Genetic Diversity Evaluation of *Bacillus* isolates using Randomly Amplified Polymorphic DNA molecular marker

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Abstract

The explorations of the eco-friendly approaches to pest and pathogen management have given birth to biological control agents and Bacillus being an endospore former, they are appealing candidates for biocontrol which provide them the ability to tolerate heat and desiccation. The knowledge concerning the behavior of these Bacilli as antagonists and genetic analysis is essential for their effective use and the commercialization. The present study was focused on the analysis of the genetic diversity of rhizobacterial isolates of Bacillus with antifungal activity using PCR based RAPD technique, which could result in identification of strains. The in vitro antifungal assay and phenotypic characterization was not sufficient to differentiate between the antagonistic isolates, as the biochemical and enzymatic fingerprints were identical. Twenty isolates were selected for the present study and characterized by various biochemical tests and enzyme production. 18 RAPD primers of OPB series were found to be polymorphic. The fingerprints obtained in all the 18 primers have remarkably indicated the polymorphic bands. From the dendrogram generated using the NTSYSpc programme, the genetic diversity is evident based on the percentage similarity between the isolates. Five major clusters were obtained at 61% similarity level.

Keywords: *Bacillus, R. solani*, *F. oxysporum,* Antagonism, RAPD PCR, Genetic Diversity.

Abbreviations: PCR- Polymerase Chain Reaction, RAPD – Randomly Amplified Polymorphic DNA, spp. - species

Introduction

Rhizoctonia solani Kuhn and *Fusarium oxysporum* are the pathogens associated with the root rot & damping off and wilting in cotton respectively. Individually both the fungi are pathogenic to several commercial crops in India and worldwide. In presence of host debris, *R. solani* is difficult to manage by chemical means or by breeding for resistance and *F. oxysporum* is almost impossible to eradicate once it establishes in the field soil. The explorations of the eco-friendly approaches to pest and pathogen management have given birth to biological control agents, which has been studied for over 65yrs [1, 2]. Several studies have been reported the ability of various bacteria for providing substantial disease control caused by soil borne and seed borne plant pathogen.

Biocontrol agents are not limited to a specific bacterial group; however, given the diversity of the rhizosphere microflora, it is probable that the full spectrum of potentially effective strains has barely been explored, many of these being effective under field conditions. Two fungal antagonists viz. *Trichoderma* and *Gliocladium* species and two bacteria viz. *Pseudomonas* and *Bacillus* have extensively been used for the control of root rot and an Indian strain of *T. harzianum* was reported to be promising biocontrol agents for *F. oxysporum* [3, 4]. The gram positive bacteria, among *Bacillus* spp., especially *B. subtilis* A13 have been explored with the antagonistic activity against the phytopathogenic fungi and tested on a wide variety of plant species for ability to control diseases [5, 6]. The *Bacillus* are appealing candidates for biocontrol as they produce endospores, which provide them the ability to tolerate heat and desiccation [7]. Though they have been investigated as biological control agents, the knowledge concerning the behavior of these *Bacilli* as antagonists and genetic analysis is essential for their effective use and the commercialization.

The so far identified antifungal bioagents, *Pseudomonas* have been intensively investigated with regard to the production of antimicrobial metabolites, genetic analysis and regulation of some metabolites and ecological fitness of soil [8-11]. The relationship between the rhizosphere environment and genetic diversity of local microbial populations need to be studied for evaluating the effect of microbial inoculation on the preexisting balance in indigenous populations [12]. The diversity study of antifungal rhizobacteria using fingerprint techniques is important not only for understanding, their ecological role in the rhizosphere, but also for the (i) registration and patenting biocontrol strains, (ii) recognition of the strains, (iii) ecological characterization [13, 14]. The advent of molecular biology in general and the PCR reaction in particular have greatly facilitated genomic analysis of microorganisms, provide enhanced capability to characterize and classify strains and facilitate research to assess the genetic diversity of populations [15] and RAPD technique based on PCR has been on the most commonly used molecular technique to develop DNA marker as it do not require prior knowledge of the genome subjected to analysis. RAPD-PCR markers have been

found to be efficient in demonstrating the DNA polymorphism and have been used extensively to assess the diversity or nature of phytobacteria [16, 17].

The present study focused on the genetic diversity analysis of rhizobacillus isolates with antifungal activity using RAPD technique that could be considered as a major step towards the development of identifiable DNA markers of the isolates.

