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Molecular Identification and Characterization of *Bacillus* Antagonist to Inhibit aflatoxigenic *Aspergillus flavus*

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A B S T R A C T

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* using PCR based RAPD technique and selection of best biocontrol antifungal *Bacillus* strain with aflatoxin producing *Aspergillus* by antagonism on PDA medium. About 16 different strains of bacteria were isolated from healthy and infested rhizosphere of groundnut using N-agar medium. The isolates were identified based on morphological and microscopic characters such as colony color, shape, size, margin, opacity, texture, elevation, pigmentations, Gram staining and spore staining. Bacterial isolate JND-KHGn-29-A and JND-KSGn-30-L were recorded to be a best antagonist as of its ability to inhibit most toxic fungus *A. flavus* JAM-JKB-BHA-GG20 (58.20 %) after screening with 16 *Bacillus* isolates. The best antagonist bacterial isolate JND-KHGn-29-A also evidenced with nitrate reduction and sederophore as PGPR activity. The genetic diversity was studied among bacterial 16 bacterial isolates by using RAPD markers. Out of 38 primer, total 10 primers showed amplification. The highest numbers of 19 bands were produced by OPA-07 primer and lowest 1 band was produced by OPJ-07. The similarity index values generated by Jaccard's similarity coefficient and dendrogram grouped all bacterial isolates into two main clusters at 61% similarity. The best and least bacterial antagonist were grouped into different clusters depicting genetically difference between isolates. The 16S rDNA study revealed that the best and least antagonist bacterial isolates JND-KHGn-29A and JND-KHGn-29B were identified as *Bacillus subtilis.*

Introduction

The rhizosphere is a complex system in which beneficial plant microbe interactions play vital role in agriculture to sustain the plant growth and productivity. Plant growth promoting rhizobacteria (PGPR) exert the positive effect on plant growth through various mechanisms either directly or indirectly (Joseph *et al*., 2007). The *bacillus* bacteria play vital role in plant health by direct and indirect activities. The direct activity attributed by increased uptake of nitrogen (Kennedy *et al*., 2004) phytohormones synthesis (Hayat *et al*. 2008 a, b) solubilization of phosphorus and siderophore production (Pidello, 2003) while indirect activity include realise of phytoharmones like secondary metabolites *viz.* HCN, ammonia, antibiotics, and volatile metabolites (Owen

and Zlor, 2001). A large number of researchers have reported significant increases in productivity of important agronomic crops by inoculation with PGPR (Bashan *et al*., 2004). The ability of the antagonistic rhizobacteria is highly influenced by their morphological characteristics to inhibit the pathogens.

RAPD-PCR technique has been proposed as a tool for generating taxon-specific markers with different specificities (Kim *et al*., 2007). RAPD-PCR analyses have been shown to be suitable for generating strain and species-specific amplification profiles (Ronimus *et al.*, 1997). Jeyaram *et al.* (2008) used RAPD-PCR analyses for confirming 82 *B. subtilis* strains from Hawaijar, a traditional Indian fermented soy food. Torriani *et al*. (2001) used RAPD-PCR for species differentiation among similar *Lactobacillus plantarum, L. pentosus* and *L. paraplantarum*. DNA-based identification methods such as 16S rRNA gene sequencing have been used widely for the purpose of identification and typing of microorganisms isolated from natural environments including fermented foods (Levine *et al*., 2005).

The cultivated groundnut (*Arachis hypogaea* L.) is the most important oilseed crop and its kernels are also eaten raw, boiled or roasted. After the crop harvest, haulm and the expeller oil cake is used for animal feed. Aflatoxin contamination in groundnut seed is a major problem affecting the export. Aflatoxin contamination of the seed by *A. flavus* can occur during pre-harvest, during harvest and drying in the field, and during transportation and storage. The objectives of the present study was to evaluate the best bacterial biocontrol agent using *in vitro* antagonism against toxinogenic *A. flavus* and study the molecular diversity, microbial identification using 16S rRNA of the antagonist isolated from healthy and infested rhizosphere of groundnut. Furthermore, to confirm their plant growth promoting activities for the conventional use of commonly applied fertilizers and pesticides.

Materials and Methods

The present study was conducted to isolate native strains of rhizobacteria from healthy and infested rhizosphere of groundnut.

Collection of soil samples and isolation of rhizospheric bacteria

Rhizosphere soil was collected from groundnut fields healthy and infested with fungal disease like stem rot, color rot *etc.* Soil samples were collected from 16 rhizospheric soils of different field crops. For the isolation of native rhizobacteria 1g of soil was suspended in 90 ml distilled autoclaved water. Serial dilution agar plate method was used for further processing of the prepared soil suspension, Suitable dilutions were plated on N-agar media. All the plates were incubated for 2 days at 28˚C (Aneja, 2002). Well isolated pure bacterial colony were selected and transferred on freshly prepared N-agar media and stored at low temperature in refrigerator till further use (Alemu, 2013).

Morphological characteristics of bacterial isolates

Morphological characteristics of the colony of each isolate were examined on the NA-agar plates after incubated for 3 days at 28^0C. Then colony characterization of N-agar media was carried out *viz*., size, shape, margin, elevation, texture, opacity and pigmentation.

Microscopic examination of bacterial isolates

Standard microbiological methods were used to fix the cells to slides for Gram staining and

observed under Zeiss Axiocam Imager, model Z 2. Endospore staining was carried out by the method of Aneja (2003).

In vitro antagonism of bacterial isolates against aflatoxinogenic *A. flavus*

To derive best biocontroller, all bacterial isolates were subjected to *in vitro* antagonism with highly virulent and aflatoxigenic *Aspergillus* strain. The most responsive fungal isolate was cultivated in petriplate with 20 ml of Potato Dextrose Agar for seven days. Discs of 5 mm diameter were cut and removed from the growing borders of the colonies and transferred to another petriplates with Potato Dextrose Agar. Aflatoxicity of isolated pathogen was tested using biochemical method. In this method, the reverse side of colonies of toxin producing strains on potato dextrose agar (PDA) medium turns from yellow to pink immediately after exposure to ammonium hydroxide vapor. The test fungus was placed in the each center of the petriplate and approximately 3cm away bacterial isolates. The bacterial isolates were spread in round shape around the bid of the fungus. Control plates were maintained only with pathogen. All the inoculated plates were incubated at 28 ± 2^0 C temperature and observed after ten days for growth of antagonist bacteria and test fungus (Reddy *et al.,* 2008). The experiment was conducted in completely randomized design with three replications. At the end of incubation period, radial growth of pathogen *A. flavus* was measured and Index of antagonism was determined by following the method of Zarrin (2009) as depicted below

% Growth Inhibition = C-T/C*100

Where,
C = colony diameter of pathogen in control
T = colony diameter pathogen in inhibition plate

Defense related and plant growth promoting (PGPR) activity of bacterial isolates

Bacterial isolates were grown in 250 ml conical flasks containing 100 ml of LB broth for 48 h on a rotary shaker at 28 °C. Cells were taken by centrifugation at 10,000 g for 10 min at 4°C. The pellet was re-suspended in 100 ml of sterile distilled water (density measured as 1 at 600λ).

