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# Genetic Diversity and Biocontrol Potential of Rhizospheric Microbes Isolated from Tomato and Maize

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**Sixteen potential microbial agents consisting of seven bacterial isolates and nine *Trichoderma* isolates were used in study. Among studied 16 microbial culture isolates Bsp 3/aM, 2apa, Mys 21/a, Has1/c, T2, M5, M29 and M21 showed antagonism against *Aspergillus flavus* and *Fusarium verticillioides* and these isolates were positive for root colonization in maize seedlings. Rhizobacterial and *Trichoderma* sp. diversity was analyzed using sequence and molecular marker based analysis, which failed to group isolates based on antagonism, region of origin and root colonization. Interestingly, in ITS and ISSR diversity analysis two *Trichoderma* isolates T8 and M5 showed low diversity indicating them to be closely related isolates. The study highlighted use of diverse microbial agents showing antagonism and root colonizing potential which can be used in future for field applications and Screening microbial agents with molecular markers will prevent use of closely related isolates and enhance use of diverse and distinct isolates in biocontrol research.**

**Key words:** *Trichoderma*, rhizobacteria, maize, genetic diversity, antagonism and root colonization.

Maize is third largest cereal crop grown in world and is a source for food, feed and also serves as raw material for various industrial products[1]. Maize is susceptible to fungal infection during crop growth or at kernel storage conditions[2]. Major fungi infecting maize seeds are *A. flavus* and *F. verticillioides* which are known to produce aflatoxins and fumonisins[3]. Aflatoxins are potent natural carcinogens known to human kind and fumonisins are known to cause equine leukoencephalomalacia[4-6]. Similarly, aflatoxins and fumonisins are known to inhibit growth parameters like seed germination, seedling growth, root elongation, chlorophyll, carotenoid synthesis and reduce protein, nucleic acid and lipid contents in plants[7-8]. The methods used to manage toxins have their own drawbacks *viz.*, chemical fungicides have residual carry over issues and environmental side effects, building of host plant resistance has not been successful and agro environmental management has not been economical[9-13]. These problems associated with traditional methods are making way for alternative methods like seed treatment with biocontrol agents[14]. Beneficial traits shown for plant growth and protection by microbes may include antagonism, root colonization, antibiosis, parasitism, induction of resistance and production of growth regulators[15]. The usage of the microbial agents with multi beneficial traits is

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gaining importance for plant disease protection and growth promotion[16]. The advantage of using root colonizing and antagonistic microbial agents is that they have better survival rate in harsh environmental conditions as plants provide nutrition for growth and antagonistic nature of microbial agents inhibits the competitive microbes[17]. Antagonistic and root colonizing *Pseudomonas* and *Trichoderma* sp. have been used as seed treatment followed by foliar treatment to reduced incidence of *A. flavus* in soil and geocarposphere of groundnut[18].

The sequencing of 16S rDNA region in bacteria and internal transcribed spacer (ITS) region in fungi has become a method of choice for taxonomic identifications[19-21]. The microbes of same species collected from different or same geographical locations are known to show variation at biochemical and molecular levels[22,23]. Nowadays, sequences of 16S rDNA and ITS regions are being routinely used to resolve inter and intra species variability[24-26]. Likewise, Random Amplified Polymorphic DNA (RAPD) in bacteria and Inter Simple Sequence Repeats (ISSR) in fungi have been used to determine inter and intra species variability in microbes[27-30]. The application of DNA molecular markers like RAPD and ISSR have been used extensively to detect the duplicate entries or clones in the germplasm of plants[31,32]. However few studies of microbial culture collections have been screened for the duplicates entries or clones[33]. The present study was conducted inorder; (1) to screen selected microbial agents for antagonism and root colonization of maize and (2) molecular diversity analysis for establishing relation between isolates .

## MATERIALS AND METHODS

### Microbial agents and seed samples

All rhizospheric isolates consisting of *Trichoderma*, *Bacillus*, *Serratia*, *Pseudomonas* species and mycotoxigenic strains of *A. flavus* and *F. verticillioides* were collected from culture collection centre of the Department of Biotechnology, University of Mysore, India (Table 1.). All rhizobacterial strains were originally isolated from rhizosphere of tomato Hariprasad et al. (2014)[16] and *Trichoderma* sp. isolates were isolated from rhizospheres of maize. Maize cv. Hema seeds were collected from the National Seed Centre, located in Bangalore. All bacterial and fungal isolates were routinely sub-cultured on nutrient agar (NA) and potato dextrose agar (PDA) medium, respectively.