Material and Method

Isolation and identification of the Bacillus spp:

The soil samples used in present study for the isolation of *Bacillus* spp. were collected from cotton cultivating farms of Vidarbha region of Maharashtra, India, from different sites viz. from rhizosphere of cotton plant and bulky soil in the farming fields. The roots along with the adhered soil on the root surface were allowed to dry overnight at room temperature and was collected in a fresh sterile container. A pinch of soil sample was suspended in 100µl sterile distilled water, incubated in water bath at 80ºC for 10min and a loopful suspension was streaked on nutrient agar plate. The plates were incubated at 28ºC for 24 to 40hrs. & the colonies appeared on the media were checked for the grams nature, motility and catalase enzyme production. The selected isolates were stored in nutrient broth with 15% glycerol at -70ºC. The biochemical characterization was done according to the Bergey's manual of determinative Bacteriology, but only relevant biochemical tests were considered for this study.

In vitro antifungal assay for antagonism:

The isolates identified as *Bacillus* were tested for their antagonistic activity *in vitro* against *Rhizoctonia solani* and *Fusarium oxysporum* using dual culture method. A fungal disc of five day old mycelial mat of 1cm diameter was placed in the centre of the petri plate containing corn meal agar and nutrient agar medium in 2:1 ratio to facilitate the fungal as well as bacterial growth and the 24hr old *Bacillus* culture was single streaked at a distance of approx. 3cm from the fungal disc. The plate was incubated at 28ºC and observed after every 24hrs for any inhibition of mycelial growth.

The isolates were divided into 3 types based on their antagonistic activity in which type I included all those isolates which gave a remarkable antagonism towards both the fungi. The isolates, which have given the antifungal activity against either of the fungi or less remarkable antagonism, were categorized into type II and the isolates without any antagonistic property against any of the fungi were classified under type III. The selected isolates were further used for the isolation of genomic DNA.

DNA isolation and PCR assay using RAPD markers:

DNA was extracted from 24hr old culture grown in Luria Burtani (LB) medium using a modified method of DNA isolation [18] and the quality and quantity was determined by agarose gel electrophoresis with known amounts of DNA marker (Hyperladder, Biolin) as a standard. Twenty RAPD primers of OPB series were used for the analysis. The PCR programme was set with initial denaturing step of 6 min. at 94°C, followed by 35 PCR cycles (denaturing at 94°C for 45 sec, a primer annealing at 36°C for 1 min, and primer extension at 72°C for 1 min). A final extension of 10 min at 72°C was carried out for polishing the ends of PCR products. The DNA amplification was carried out in Biometra UNO thermoblock thermalcycler and the PCR products were analyzed by electrophoresis on 1.5 % agarose gel containing 0.1µg/µl of ethidium bromide and were documented with Alpha Innotech digital gel documentation system.

Data analysis:

Comparison of genotypes was carried out based on the presence or absence of fragment produced by RAPD PCR amplification. '1' was designated for presence of fragments and '0' for the absence of fragments. Pair wise Jaccard's similarity coefficients [19] were calculated. Cluster analysis was performed for the molecular data based on UPGMA method [20] and SAHN using NTSYS-PC [21].

Results and Discussion

The phenotypic and genotypic characterization of the antifungal *Bacillus* isolates was studied to demonstrate the diversity amongst the rhizospheric and bulk farm soil *Bacillus* isolates. The *in-vitro* dual culture assay was performed for detection of antifungal *Bacillus* isolates and RAPD PCR analysis was done for the genetic diversity study.

Isolation, identification and screening of antagonistic Bacillus isolates:

The heat resistant, gram-positive aerobic rods, catalase positive and endospore forming isolates were selected and maintained on nutrient agar slants. The isolates obtained were assayed for their antagonistic activity against *Rhizoctonia solani* and *Fusarium oxysporum* by dual culture method and the total sixteen isolates produced a clear zone around the bacterial growth (Fig 1) indicating the inhibition of the fungal growth. Eight isolates were marked with strong antagonism against both the phytopathogens, while rest of the isolates shown strong antagonism against either of the phytopathogen. Apart from these 16 isolates, eight isolates selected were showing very less or absolutely no antifungal activity of which four isolates were selected for the comparative study. Different patterns of *in vitro* inhibition of fungal growth were obtained. The aggressive antagonism pattern was marked for those isolates, which inhibited the fungal growth *in vitro* only after 48hrs, resulting into formation of a clear zone around the bacterial growth, the reason may be production of extracellular antifungal component. Some isolates found to inhibit the fungal

growth only after their contact, which indicate that attachment of bacterial cell on mycelium is necessary for the inhibition of fungal growth. Thus overall twenty four isolates were selected for the present study as listed in table 1. The isolates were characterized by various biochemical tests and for enzyme production (Table 1).