Siderophore production

Siderophore production was assayed by spot inoculation of bacterial isolates in the CAS agar medium (Clark and Bavoil, 1994). The plates were incubated at 28°C for 5 days. Siderophore production was observed by the development of orange halo around the colonies.

Indole acetic acid (IAA) production

The bacterial isolates were inoculated for determination of IAA like substances in 100 ml of N broth supplemented with tryptophan 0.1mg.ml^{-1}. The cultures were incubated at 28±2^o C for 3 days (72 hr) with occasional shaking. After incubation, the cultures were centrifuged at 10,000 rpm for 10 min. Two millilitres of freshly prepared Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) was added to 1 ml of culture supernatant. The reaction mixture was incubated at 30ºC for 30 min. Development of pink colour indicates the production of IAA (Aneja, 2003).

Phosphate solubilisation

Phosphate solubilization test of isolated bacterial isolates was carried out as described by Ravikumar (2002). The plates were prepared with Pikovskaya's medium. The isolates were streaked on the plates and

incubated in an incubator at 28°C for 7days. The plates were observed for the clear zone around (Light bluish) the colonies and consider positive for phosphate solublizing activity.

Nitrate reduction

The bacterial isolates were checked for nitrate reduction. The medium containing beef extract (3.0gm), geletin peptone (5.0gm), KNO3 (1.0gm) and deionised water (1000ml) was prepared and heated gently. Then, 20ml broth was taken in sugar tubes and Durham's tube was added inverted and autoclaved. After autoclaving each tube were heavily inoculated and incubated for 48 hrs. Two drops of reagent A (N, N- dimethylphenolpthalamine (0.6ml) and 5N acetic acid (100 ml) and reagent B (sulphanilic acid-0.8gm) and acetic acid (100ml) were added in one test tube and then 1ml broth was added in it. The positive test was confirmed by appearance of red color within two minutes where as negative test was confirmed by adding zinc dust for visualizing red color in same tubes.

Molecular characterization of antagonist bacterial isolates

Isolation of genomic DNA

The genomic DNA was isolated from overnight culture in nutrient broth by the method of Martinez *et al*. (2002). Cells were recovered by centrifugation at 13,000 ×g for 3 min. Cell pellet was resuspended in 1 ml of 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, and 400 µg/ml proteinase K (20 mg/ml) and incubated for 30 min at 55 °C. Total DNA was isolated using method described by Amer *et al*. (2011)**.** The aqueous upper layer was transferred into a fresh tube and same volume of isopropanol was added. DNA was precipitated by centrifugation at 13,000 ×g for 20 min at 4 °C followed by washing with 70% (v/v) ethanol, dried under vacuum, and resuspended in 50 µl sterile water.

RAPD-PCR analysis

RAPD-PCR assays were performed in 15 µl reaction volume and each tube contained Taq DNA polymerase, 10 pmol primers, and 1 µg of template DNA. PCR was done using Thermal cycler (Veriti, Model 96 well thermo cycler) and amplification conditions included an initial denaturation step at 94 °C for 4 min, 35 cycles of 94 °C for 1min., 36 °C for 15 1min., 72 °C for 2 min, and final extension at 72 °C for 10 min (Archana *et al*., 2007**).** RAPD-PCR products were analyzed by agarose gel (1.5%) electrophoresis with a molecular size marker (1 kb DNA ladder). DNA bands were visualized under UV light and banding patterns of amplified DNA was scored as present or absent in binary matrix. The RAPD data were subjected to statistical analysis for the calculation of Jaccard's similarity coefficient and cluster analysis by UPGMA (unweighted pair-group method with arithmetic averages) using NTSYSpc-2.02i software.

PCR amplification of *B. subtilis* species-specific 16S rRNA

Species-specific primer set for *B. subtilis* Bsub5F (5'- AAGTCGAGCGGACAGATG G-3') and Bsub 3R (5'- CCAGTTTCCAATGACCCT CCCC -3') were used. PCR was performed using a Thermal cycler (Veriti, Model 96 well thermo cycler). The reaction mixture (50 µl) contained 1 µg of template DNA, 1 µl of each primer (10 pmol), 5 µl of dNTP (0.25 mM), and 0.5 µl of Taq DNA polymerase. The following thermal cycling conditions were used: initial denaturation step at 94 °C for 2 min and 30 cycles consisting of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 min and primer extension at 72 °C for 1 min.

PCR products were analyzed by agarose gel (2%) electrophoresis with a molecular size maker (100 bp DNA ladder). Bacterial isolates were identified based on 16S rDNA sequencing using MicroSeq®500 16S rDNA bacterial identification kits (PN 4346298) as per manufacture protocol, by using 3130XL gene sequencer. The 16S rDNA gene was amplified by using PCR and sequencing kit supplied by Invitrogen Pvt. Ltd., USA. The PCR and sequencing reaction were carried out as per the protocol described in the above said kit. Therefore, amplified amplicon from bacillus specific primer set were further taken for sequencing. The obtained sequences were BLAST on NCBI data base.

Data were statistically analyzed by analysis of variance technique and comparison among means was made by completely randomized design (CRD) for study in the significance of various data (Fisher and Yates, 1948).

Results and Discussion

Morphological characteristics of bacterial isolates

Total 16 different strains of bacteria were isolated from healthy and infested rhizosphere of groundnut and colony color, shape, size, margin, opacity, texture, elevation and pigmentations of all sixteen isolates were determined by observing the plates after 7 days on N agar medium (Table 1).

In vitro antagonism of bacterial isolates with virulent *Aspergillus* biocontrol agent

All the bacterial isolates were screened with JAM-JKB-BHA-GG20 most toxic isolate of *Aspergillus flavus* fungus. Growth inhibition of *Aspergillus flavus* during *in vitro* interaction with biocontrol bacterial agents were recorded at 7 DAI (Table 2). The antagonist result depicted that the bacterial isolate T1 (JND-KHGn-29-A was the best antagonist inhibiting highest growth (58.20 %) of test pathogen A. *flavus* followed by isolate T15 (JND-KSCa-22) (48.04 %), T16 (JND-KSCa-23) (45.30 %) and T4 (JND-KSGn-30-B) (47.80 %). Whereas, bacterial isolate T2 (JND-KHGn-29-B) (0.00 %) evidenced as least antagonist among 16 bacterial isolates followed by isolates T6 (JND-KSGn-30-D), T8 (JND-KSGn-30-F), T3 (JND-KSGn-30-A), T12 (JND-KSGn-30-J) and T10 (JND-KSGn-30-H), against toxigenic *A. flavus* isolate JAM-JKB-BHA-GG20 (Table 2, Fig. 1 and Fig. 2).