### *In vitro* antagonism assay

To study the antagonistic nature of selected biocontrol agents, dual culture technique was employed [34]. The interactions were studied in 90 mm Petri plates containing PDA. Four different bacterial isolates were point inoculated equidistantly along the edge of each plate and were allowed to grow for 24 h at 35 ± 2 °C. A 6-mm diameter agar disc of either *A. flavus* or *F. verticillioides*, from a 7-day-old culture, was placed in the center of each plate.

For the evaluation of *Trichoderma* isolates, a disc of each isolate (6 mm diameter) was taken from the edge of an actively growing colony and was transferred to one edge of each Petri plate. A similar size disc of either *A. flavus* or *F. verticillioides* was placed at the opposite edge. Control plates for each pathogen were also inoculated with agar plugs in similar orientations.

The plates were incubated for 7 days at 28 ± 2 °C, and the zone of inhibition was recorded for microbial agents studied. The experiments were conducted in triplicate and repeated twice.

### Root colonization of microbial agents

Rhizobacterial and *Trichoderma* inoculums were prepared and concentration of inoculums was adjusted to $1 \times 10^8$ cfu/ ml and $1 \times 10^8$ conidia/ ml, respectively according to Niranjana et al. (2009)[34]. Two hundred grams of maize seeds were weighed and treated with 1000 ml of microbial culture suspension containing 0.5% carboxymethyl cellulose (CMC) to facilitate adherence of the biocontrol agent to seeds. The seeds were incubated for 6 h at 28 ± 2 °C in a rotary shaker incubator at 150 rpm. Seeds treated with sterile distilled water (SDW) amended with 0.5% CMC served as a control.

Fifty maize seeds were placed equidistantly in a uniform layer of sterile moist sand and covered with sand. The setup was incubated under greenhouse conditions. The sand was kept moist by watering regularly to keep the sand moist. Observations relating to root colonization were analyzed by harvesting roots after 15 days. The roots were suspended in 45 ml of SDW and shaken for 30 min in rotary shaker (150 rpm) to suspend bacterial and fungal

propagules. 10 µl of serial dilution of these suspensions was spread on the Kings B agar medium and PDA plates and observed regularly. The fungal and bacterial isolates were identified based on their morphological and cultural characters.

**Genomic DNA extraction**

The bacterial DNA was extracted using bacterial DNA extraction kit GeNeiPure™ DNA Purification Kit (GeNei, India), similarly fungal genomic DNA was isolated using a Hi PurA™ Plant Genomic DNA Miniprep Purification Spin Kit (HiMedia, India). The extraction of genomic DNA was carried according to the manufacturer's instructions. After extraction of DNA concentrations were measured using NanoDrop* 2000/2000c spectrophotometer (Thermo Scientific, USA). The presence and quality of extracted DNA from microbial samples were analyzed by electrophoresis on 1% agarose gel (w/ v). The DNA gel was documented using GelDoc™ $XR^+$ with image lab TM software (Bio-Rad, Canada).

**ITS and ISSR analysis of *Trichoderma* isolates**

Target regions of the rDNA ITS region were amplified symmetrically using primers ITS 1 and ITS 4 according to White et al. (1990)[35]. ITS regions of *Trichoderma* sp. were sequenced at Eurofins, Bangalore and sequences were aligned using ClustalW Version 1.7[36]. The aligned sequences were used for construction of phylogenetic tree based on maximum likelihood method in Mega 5.0 software[37].

For ISSR PCR analysis, a total of 10 primers were synthesized from Sigma Aldrich (Bangalore) and were further used for PCR amplification of inter simple sequence repeats within the genome of studied *Trichoderma* isolates according to Chandrika and Rai (2009)[38]. Annealing temperature was primer specific (Table 2). Amplified products were then separated in a 1.2% in agarose gel electrophoresis with 1X TBE buffer.

**16S rDNA and RAPD analysis of bacteria**

The sequences of 16S rDNA region of studied bacteria were retrieved from GenBank and aligned using ClustalW Version 1.7[36]. The aligned sequences were used for determination of phylogenetic tree based on maximum likelihood method in Mega 5.0 software[37].