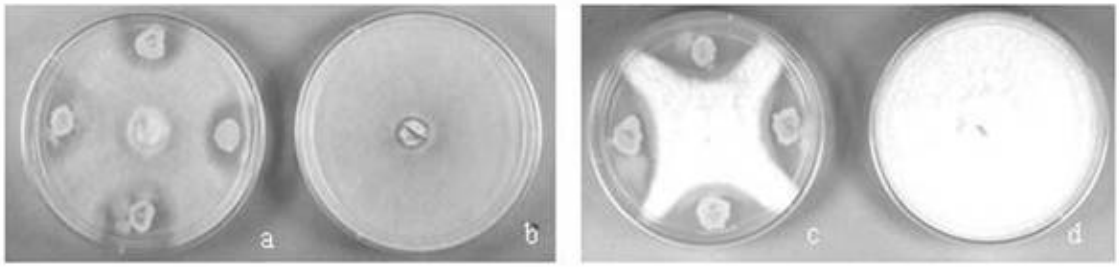

Figure 1: *In Vitro* Antifungal Assay by Dual Culture method. **a**: *Bacillus* isolate (BP-5) against *Rhizoctonia solani*; **b**: Control plate of *Rhizoctonia solani*; **c**: *Bacillus* isolate (BP-5) against *Fusarium oxysporum*; **d**: Control plate of *Fusarium oxysporum*.

Genomic DNA isolation, RAPD PCR and genotypic characterization:

A good quality genomic DNA isolated from the *Bacillus* isolates was used for the RAPD PCR amplification with twenty primers of OPB series. Out of which 18 primers except OPB 02 and OPB 03, were found to be polymorphic. The primers OPB 10, OPB 11, OPB 12, OPB 13, OPB, 15, OPB 17, OPB 18 and OPB 20 effectively amplified genomic DNA in all 24 samples. Only strong bands were considered for binary counting avoiding the blurred faint bands to generate the precise dendrogram. Total 1586 bands were scored on the 1.5 % agarose gel with all the eighteen primers. The per sample amplification has generated a single scorable band to a maximum of 12 bands of 400bp to 4 kb. The polymorphic bands obtained in all the 18 primers remarkably revealed the polymorphism among the *Bacillus* isolates (Fig 2).

Table 1. Origin, antagonistic property and important biochemical characteristics for identification of Bacillus isolates.

Sr. No.	Isolate No.	Soil No.	Antagonism level	Glucose (Acid/Gas)	Acetoin	Starch hydrolysis	Gelatin hydrolysis	Mannitol	Lecithinase
1.	BP-1	Soil No.I (Yavatmal)	+++	+/-	+	+	+	+	-
2.	BP-2		+++	+/-	+	+	+	+	-
3.	BP-3		++	+/-	++	+	+	+	-
4.	BN-4		-	+/-	-	+	+	+	-
5.	BP-5		+++	+/-	+	+	+	+	-
6.	BP-6	Soil No. II (Nagpur)	++	+/-	+	+	+	+	-
7.	BN-7		-	+/-	-	+	+	-	+
8.	BP-8	Soil No. IV (Nagpur)	++	+/-	-	+	+	-	+
9.	BP-9	Soil No. V (Nagpur)	+++	+/-	+	+	+	+	+
10.	BP-10		+++	+/-	+	+	+	+	-
11.	BP-11		++	-/-	-	-	+	-	+
12.	BN-12		-	+/-	-	-	-	+	-
13.	BP-13	Soil No. VI (Nagpur)	+++	+/-	+	+	-	+	-
14.	BP-14	Soil No. VII (Yavatmal)	+++	+/-	++	+	+	+	-
15.	BP-15		++	+/-	-	+	+	-	+
16.	BN-16		-	+/-	-	+	+	+	-
17.	BN-17	Soil No. IX Nagpur	-	+/-	-	-	+	+	-
18.	BN-18	Soil No. XII Nagpur (Rhizoshere)	-	+/-	-	+	+	+	-
19.	BP-19	Soil No. XII Nagpur (Rhizoshere)	++	+/-	-	+	+	+	-
20.	BP-20	Soil No. XII Nagpur (Rhizoshere)	+++	+/-	+	+	+	+	-
21.	BN-21 (*B.cereus*)	RGBC, Nagpur	-	+/-	+	+	-	+	+
22.	BN-22	RGBC,	-	+/-	-	-	-	+	-