Assay of defense related substances and PGPR activities

All 16 different strains of bacteria, isolated from healthy and infested rhizosphere of groundnut. All the bacterial isolates were screened for gram's staining and their defense related substances (Table 5). The observations were recorded as presence (+) or absence (-) of defence related substances. All isolates act differently to defence related substances. The bacterial isolate JND-KHGn-29-A was found to have nitrate reduction and sederophore activity. The best antagonist bacterium was identified as *Bacillus* after its colony characterization by gram's staining (+ ve) and spore forming.

PGPRs bear inhibitory effects for various pathogens on plant growth and development in the forms of biocontrol agents. The PGPR activities vary with the bacterial species and also with the physico-chemical conditions of the rhizosphere. (Glick and Bernard, 2012). Biocontrol of plant diseases, especially of fungal origin, has been achieved using microorganisms *Pseudomonas* sp. nd *Bacillus* sp (Ligon *et al.,* 2000). Raaijmakers *et al.* (2002) examined IAA production by test isolates of *Bacillus* spp. The results were contradictory with our results that, best antagonist bacterial isolate JND-KHGn-29-A showed negative IAA test and least antagonist

Bacillus JND-KHGn-29-B bacterial isolate showed positive IAA test.

In present study best antagonist *Bacillus* isolate JND-KHGn-29-A (isolate no. 1) was found to have better nitrate reduction activity. Similar nitrate reductases activity of *Bacillus* was reported by Nakano *et al.* (1998). *B. subtilis* can use nitrite or nitrate as a terminal acceptor of electrons. Production of siderophore by best antagonist *Bacillus* isolate JND-KHGn-29-A was observed. The production of siderophore by rhizobacteria has been confirmed by previous studies (Noori and Saud, 2012). A direct correlation was found to exist between siderophore production and antifungal activity (Raval and Desai, 2012). The sederophore create iron limiting conditions for pathogenic fungus and prevents it from invading and colonizing the plant roots (Meyer and Stintzi, 1998). The similar results were also obtained in the present study corresponding to siderophore production with greater antagonistic activity of the *Bacillus* isolate JND-KHGn-29-A and JND-KSGn-30-L.

Molecular diversity of bacterial isolates using RAPD

The polymorphisms can be detected by the use of random amplified polymorphic DNA (RAPD) which does not require prior knowledge of the genome. The RAPD has been commonly used for fingerprinting of biocontrol agents Chapon *et al*. (2002). In the present investigation, amplified products were observed when the genomic DNA of bacterial isolates was subjected to RAPD analysis using 38 random decamer primers.

Initially total 38 primers were screen for polymorphism using genomic DNA of isolates. Out of total 38 primer total 10 primer gave amplification which were further selected for amplification of genomic DNA of 16 bacterial isolates. The highest numbers of 19 bands were produced by OPA-07 primer followed by 15 bands of OPK-03 primer. The lowest 1 bands were produced by OPJ-07. The largest fragment of 3798 bp and the smallest fragment of 116 bp were amplified by OPH-15 primer (Table 6). The polymorphism information content (PIC) was calculated for each primer and it was varied between 0.84 (OPD-03) and 1.00 (OPA-07, OPA-18, OPH-15, OPJ-07, OPK-03, OPG-08, B1, OPO-06 and OPD-03) with an average of 0.95 per primer. The details of polymorphism pattern of individual primer are given in (Table 4).

Cluster analysis of RAPD

The similarity index values generated by Jaccard's similarity coefficient among 16 bacterial isolates based on RAPD data showed the similarity coefficient ranging from 0.5446 to 0.8911 (54.46 % to 89.11%). The more genetic similarity (89.11%) was observed between isolate 12 (JND-KSGn-30-J) and isolate 7 (JND-KSGn-30-E) followed by (86%) between isolate 10 (JND-KSGn-30-H) and isolate 9 (JND-KSGn-30-G), whereas lowest genetic similarity (54.46%) was observed between isolate 2 (JND-KHGn-29-B) and isolate 1 (JND-KHGn-29-A), isolate 9 (JND-KSGn-30-G) and isolate 1 (JND-KHGn-29-A) and isolate 9 (JND-KSGn-30-G) and isolate 2 (JND-KHGn-29-B) followed by 55% genetic similarity between isolates 14 (JND-KSGn-30-L) and isolate 8(JND-KSGn-30-F).

The similarity index values generated by Jaccard's similarity coefficient were used to construct dendrogram using UPGMA method was depicted in Fig. 3. The dendrogram grouped all bacterial isolates into two main clusters at 61% similarity *viz.* cluster I and cluster II. Cluster I was again sub divided into cluster IA and cluster IB at 61.8 % similarity (Fig. 3).

Table.1 Morphological characterization of bacterial isolates collected from groundnut rhizosphere

Crop name	Rhizosphere Condition	Code Name	Colony Shape	Size	Color	Margin	Opacity	Texture/ Consistency	Elevation	Pigmentation
Ground nut	Healthy	JND-KHGn-29-A	irregular	medium	white	Undulate	opaque	brittle	flat	no
Ground nut	Healthy	JND-KHGn-29-B	circular	tiny	white	Entire	opaque	dry	raised	no
Ground nut	SICK	JND-KSGn-30-A	circular	tiny	yellowish	Entire	opaque	dry	raised	no
Ground nut	SICK	JND-KSGn-30-B	irregular	medium	white	Undulate	opaque	brittle	flat	red
Ground nut	SICK	JND-KSGn-30-C	irregular	small	white	Curled	opaque	dry	umbonate	no
Ground nut	SICK	JND-KSGn-30-D	filamentous	large	white	Filiform	opaque	dry	flat	no
Ground nut	SICK	JND-KSGn-30-E	circular	small	white	Entire	opaque	moist	raised	no
Ground nut	SICK	JND-KSGn-30-F	irregular	large	white	Curled	opaque	dry	umbonate	red
Ground nut	SICK	JND-KSGn-30-G	circular	tiny	white	Entire	opaque	moist	umbonate	no
Ground nut	SICK	JND-KSGn-30-H	irregular	large	white	Undulate	opaque	dry	flat	red
Ground nut	SICK	JND-KSGn-30-I	irregular	medium	white	Undulate	opaque	brittle	flat	no
Ground nut	SICK	JND-KSGn-30-J	irregular	large	yellow	Curled	opaque	dry	umbonate	yellow
Ground nut	SICK	JND-KSGn-30-K	irregular	large	white	Undulate	opaque	buttery	raised	red
Ground nut	SICK	JND-KSGn-30-L	irregular	large	white	Undulate	opaque	brittle	flat	cream
Castor	SICK	JND-KSCa-22	circular	small	white	Entire	opaque	viscous	convex	no
Castor	SICK	JND-KSCa-23	circular	small	white	Entire	opaque	viscous	convex	no