Ten RAPD primers synthesized at Sigma Aldrich (Bangalore) were used for amplification of DNA samples to obtain reproducible RAPD finger prints [39,40]. The PCR reaction mixture included 50 ng of genomic DNA, 10 pM of primer, 1.0 U of Taq DNA polymerase, 2 µl of 10× PCR buffer, 1.5 mM of $MgCl_2$, 0.2 Mm dNTPs, and the remaining volume was made up to 20 µl with nuclease free water. A total of 35 PCR cycles were performed with the following temperatures: initial denaturation at 94°C for 5min, denaturation at 94°C for 1 min, annealing at 40°C for 1 min, extension at 72°C for 1min, and final extension at 72°C for 5min. Amplified products were then separated in a 1.2% agarose gel using 60 V $cm^{-1}$ electrophoresis in 1X TBE buffer (Table 3).

**RAPD and ISSR Data analysis**

Reproducible amplified and unambiguous ISSR and RAPD fragments were scored for the presence (1) or absence (0) in ISSR and RAPD patterns. The presences of similar bands in all isolates were referred to as monomorphic, whereas those that differed were referred to as polymorphic. Clustering analysis of the strains was performed using the UPGMA functionality in TFPGA.

## RESULTS

***In vitro* antagonism assay**

Among seven bacteria screened, maximum inhibition to *A. flavus* and *F. verticillioides*, was recorded by Has 1/c while, 9/e and Bag 6/a isolates did not inhibit test fungi. Further, among the *Trichoderma* isolates, T2 exhibited a maximum growth inhibition of *A. flavus* and *F. verticillioides* and all other *Trichoderma* isolates showed varied protection and M3 isolate offered least inhibition of test fungi (Table 1; Figure 1).

**Root colonization by microbial isolates**

The root colonization assay for bacteria was performed by plating maize root suspensions on Kings B agar medium plates. Among seven bacterial isolates, four isolates namely Bsp 3/aM, 2apa, Mys 21/a and Has1/c were successfully isolated from the root suspensions plated on KB medium. The root colonization ability of *Trichoderma* isolates were done by plating maize root suspensions on PDA plates. Among the nine *Trichoderma* isolates, M29, M5, T2 and M21 showed presence of colonies in PDA medium (Figure 2).

**ITS and ISSR diversity analysis of *Trichoderma* isolates**

Maximum likelihood tree was generated using MEGA 5 software for the ITS sequences of *Trichoderma* isolates and were grouped into two major clusters, Cluster I and Cluster II, respectively. The Cluster I contained M21, M29, M43, T2, T9, T8 and M5 isolates. The Cluster II contained two isolates T13 and M3. Each node of the phylogenetic tree was well supported with high boot strap values (Figure 3).

The entire selected *Trichoderma* sp. isolates were analyzed using 10 ISSR primers to investigate the degree of genetic diversity among and within the isolates. The ISSR primers generated very distinct amplification products, showing considerable variability among the isolates. A total of 70 bands consistent in repeated amplification were scored from all isolates. The number of ISSR fragments produced per primer varied, with the number of polymorphic bands ranging from three to eleven (Table 2). The size of the PCR fragments

**Table 1.** List of isolates used in the study for inhibition of mycotoxigenic fungi and root colonization

| Species identification | Biocontrol agent | GenBank Accession | *A. flavus* Inhibition | *F. verticillioides* Root colonization | *In vitro* |
|---|---|---|---|---|---|
| Bacteria | | | | | |
| *Bacillus* sp. | Bsp 3/aM | KJ941327 | +++ | +++ | + |
| *Bacillus cereus* | Kol-2/B | HM229803 | + | + | - |
| *Pseudomonas aeruginosa* | 2apa | HM229797 | ++ | ++ | + |
| *Pseudomonas fluorescens* | Mys 21/a | HM229810 | + | ++ | + |
| *Pseudomonas putida* | Has 1/c | HM229805 | +++ | +++ | + |
| *Serratia* sp. | Bag 6/a | HM229811 | + | + | - |
| *Serratia marscens* | 9/e | FJ897544 | + | + | - |
| *Trichoderma* spp. | | | | | |
| *Trichoderma atroviride* | M-3 | JX422012 | ++ | ++ | - |
| *Trichoderma asperellum* | M-5 | KC213819 | +++ | +++ | + |
| *Trichoderma asperellum* | M- 21 | JX422011 | ++ | ++ | + |
| *Trichoderma asperellum* | M-29 | JX422010 | + | + | + |
| *Trichoderma asperellum* | M-43 | JX422009 | + | + | - |
| *Trichoderma asperellum* | T-2 | JX422015 | +++ | +++ | + |
| *Trichoderma asperellum* | T-8 | JX422014 | ++ | ++ | - |
| *Trichoderma asperellum* | T-9 | JX422013 | + | + | - |
| *Trichoderma asperellum* | T-13 | JX422016 | ++ | ++ | - |