	(B. cereus)	Nagpur							
23.	BP-23 *(B. thuringiensis* B-17)	RGBC, Nagpur	+	+/-	+	+	+	+	+
24.	BP-24 *(B. thuringiensis* -IPS80)	RGBC, Nagpur	+	+/-	+	+	+	+	+
25.	Blank	-	-	-/-	-	-	-	-	-

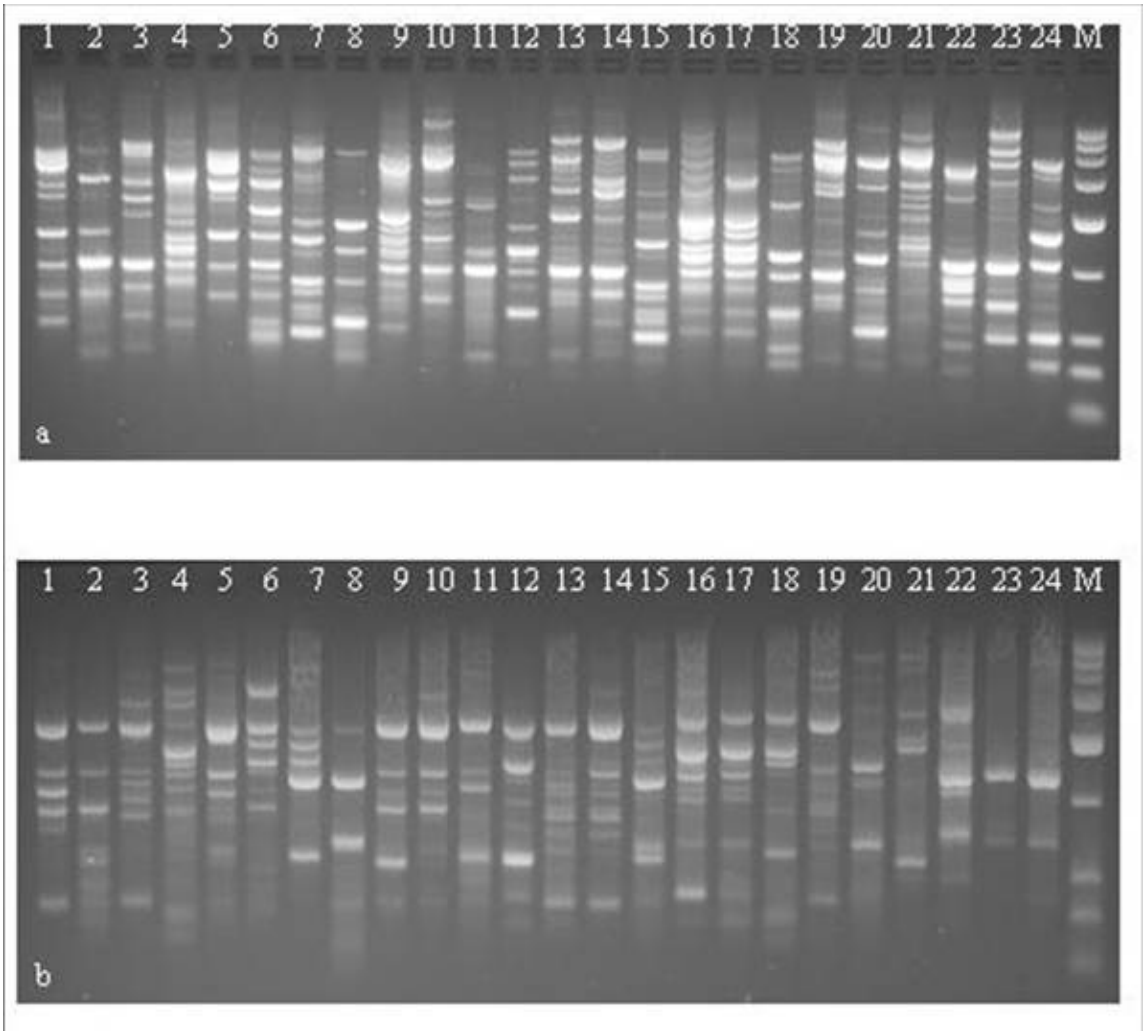

Figure 2: RAPD profiles of *Bacillus* isolates obtained with **a**: OPB 17 & **b**: OPB 18, 1 to 24 *Bacillus* isolates (Table 1), M- Marker DNA.

