Th5 of T. harzianum found to have the maximum inhibiting effect on the growth of Fusarium oxysporum (78.93%)which directly corresponded to their mycelial growth rate of 17.00 mm against the target fungus. The isolate Th 3 of T. harzianum found to have the maximum inhibition zone against Xanthomonas campestris pv. Vesicatoria 24.44 mm. Among the T. viride isolates was not more aggressive against both pathogens.

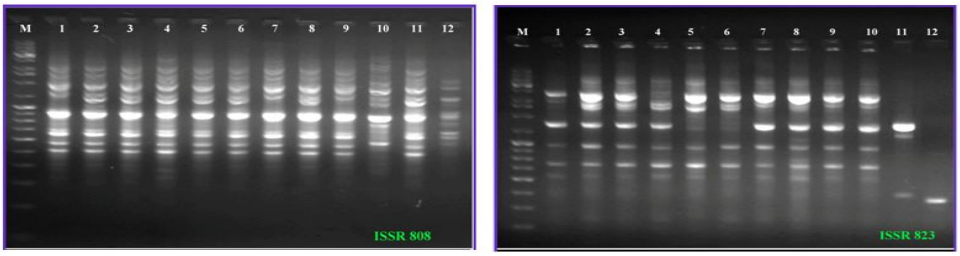
Morphological characterization

The growth patterns of Trichoderma isolates after three days of incubation at 25°C showed significant differences in nature of culture growth and sporulation patterns (Table 1 and Plate.1) The conidial wall patterns and shape were rough and subglobose among T. harzianum, while they were smooth and globose to obovoid among T. viride. The growth characters of culture and sporulation patterns varied within and between the species.

Table No. 1: Morphological characters of different isolates of Trichoderma on PDA.

Isolate	Colony colour	Reverse Colony colour	Mycelial form	Mycelial colour	Conidiation	Colony growth (cm/day)	Chlamydospores
Th 1	Green to dark green	Yellow	Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 2	Green to dark green	Yellow	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 3	Green to dark green	Light yellow	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Present globose
Th 4	Green to dark green	Creamish	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 5	Green to dark green	Light yellow	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 6	Green to dark green	Yellow	Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 7	Green to dark green	Yellow	Arachnoid	Watery white	Ring like zones	8-9 in 3days	Present globose
Th 8	Green to dark green	Light yellow	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 9	Green to dark green	Light yellow	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Th 10	Green to dark green	Creamish	Floccose to Arachnoid	Watery white	Ring like zones	8-9 in 3days	Not observed
Tv 1	Dark green	Deep yellow	Floccose to Arachnoid	Watery white	Concentric zones	9-10 in 5 days	Not observed
Tv 2	Dark green	Deep yellow	Arachnoid	Watery white	Concentric zones	9-10 in 5 days	Not observed





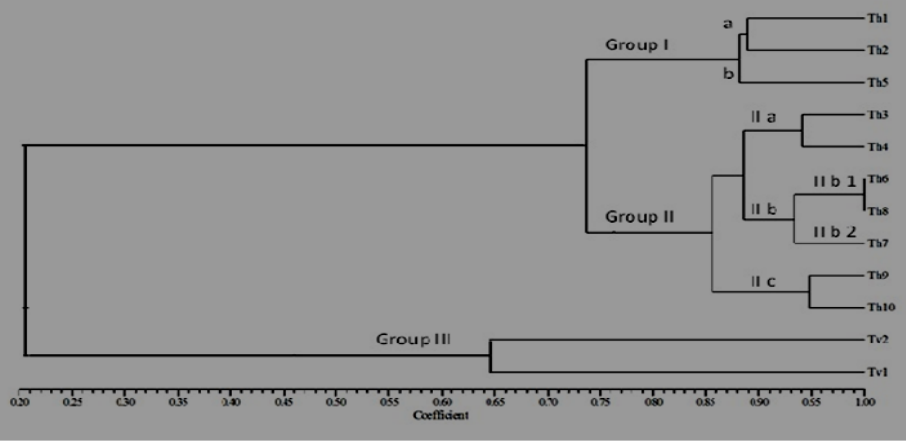
Molecular characterization

The genomic DNA of twelve different isolates of Trichoderma was subjected to PCR amplification using nineteen Inter Simple Sequence Repeat primers.