Notes: + = showed inhibition or root colonization; - = showed no inhibition or no root colonization

**Table 2.** List of ISSR primers used in analysis of *Trichoderma* isolates

| S. No | Primer Name | Sequence | Annealing (°C) | Monomorphic bands | Polymorphic bands | Polymorphism (%) |
|---|---|---|---|---|---|---|
| 1 | ISSR02 | $(CT)_7AC$ | 40 | 2 | 6 | 75 |
| 2 | ISSR03 | $(CT)_7GC$ | 43 | 1 | 8 | 88.8 |
| 3 | ISSR04 | $(CA)_6AC$ | 43 | 2 | 9 | 81 |
| 4 | ISSR05 | $(CA)_6GT$ | 43 | 0 | 8 | 100 |
| 5 | ISSR06 | $(CA)_6AG$ | 43 | 2 | 8 | 80 |
| 6 | ISSR07 | $(CA)_6GC$ | 43 | 0 | 7 | 100 |
| 7 | ISSR09 | $(GT)_6GG$ | 40 | 0 | 4 | 100 |
| 8 | ISSR10 | $(GA)_6CC$ | 40 | 0 | 5 | 100 |
| 9 | ISSR12 | $(CAC)_3GC$ | 40 | 1 | 4 | 80 |
| 10 | ISSR13 | $(GAG)_3GC$ | 46 | 0 | 3 | 100 |

**Table 3.** List of RAPD primers used in analysis of bacterial isolates

| S. No | Primer Name | Sequence | Annealing (°C) | Monomorphic bands | Polymorphic bands | Polymorphism (%) |
|---|---|---|---|---|---|---|
| 1 | PM1 | GCCCCTGGAG | 40 | 1 | 7 | 87.5 |
| 2 | PM2 | AACGGGCAGG | 40 | 2 | 7 | 77.7 |
| 3 | PM3 | GGCTGCGGTA | 40 | 1 | 6 | 85.7 |
| 4 | PM4 | GCGGAGGTCC | 40 | 2 | 5 | 71.4 |
| 5 | PM5 | CGACGCCCTG | 40 | 0 | 5 | 100 |
| 6 | PM6 | GCGTCGAGGG | 40 | 1 | 8 | 88.9 |
| 7 | CRA22 | CCGCAGCCAA | 40 | 3 | 9 | 75 |
| 8 | CRA23 | GCGATCCCCA | 40 | 1 | 6 | 85.7 |
| 9 | CRA25 | AACGCGCAAC | 40 | 0 | 8 | 100 |
| 10 | CRA26 | GTGGATGCGA | 40 | 0 | 6 | 100 |

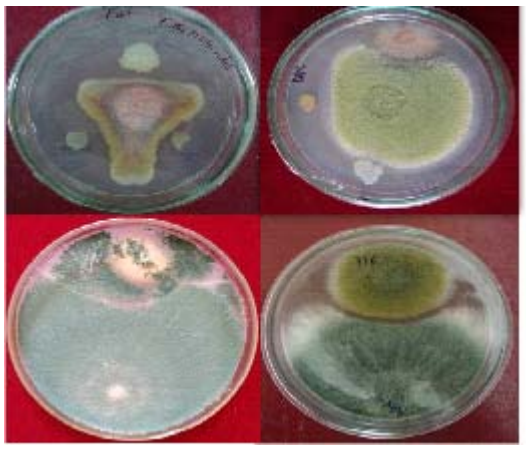

**Fig. 1.** Antagonism of selected Rhizobacteria and Trichoderma against *Fusarium verticillioides* and *Aspergillus flavus*

**Fig. 2.** Root colonization potential of a) rhizospheric bacterial isolates b) *Trichoderma* sp. isolates

generated ranged from 160 to 1200 bp (Figure 4). The distance matrix of genetic relatedness between *Trichoderma* sp. isolates ranged from 0.0 to 0.35. The dendrogram of ISSR analyses grouped the selected *Trichoderma* isolates into two clusters. Cluster I contained 4 isolates namely T8, M5, T13 and M3) and Cluster II contained 5 isolates namely T2, T9, M29, M43 and M21. Further, detailed analysis of Cluster I revealed isolates T8 and M5 were similar with the genetic distance 0.0 (Figure 5).