Table.2 Percent growth inhibition of *A. flavus* by Bacterial antagonists

Isolate No.	Treatment	% Growth Inhibition 7 DAI
T1	JND-KHGn-29-A X Pathogen-AFvs*	58.20
T2	JND-KHGn-29-B X Pathogen -AFvs	0.00
T3	JND-KSGn-30-A X Pathogen-AFvs	6.04
T4	JND-KSGn-30-B X Pathogen-AFvs	47.80
T5	JND-KSGn-30-C X Pathogen-AFvs	25.82
T6	JND-KSGn-30-D X Pathogen-AFvs	2.20
T7	JND-KSGn-30-E X Pathogen-AFvs	8.79
T8	JND-KSGn-30-F X Pathogen-AFvs	5.00
T9	JND-KSGn-30-G X Pathogen-AFvs	20.88
T10	JND-KSGn-30-H X Pathogen-AFvs	7.14
T11	JND-KSGn-30-I X Pathogen-AFvs	22.53
T12	JND-KSGn-30-J X Pathogen-AFvs	21.43
T13	JND-KSGn-30-K X Pathogen-AFvs	6.04
T14	JND-KSGn-30-L X Pathogen-AFvs	50.27
T15	JND-KSCa-23 X Pathogen-AFvs	48.04
T16	JND-KSCa-22 X Pathogen-AFvs	45.30
T17	Control = Pathogen	0.00
	S.Em.±	0.444
	C.D. @ 5%	1.275
	C.V. %	3.783

* *A. flavus* JAM-JKB-BHA-GG20 (Isolate-3) - most toxic to produce aflatoxin

Table.3 Characterization of bacterial isolates for PGPR activity

Isolate No.	Bacterial Isolates	IAA	Gram's staining	PSB	Spore-staining	Nitrate reduction	Sederophore
1	JND-KHGn-29-A	-	+	-	-	+	+
2	JND-KHGn-29-B	+	-	-	-	-	-
3	JND-KSGn-30-A	-	-	+	-	+	+
4	JND-KSGn-30-B	+	+	-	-	-	
5	JND-KSGn-30-C	-	-	+	-	+	+
6	JND-KSGn-30-D	+	+	-	+		-
7	JND-KSGn-30-E	-	-	+	-	+	+
8	JND-KSGn-30-F	+	+	-	-	-	-
9	JND-KSGn-30-G	-	-	+	+	-	-
10	JND-KSGn-30-H	-	+	-	-	-	-
11	JND-KSGn-30-I	-	-	-	-	-	+
12	JND-KSGn-30-J	-	-	-	+	-	+
13	JND-KSGn-30-K	-	+	-	+	-	-
14	JND-KSGn-30-L	-	-	-	-	-	+
15	JND-KSCa-23	+	-	-	-	-	-
16	JND-KSCa-22	-	-	+	-	-	-

Table.4 Polymorphism of 16 bacterial isolates generated with different RAPD primers

Sr. No.	RAPD Primer	Bend Size (bp)	Total No. of Bends (A)	Polymorphic Bands (B)			Mono-Morphic Bend	% Poly-Mor Phism (B/A)	PIC*	RPI
				S	U	T				
1	OPA-07	233-1678	19	14	5	19	0	100.00	1.00	19.00
2	OPA-18	199-2174	14	12	2	14	0	100.00	1.00	13.98
3	OPH-15	116-3798	7	7	0	7	0	100.00	0.89	6.22
4	OPJ-07	253	1	1	0	1	0	100.00	0.89	0.89
5	OPK-03	213-2335	15	8	7	15	0	100.00	1.00	14.98
6	OPG-08	207-1428	11	5	6	11	0	100.00	0.98	10.80
7	B1	138-1359	12	12	0	12	0	100.00	0.99	11.88
8	OPO-06	240-1792	10	8	2	10	0	100.00	0.94	9.38
9	OPC-13	178-1102	8	7	0	7	1	87.50	0.99	7.91
10	OPD-03	345-1205	3	3	0	3	0	100.00	0.84	2.53
Total			86.00	61.00	24.00	85.00	1	887.50	8.52	83.58
Average			10	7.3	2.6	9.44	0.11	98.61	0.95	9.29

S = Shared; U = Unique; **T** = Total Polymorphic Bands;
PIC = Polymorphism Information Content; **RPI** = RAPD Primer Index

Table.5 Unique RAPD markers associated with antagonistic bacterial isolates

RAPD Primers	Bacterial Isolates															
	JND-KHGn-29-A	JND-KHGn-29-B	JND-KSGn-30-A	JND-KSGn-30-B	JND-KSGn-30-C	JND-KSGn-30-D	JND-KSGn-30-E	JND-KSGn-30-F	JND-KSGn-30-G	JND-KSGn-30-H	JND-KSGn-30-I	JND-KSGn-30-J	JND-KSGn-30-K	JND-KSGn-30-L	= JND-KSCa-22	JND-KSCa-23
OPA-18	1950	1135	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA-07	966	875	-	-	493	-	-	-	-	-	-	-	-	-	-	-
	191	-	-	-	331	-	-	-	-	-	-	-	-	-	-	-
OPK-03	1642	305	-	-	-	-	-	-	-	-	-	-	2308	-	-	-
	671	-	-	-	-	-							1050			
	362	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	168	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPG-08	650	1196	-	-	-	-	-	-	-	-	-	-	-	1001	-	701
	-	401	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	281	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B1	-	1555	-	-	-	-	-	-	-	-	-	-	1792	-	-	-
Total No.	8	7	0	0	2	0	0	0	0	0	0	0	3	1	0	1

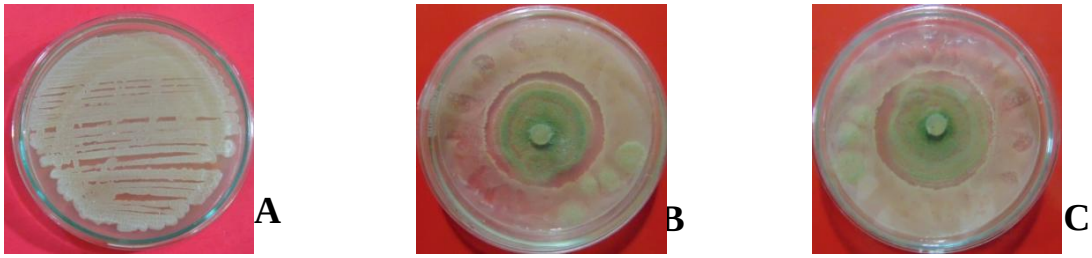
Table.6 Molecular identification of bacterial isolates *B. subtilis* using 16 S rRNA gene specific primers and their blast results