The genetic diversity between the isolates is evident from the dendrogram generated using the NTSYSpc programme (Fig 3). Five major clusters were obtained at 61% similarity level, separating the strong antagonists (Cluster I) from rest of the isolates, sharing a similarity coefficient of 0.79 which is quite high enough to share the same species [22, 23]. Even though the isolates in cluster I were from two different origins (two isolates from Nagpur district and three from Yavatmal district.), they share the common phenotypic characteristics like production of acid and acetoin from glucose, hydrolysis of starch and gelatin and growth at 10°C and 55°C and classifies them under *B. subtilis* group.

Two clusters that shared the highest similarity level of 70% were found to be clusters II and III, in which cluster II consisting of four isolates, two with aggressive antagonism (BP13 & BP 14) and two with less antagonism (BP3 & BP 19). All the four isolates of cluster II had different origin sites viz. BP19 was the rhizospheric isolate from Nagpur region, and other three were from bulky soils of different areas of Yavatmal and Nagpur, showing the similarity between the wide isolates. The isolate BP3 and BP19 were more similar in antifungal activity, but biochemical reactions had shown BP19 being most different from the rest of the three, whereas revealed from the dendrogram BP3 was the most diverse sharing 76% similarity with the other three isolates. BP14 which shares approximately 85% genetic similarity with BP19 was found to be more similar to BP3 in sharing common biochemical reactions.