**16S rDNA and RAPD based diversity of rhizobacteria**

Maximum likelihood tree was generated using MEGA 5 software for 16S rDNA sequences. The selected bacterial isolates were clustered into two major clusters namely , Cluster I and Cluster II. Cluster I contained Has-1/c, strain 9/e, Kol-2/b and Bag-6/a isolates, while Bsp3/a, Mys-21/a and 2apa isolates were grouped in Cluster II. All the nodes of the phylogenetic tree were well supported with high boot strap values (Figure 6).

To investigate the degree of genetic diversity within bacterial isolates 10 RAPD primers were used. RAPD primers generated very distinct amplification products, among the isolates. A total of 78 bands were scored consistently with repeated amplification from all the isolates. The number of RAPD fragments produced per primer varied, with the number of polymorphic bands ranging from five to twelve (Table 3). The size of the PCR fragments generated ranged from 160 to 1100 bp (Figure 7). In order to assess the genetic relatedness between bacterial isolates, the distance matrix was calculated based on the obtained fingerprints and was found to range from 0.0 to 0.45. This indicates wide genetic diversity among and within bacterial isolates. Using TFPGA software, a phylogenetic analysis was carried out and a dendrogram was constructed. The dendrogram of RAPD analyses grouped bacterial isolates into two clusters. The

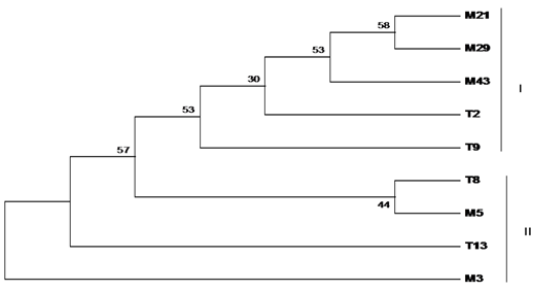


**Fig. 3.** Molecular Phylogenetic analysis of *Trichoderma* ITS sequences by Maximum Likelihood method by MEGA 6

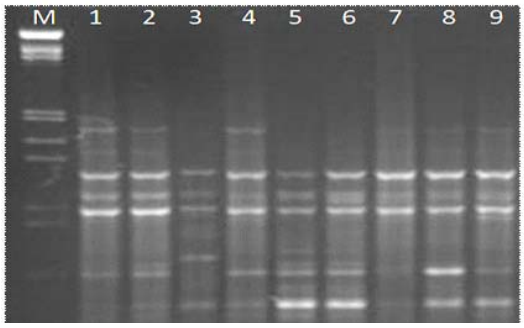


**Fig. 4.** ISSR profile of the nine *Trichoderma* isolates generated by using the primer ISSR04**.** Lane M. DNA marker, Lane 1 – 9 (M21, M29, T13, M43, M3, T8, M5, T2 and T9 , respectively)