Sr. No.	Isolate Code	Amplification product (Fragment size in bp)	Sequence obtained (bp)	Blast Identities (%)	Identification/ Accession no.
1	JND-KHGn-29-A	600	582	98	*Bacillus subtilis*/ KU984480
2	JND-KHGn-29-B	600	547	96	*Bacillus subtilis*/ KU984481
3	JND-KSGn-30-A	600	587	90	*Bacillus subtilis*/ KU984482
4	JND-KSGn-30-B	600	578	96	*Bacillus subtilis*/ KU984483
5	JND-KSGn-30-C	600	643	99	*Bacillus subtilis*/ KU984484
6	JND-KSGn-30-D	600	609	98	*Bacillus subtilis*/ KU984485
7	JND-KSGn-30-E	600	619	98	*Bacillus subtilis*/ KU984486
8	JND-KSGn-30-F	600	535	94	*Bacillus subtilis*/ KU984487
9	JND-KSGn-30-G	600	579	97	*Bacillus subtilis*/ KU984488
10	JND-KSGn-30-H	600	623	95	*Bacillus subtilis*/ KU984489
11	JND-KSGn-30-I	600	604	98	*Bacillus subtilis*/ KU984490

16 S rRNA *B. subtillis* gene specific primers pair F: [5'AAGTCGAGCGGACAGATGG 3']
R: [5' CCAGTTTCCAATGACCCTCCCC 3']

Fig. 1 *In vitro* antagonism of bacterial isolates against toxic *Aspergillus flavus* (JAM-JKB-BHA-GG20) on PDA media. A: best antagonist bacterial isolate *B. subtilis* JND-KHGn-29-A on N-agar medium; **B**: antagonism after 5 days of inoculation; **C**: antagonism after 10 days of inoculation; **D**: least antagonist bacterial isolate *B. subtilis* JND-KHGn-29-B on N-agar medium; **E**: antagonism after 5 days of inoculation; **F**: antagonism after 10 days of inoculation

1. ***B. subtilis* JND-KHGn-29-A *X A. flavus* (JAM-JKB-BHA-GG20)**



2. ***B. subtilis* JND-KHGn-29-B *X A. flavus* (JAM-JKB-BHA-GG20)**

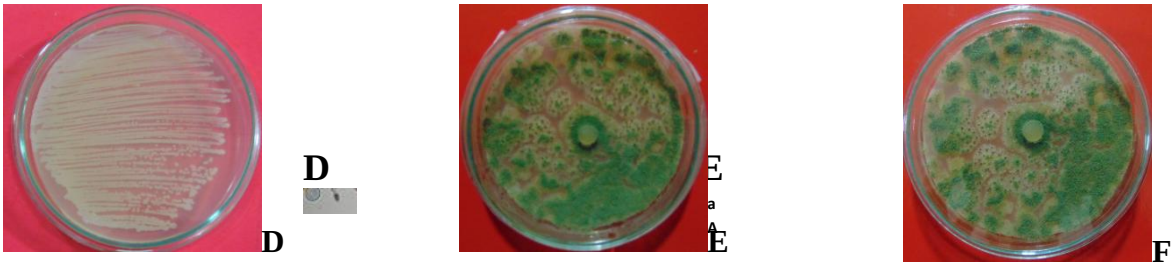


Fig.2 Percent growth inhibition of *A. flavus* JAM-JKB-BHA-GG20 (Isolate-3) by *Bacillus* strains at 7 DAI

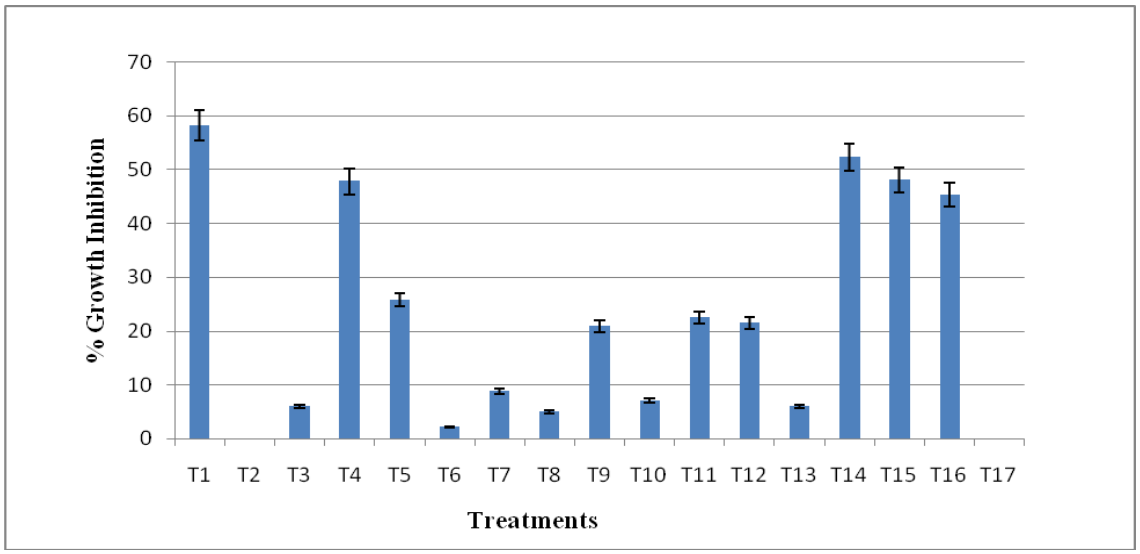


Fig.3 Dendrogram depicting the genetic relationship among the antagonists bacterial isolates based on the RAPD data (1= JND-KHGn-29-A; 2= JND-KHGn-29-B; 3= JND-KSGn-30-A; 4= JND-KSGn-30-B; 5= JND-KSGn-30-C; 6= JND-KSGn-30-D; 7= JND-KSGn-30-E; 8= JND-KSGn-30-F; 9= JND-KSGn-30-G; 10= JND-KSGn-30-H; 11= JND-KSGn-30-I; 12= JND-KSGn-30-J; 13= JND-KSGn-30-K; 14= JND-KSGn-30-L; 15= JND-KSCa-22; 16= JND-KSCa-23).