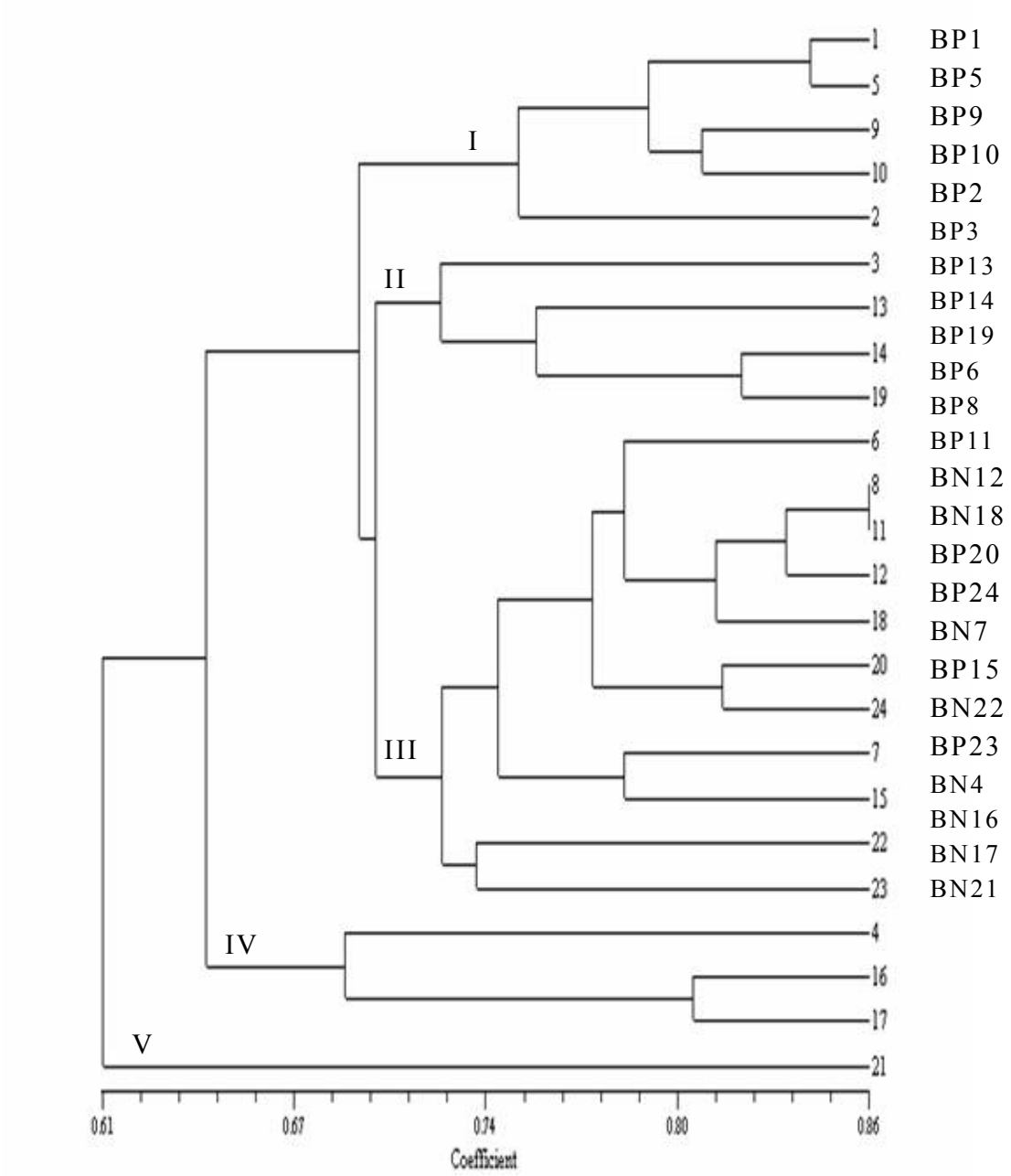

Figure 3: UPGMA Dendrogram based on the Jaccard similarity coefficient illustrating the genetic relationship among 24 *Bacillus* isolates.

Cluster III formed a major group of total 11 isolates consisting one strong antagonist (BP20), six moderate antagonists (BP-6, BP-8, BP-11, BP-15, BP23, BP24) and four absolutely ineffective isolates (BN12, BN18, BN7, BN22), as a result evidences most comparable diversity. Even though belonging to different species, the isolates BP23 (*B. thuringiensis*) and BN22 (*B. cereus*) share similarity of approx. 73%, while BP23 and BP24 were 71% similar both being the strains of *B. thuringiensis*. BP24 shared highest similarity level of 81% with BP20, which employs the best example for similarity of two distinct isolates with different phenotypic characteristics, not only for antagonism, but also the biochemical characteristics except production of acid & acetoin from glucose and sensitivity to 10°C & 55°C. BP8 and BP11 shared the highest similarity of 86% amongst all the 24 isolates. These isolates were from different origins and also differed in biochemical characteristics, but observed the similar antifungal activity. The isolates BN7 and BP15, from two different origins i.e. from Nagpur and Yavatmal respectively, were found to be genetically similar. Whereas, BP14, BP15 and BN16, the isolates of the Yavatmal were found genetically distinct in dendrogram and were distributed in three different clusters.

Cluster IV was formed of three isolates from different soil samples, all being non antagonistic and shared common biochemical properties. There was a very less difference in the similarity between cluster I, cluster II & cluster III, and share 69% genetic similarity. The overall similarity between clusters I to IV was 64.5%, whereas cluster V consisted of a single isolate BN21 separating it from rest of the groups at 39% diversity and thus making it the most diverse isolate belongs to *B. cereus*.

The genetic diversity studies on the isolates having a potential biocontrol agent done on *Trichoderma* isolates by RAPD, the profiles of which classified them in different groups according to their capacity for control of the plant pathogenic fungi [24, 25]. In this study, most of the strong aggressive antagonists shared common phenotypic characteristics and the non antagonists shared the similar phenotypic characteristics and were grouped in different clusters, cluster I and cluster IV respectively, which could provide information that most of the biocontrol agents of *Bacillus* group belong to a single species. On the contrary cluster III, which was a combination of all kinds of characteristics discussed above may not support the above statement. Hence, the *Bacillus* may share more than 60% of their genomic similarity irrespective of the species. *Bacillus subtilis*, *B. pumilis* strains from industrial and artisenal cured sausages were studied for genotyping and toxigenic potential using RAPD-PCR technique by Maturante [26] and the similarity coefficient was observed in a range of 0.12 to 1.00 among the Bacillus species grouped under *B. subtilis* and *B. pumilis*. The genetic polymorphism of *Bacillus* strains from hot spring sources by RAPD-PCR and phenotypic characteristic has been studied by Hazem [27] and resulted with five major clusters at 60% similarity. The similarity observed by Marten P et al among the biocontrol strains of *B. subtilis* was 90% [28], whereas, in our study the similarity was ranged between 61 to 86%.

Conclusion

The similarity level amongst the biocontrol isolates of *Bacillus* was observed to be very high, hence it was essential to study the isolates very carefully for providing a perfect fingerprint for its identification. The present study was an approach to provide the information about the diversity of *Bacillus* isolates of bulky soil and rhizosphere with a potential use as *R. solani* and *F. oxysporum* biocontrol agent. The biochemical and enzymatic fingerprinting alone were not enough to differentiate the isolates as many of them shared the common characteristics, whereas RAPD DNA fingerprinting has given a high degree of polymorphism and separated them into different clusters, which may prove to be a tool for providing a genetic base for identification and differentiation of antagonistic *Bacillus*.

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