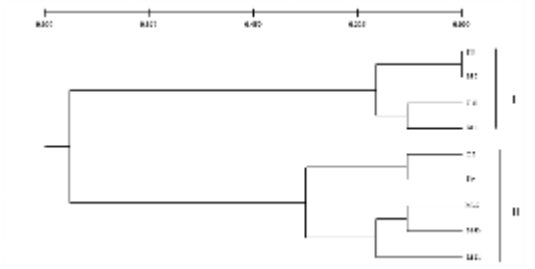


**Fig. 5.** UPGMA Dendrogram based on the ISSR marker analysis of nine different *Trichoderma* isolates

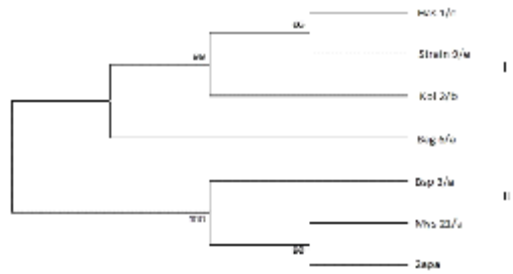


**Fig. 6.** Molecular Phylogenetic analysis of rhizobacterial 16S rDNA sequences by Maximum Likelihood method by MEGA 6

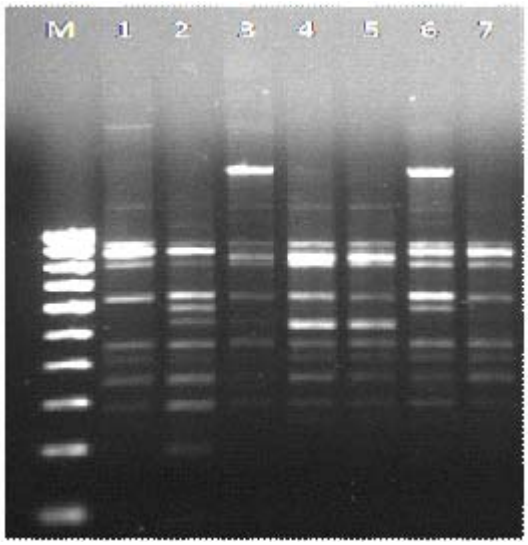


**Fig. 7.** RAPD profile of the seven rhizobacterial isolates generated by using the primer CRA22**.** Lane M. DNA marker, Lane 1 – 7 (Has 1/c, Bsp 3/a, Strain 9/e, Mys 21/a, Bag-6/a, Kol-2/b and 2apa , respectively )

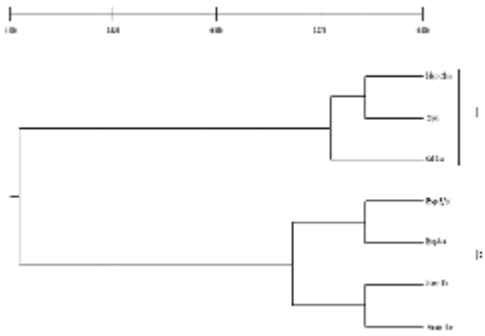

**Fig. 8.** UPGMA Dendrogram based on the RAPD marker analysis of 7 different rhizobacterial isolates.

Cluster I contained Kol 2/a, 2 apa and Bsp 3/a bacterial isolates. The Cluster II was Subclustered into Sub-clusters IIa and IIb which contained Kol 2/B, Bag6/a, Has 1/c and 9/e bacterial isolates (Figure 8).

## DISCUSSION

Control strategies aimed for reducing crop infection by phytopathogens have been widely used, based on the use of chemical pesticides[41]. The inappropriate use of chemical control agents tends to promotes water pollution, soil degradation and building of insect resistance[42]. Rhizosphere, serves diverse rich niche for microbial biocontrol agents with plant growth-promoting and protection properties[16]. The present study utilized rhizosphere microbial species like *T. atroviride, T. asperellum, P. fluorescens, S. marscens, Bacillus* sp., *Serratia* sp., *P. aeruginosa, P. putida* and *B. cereus* which have been successfully used in earlier studies for biocontrol of plant pathogens[43-45]. Among the studied bacterial and *Trichoderma* isolates Mys 21/a, Bsp 3/aM, 2apa, Has 1/c, M 5, M 21, M 29 and T 2 were positive for antagonism and root colonization. The microbial agents with antagonistic nature represent a promising strategy for biological control[15,46]. Knowledge concerning the mechanism of pathogen inhibition by microbial agents is important for successful application of biocontrol agents[47]. The antagonism mechanism offered by the selected microbial agents may be due to antibiosis, competition, mycoparasitism and production of cell wall degrading enzymes[48,16]. Root colonizing microbes are known to form physical barrier between the root and pathogen, thereby controlling plant pathogens[15,49-51]. In the present study, some of the tested isolates did not show antagonism against *A. flavus* and *F. verticilloides* or root colonization (Table 1). Further, these isolates may be evaluated for additional mechanisms of fungal protection like induction of resistance in plants[52,53].

The genetic diversity analysis using 16S rDNA sequences and RAPD primers for the bacterial isolates revealed that the tree generated were very similar which consisted of two major clusters. The present results are concordant with other studies, where similar genetic trees were generated using three different molecular methods like RAPD, RFLP and 16S rDNA sequences in *Herbaspirillum* bacteria[54]. The phylogenetic tree generated for *Trichoderma* sp. using ITS sequences and ISSR generated nearly similar trees which consisted of two major clusters. In case of microbial agents of few repositories/ collection centers were analyzed for duplicate entries. However, ISSR and RAPD makers have successfully used for screening of duplicates in plants and bacteria by various researchers in germplasm and microbial stock cultures[28,55,56]. The ISSR analysis of *Trichoderma* sp. of the present study indicated that T8 and M5 isolates had no diversity between them and indicated that the isolates are duplicates, which was further supported by the results of ITS sequence tree. The phylogenetic trees generated failed to separate biocontrol isolates based on their geographic origin, antagonism and root colonizing potential. Likewise, various reports on molecular markers have also failed to group on the above said parameters[57,58]. In conclusion, the present study provides an insight into abilities of biocontrol agents for mycotoxigenic fungi and screening of the isolates with molecular makers initially will prevent the usage of the similar strains or clones during in biocontrol treatments.

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