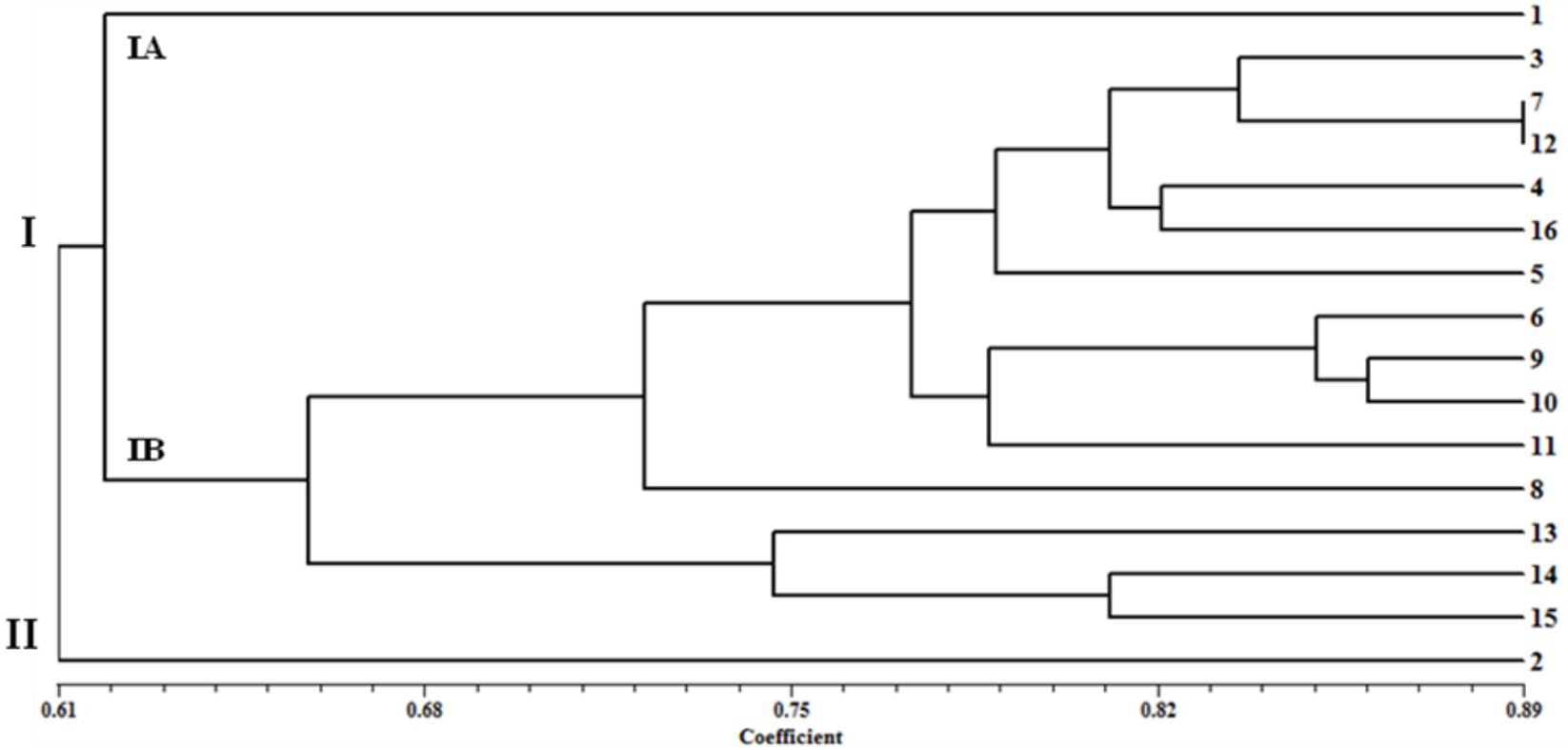
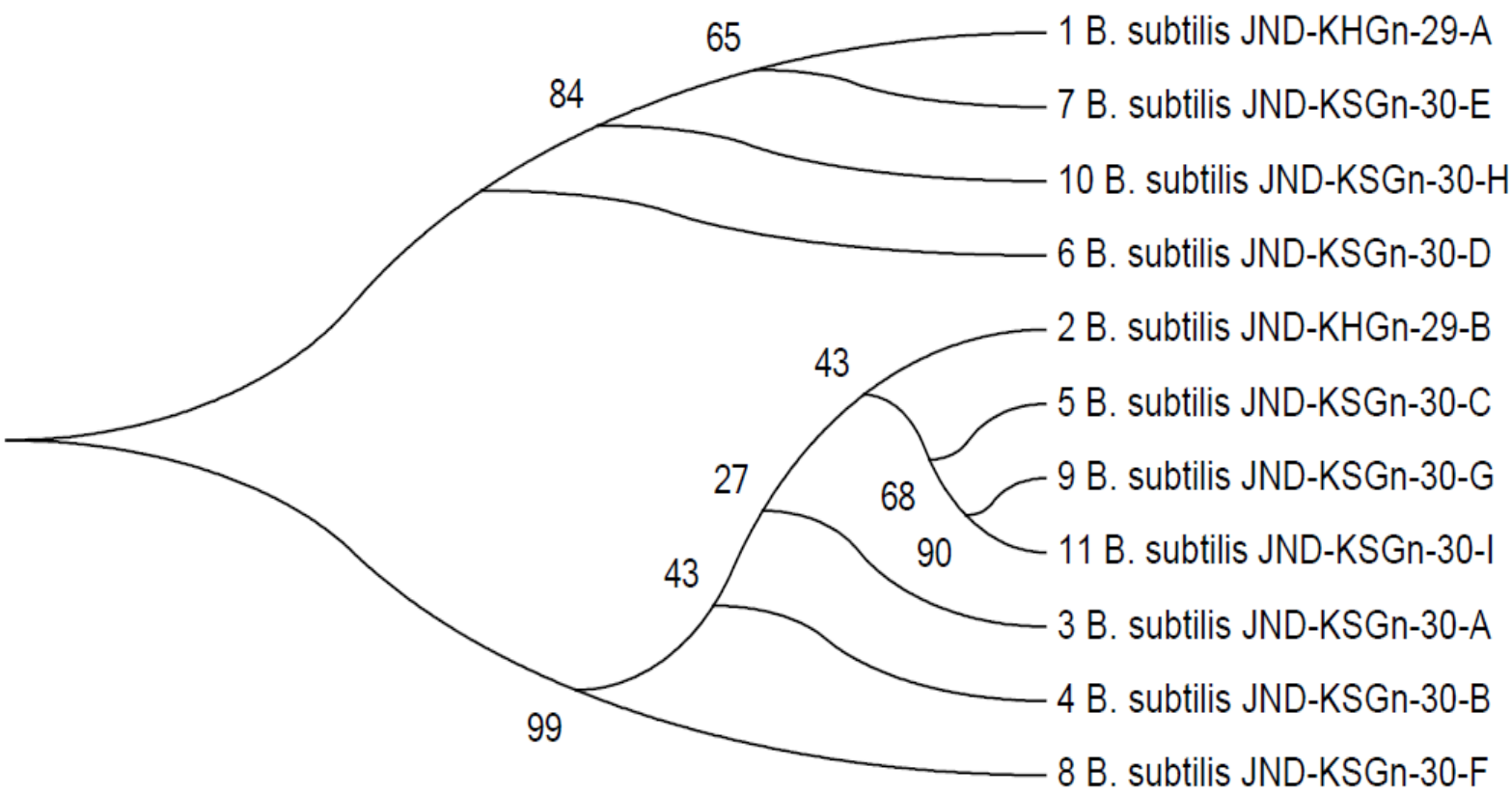