It was observed that 220 bands were generated in all the 12 different isolates, out of which 130 bands were polymorphic (59.09 %), 83 were unique bands (37.72%) while, 07 bands were monomorphic (3.18%). Polymorphic bands were observed maximum (14) in primer ISSR-821 while minimum polymorphic bands were observed in ISSR-814. Amongst all the primers the maximum amplification was observed with ISSR-821, ISSR-808 and ISSR-811 (22, 22 and 20 bands, respectively) primers, whereas least banding pattern was generated by ISSR-807 and ISSR 819 (3 polymorphic bands in each primer).

The primer ISSR-823 (Plate.2.a) showed maximum per cent polymorphism of 90.90%. All the primers showed the genetic polymorphism between the Trichoderma isolates tested. However, ISSR-808(Plate.2.b) was found to be the best primer for determination of variability among the Trichoderma isolates. This primer generated 22 bands of different molecular weight. Though all the isolates of Trichoderma were genetically variable, they share some common bands indicating phylogenetic relationship among them. Dendrogram representing the genetic relationship among the isolates based on ISSR analysis was developed and presented in Figure 3.Genetic similarity index was computed based on pooled data of ISSR profiles for isolates of Trichoderma as DICE coefficient (Nei and Li, 1979) using the similarity routine of NTSYS PC 2.0 software. The genetic similarity matrix based on the pooled data of ISSR profiles from all the 19 primers revealed that highest genetic similarity index of 1.00 was between the isolates Th-6 and Th-8. The minimum genetic similarity index 0.35 was observed in between the isolates Tv-1 and Th1.On the basis of ISSR analysis, Trichoderma isolates were classified into three broad groups; I, II, and III. Group I was clustered into two subgroups. I a consisted of two isolate viz., Th-1 and Th-2 having genetic similarity coefficient 0.89, I b consisting Th-5 isolate have genetic similarity coefficient 0.88. Group II were largest group consisting of seven isolates and was clustered in three subgroups. Subgroup II a consists of two isolate viz., Th-3 and Th-4 having genetic similarity coefficient 0.94. Subgroup II b consist of two subgroup II b 1 consist of two isolates Th-6 and Th-8 having genetic similarity coefficient 1.00 and II b 2 consists of single isolate Th-7 having genetic similarity coefficient 0.93. Subgroup II c consist of two isolates Th-9 and Th-10 having genetic similarity coefficient 0.95. Most divergent Group III consisted of two Trichoderma viride isolates Tv-1 and Tv-2 having genetic similarity coefficient 0.64. The similarity index clearly represents the phylogenetic relationship among different isolates of Trichoderma.

Fig. 3. Dendrogram representing the phylogenetic relationship among different isolates of *Trichoderma* based on ISSR analysis.



IV. Discussion

In pursuit for a probability of corroborating the myco-parasitic antagonism ability of Trichoderma with respect to their morphological and molecular characters the current study was undertaken. Such corroboration will certainly be a needful aspect in elucidating the molecular mechanism involved in mycoparasitic antagonism. Dual petri-plate marriage of Trichoderma with pathogen Fusarium oxysporum, and similarly inhibition zone technique endorsed the antagonism effect of T. harzianum and T. viride. T. harzianum isolates were more aggressive than T. viride in their antagonistic effect. The molecular and morphological markers could not establish any variation within T. harzianum isolates instead grouped all the isolates into one cluster. None of the markers were able to identify distinct variation among T. harzianum isolates and also it is prominent that these isolates exhibited less variation in their antagonism. Therefore it can be interpreted that these isolates taken for study had no much dissimilarity among them genetically. The quantitative and qualitative characters taken for morphological characterization were able to differentiate T. viride isolates into one cluster.

Though the morphological characterization had resulted in classifying the Trichoderma isolates, it can give only a broader picture. Waalwijk et al. (1996) indicated the diffi-culties to distinguish species based on morphology alone. Seaby (1996) was also of the same opinion where he reported that the morphological traits are subjected to environmental influence and can vary substantially from culture to culture. Thus to improve the reliability of morphological characters and to resolve the ambiguities the characterization should be complemen-ted with molecular data.

This authenticates the soundness of our perception that the bioefficacy of these two isolates were manifested at the molecular level which made them to distinguish from other T. viride isolates. Conversely Goes et al. (2002) reported that there was no relationship between the polymorphism showed by the Trichoderma isolates and their hardness against R. solani based on RAPD marker. Similar kind of result was reported by Shalini et al. (2006) and Shanmugam and Sharma (2008). However the results of molecular markers employed in this present study was complementary and confirmatory in nature which sub-stantiates our corroboration between bioefficacy of Trichoderma isolates and molecular characters. Thus we proceed to conclude that molecular marker ISSR can be used as a diagnostic kit to identify a superior Trichoderma strain for its commercial application.

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