Fig. 4: Dendogram depicting the genetic relationship among the bacterial isolates based on sequencing data corresponding to 16S rRNA region



Cluster IA consist of only one bacterial isolate, which was found to be the best antagonist among all 16 bacterial isolates against most toxic and virulent *Aspergillus flavus, an* identified fungus isolate. Cluster IB grouped most of the bacterial isolates *viz.* isolate 3,7,12,4,16,5,6,9,10,11,8,13,14 and 15. From cluster B, it was observed that isolate JND-KSGn-30-E and isolate JND-KSGn-30-J were closely related at 89% (Fig. 3). Cluster II consist of only one isolate *i.e.* isolate JND-KHGn-29-B.

The isolate JND-KHGn-29-B was found least antagonist among all 16 bacterial isolates against most toxic and virulent *Aspergillus flavus* and it also showed highest genetic dissimilarity with other isolates (Fig. 3). Archana *et al*. (2007) reported 61% similarity level after screening 21 isolates with 18 RAPD primers. Prasad (2014) found 56.25% polymorphism between selected *Bacillus cereus* species, an enterotoxic pathogenic strains of *Bacillus* from gut region of local tropical fishes by using 10 primers of the OP series. The results of present study suggest that RAPD primers are effective tool for discremating rhizobacteria in the development of bio-inoculants for disease management in crop plants as the primers were able to distinguish most antagonists and least antagonist bacterium.

Unique RAPD markers associated with antagonistic bacterial isolates

RAPD markers associated with 16 bacterial isolates were tabulated in Table 5. Out of 38, total 5 primers produce 24 unique bands to identify 16 bacterial isolates. Total 5 primers generate 24 specific unique amplicons *viz.* the primer OPA- 7 was able to produce 4 unique amplicons within 3 bacterial isolates *i.e.* 2 unique amplicons in isolate 1 of size 966bp and 191bp and isolate 5 of size 493 and 331 and 1 amplicons in isolate 2 of size 875. The primer OPA-18 generates 2 unique amplicons with in two isolates *i.e.* isolate 1 (1950bp) and 2 (1135). The primer OPK-3 was able to amplify highest 7 amplicons within 16 isolates *i.e.* 4 amplicons in isolate 1 (1642bp, 671bp, 362bp and 168bp) followed by 2 amplicons in isolate 13 (2308 and 1050) and 1 unique amplicon in isolate 2 (305bp). The primer OPG-08 was able to amplify 6 amplicons within 16 isolates *i.e.* 3 amplicons in isolate 2 (1196bp, 401bp and 281bp) followed by 1 amplicons in each isolate *i.e.* isolate 1 (650bp), isolate 14 (1001bp) and isolate 16 (701bp) respectively (Table 5).

Gun-Hee *et al*. (2009) identified RAPD primers which produced common bands of 0.5 and 0.88 kb in size with *B. subtilis* strains. All *B. amyloliquefaciens* strains generated 1.1 and 1.5 kb bands together with 0.5 kb fragment whereas *B. licheniformis* strains produced 1.25, 1.70, and 1.9 kb bands with an occasional 0.5 kb band. The 0.5 kb fragment, the major band for *B. subtilis* strains, was an internal part of a ytcP gene encoding a hypothetical ABC-type transporter. Fevzi (2001) performed RAPD profiling which revealed the diversity in the *Actinomycete*s. The number of polymorphic bands observed for each isolates was between 3 and 1 with size ranging from 100 to 2000 bp. All the nine isolates characterized on the basis of the RAPD molecular markers produced highly polymorphic patterns. This study help in understanding the difference in bandaing pattern of most antagonists and least antagonist bacterium. No certain reports are avalaable similar to the result of present study which clearly differentiate most antagonist and least antagonist bacterium.

Molecular identification of bacterial isolates using 16S rRNA gene sequencing

The 16S rRNA is a component of the 30S small subunit of prokaryotic ribosomes. The

genes coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene (Jamal *et al.*, 2013). In present study molecular identification of bacterial strain, PCR was conducted with *Bacillus* specific universal primers of 16S rRNA region (primer pair bsub 5 F [5' AAGTCGAGCGGACAGATGG 3'] - bsub 3 R [5' CCAGTTTCCAATGA CCCTCCCC 3']). The specific primers were able to amplify a single amplicon of 600 bp in 11 isolates out of 16 isolates which was further processed for analysis. Therefore, amplified amplicon from *Bacillus* specific primer set were further taken for sequencing. The obtained sequences were BLAST on NCBI data base. All the BLAST result matches 98% similarity towards *Bacillus subtilis*. Therefore, molecular result supports the result obtained from colony characterization. The best antagonist and least antagonist bacterial isolate JND-KHGn-29-A and JND-KHGn-29-B were identified as *Bacillus subtilis* based on 16S rRNA sequence and both isolates were derived from same healthy rhizospere of groundnut field (Table 6). Therefore, the bacterial isolate JND-KHGn-29-A was identified as *Bacillus subtilis*. Hall *et al.* (2003) used the internal transcribed spacers between the 16S and the 23S ribosomal RNA genes to discriminate species of the 16S rRNA group I of the genus *Bacillus* by PCR.

Sequenced based phylogenic analysis of 16S rRNA region

In the present study, determined the 16S rRNA gene sequence of 11 isolates form healthy and infected rhizospare of groundnut field. Using BLAST search, it was found that all strains belonged to species *Bacillus subtilis*. The identities of the 11 *Bacillus* isolates were determined by comparing them to the available 16S rRNA sequences found in Genbank and with high-scored rRNA sequences in BLAST searches. BLAST similarity scores ranged between 97% to 100% (Table 6).

The evolutionary history inferred using the Neighbor-Joining method grouped all 11 analyzed strains in 02 the cluster with a high supported bootstrap. The cluster I grouped 4 isolates (1. JND-KHGn-29-A, 7. JND-KSGn-30-E; 10. JND-KSGn-30-H and 6. JND-KSGn-30-D) and cluster II encompassed 7 isolates (2. JND-KHGn-29-B; 5. JND-KSGn-30-C; 9. JND-KSGn-30-G; 11. JND-KSGn-30-I, 3. JND-KSGn-30-A; 4. JND-KSGn-30-B and 8. JND-KSGn-30-F) (Fig. 4).

The 11 *Bacillus* isolates were clustered based on their antagonist property. The best antagonist bacterial isolate 1 (JND-KHGn-29-A) was grouped in cluster I and least antagonist bacterial isolate 2 (JND-KHGn-29-B) was grouped in cluster II (Fig. 4).

Jamal *et al.* (2013) reported 16S sequence size for the 26 isolates, of *Bacillus* strains grown around *Rhazya stricta* roots, ranged between 995 to 1233 nt, while their counterparts in the Genbank ranged between 1153-1559 nt. Jang et *al.* (2009) identified potential plant growth promoting (PGP) and antagonistic activities bacterial isolates as *Bacillus* sp based on 16S rRNA gene sequence after screening seven isolates from rhizosphere of common bean growing at Uttarakhand.

In conclusion, to cope with problems associated with chemical control, an environmentally friendly way of biological control using antagonistic microorganisms is becoming more and more attentive in recent years. The morphological and microscopic characters of bacteria isolates obtained from healthy and infested rhizosphere allows screening for PGPR activity and molecular

diversity analysis with RAPD markers and 16S rRNA sequencing can be employed to distinguish and identify most antagonist and least antagonist bacterium.

References

Alemu, F. 2013. Isolation of *Pseudomonas fluorescens* from rhizospheric soil of faba bean and assessment of their Phosphate solubility: *in vitro* study. *Ethiopia. Sch. Acad. J. Biosci*., 1: 346-351.

Amer, O. E., Mahmoud, M. A., El-Samawaty, A. M. and Sayed, R. M. 2011. Non liquid nitrogen-based method for isolation of DNA from filamentous fungi. *Afr. J. Biotechnol.*, 10: 1437–1441.

Aneja, K. R. 2003. Experiments in Microbiology, Plant Pathology & Biotechnology. 4th ed. New Age Int., p.607.

Archana, G., Bhandarkar, M., Dongre, A. B. and Meshram, S. 2007. Genetic Diversity Evaluation of *Bacillus* isolates using Randomly Amplified Polymorphic DNA molecular marker. *Roman. Biotech. Lett.*, 12(3): 3277-3286.

Bashan, Y., Holguin G. and de-Bashan L. 2004. Azospirillum–plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). *Can. J. Microbiol*., 50:521–577.

Chapon, A., Morgane, B., Delphine, R., Laurie, D., Guillerm, A. and Sarniguet, A. 2002. Direct and specific assessment of colonisation of wheat rhizoplane by *Pseudomonas fluorescens* Pf29A. *Euro. J. Pl. Patho*., 109: 61-70.

Clark V.L. and Bavoil P.M. 1994. Bacterial pathogenesis. In. Abelson JN and Simon MI (Editors). Methods in Enzymology. San Diego, Academic Press. 235 p. 324-357.

Fevzi, B. 2001. Random Amplified Polymorphic DNA (RAPD) Markers. *Turk J. Biol*., 25: 185-196.

Fisher, R. A. and Yates, N. D. 1948. Statistical methods for research workers. Oliver and Boyd, Edinburg, London. 12th ed-*Bio. Monograph and Manuals*., 5: 130-131.

Glick R. and Bernard. 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Hindawi Publishing Corporation Scientifica, Volume 2012, Article ID 963401, 15 pages.

Gun-Hee K., Hwang, A. L., Jae-Young, P., Jong, S. K., Jinkyu, L., Cheon, S. P., Dae, Y. K., Yong, S. K. and Jeong, H. K. 2009. Development of a RAPD-PCR method for identification of *Bacillus* species isolated from Cheonggukjang. *Internat. J. Food Microbiol.*, 129: 282–287.

Hall, L., Doerr, K. A., Wohlfiel, S. L. and Roberts, G. D. 2003. Evaluation of the MicroSeq System for identification of *Mycobacteria* by 16S ribosomal DNA sequencing and its integrations into a routine clinical mycobacteriology laboratory. *J. Clin. Microbiol.*, 41: 1447-1453.

Hayat R., Ali S., Siddique M. T. and Chatha T. H. 2008a. Biological nitrogen fixation of summer legumes and their residual effects on subsequent rainfed wheat yield. *Pakistan J. Bot*., 40(2): 711-722.

Hayat R., Ali S., Ijaz S. S., Chatha T. H. and Siddique M.T. 2008b. Estimation of N2-fixation of mung bean and mash bean through xylem uriedetechnique under rainfed conditions. *Pakistan J. Bot.* 40(2):723–734.

Jamal S. M., Sabir, S. E. M., Abo-Aba, Ayman S., Refaei M. H., Ahmed, B. and Nabeeh, A. B. 2013. Isolation,

identification and comparative analysis of 16S rRNA of *Bacillus subtilis* grown around *Rhazya stricta* roots. *Life Sci. J.*, 10(12): 121-124.

Jang, S. K., Wen, Z., and Pei Y. Q. 2009. Discovery of marine *Bacillus* species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *J. Microbiol. Meth.*, 77:48–57.

Jeyaram, K., Singh M. W., Premarani, T., Devi, A.R., Chanu, K.S., Talukdar, N.C., Singh, M.R., 2008. Molecular identification of dominant microflora associated with 'Hawaijar'-a traditional fermented soybean (*Glycine max* L.) food of Manipur, India. *Int. J. of Food Microbiol.*, 122: 259–268.

Joseph, B., Patra, R. R., and Lawrence, R. 2007. Characterization of plant growth promoting Rhizobacteria associated with chickpea (*Cicer arietinum* L). *Int. J. of Plant Production*., 1(2): 141-152.

Kennedy, I. R., Choudary, A.I.M.A. and Kecskes, M.L. 2004. Non Symbiotic bacterial diazotrophs in a crop farming systems can their potential for plant growth promotion to be better exploited? *Soil Biol. Biochem.,* 36(8): 1229-1244.

Kim, J. S., Kuk, E., Yu, K. N., Kim, J. H. and Park, S. J. 2007. Antimicrobial effects of silver nanoparticles, *Nanomed.*, **3:** 95-101.

Levine, S.M., Tang, Y.W. and Pei, Z.H. 2005. Recent advances in the rapid detection of *Bacillus* anthracis. *Rev. Med. Microbiol.*, 16:125–133.

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitw, N. R., Hofman, D., Kempt, H. J. and van Pee, K.H. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Manag. Sci.,* 56: 688-695.

Martinez, M. A., Delgado, O. D., Breccia, J. D., Baigori, M. D. and Sineriz, F. 2002. Revision of the taxonomic position of the xylanolytic *Bacillus* sp. MIR32 reidentified as *Bacillus* halodurans and plasmid-mediated transformation of B. halodurans. *Extremophiles*., 6: 391–395.

Meyer, J. M. and Stintzi, A. 1998. Iron metabolism and siderophore production in *Pseudomonas* and related species. In Montie, ed. *Pseudomonas*. New York: Plenum Press, pp. 201-243.

Nakano, S., Peberdy, J. F., and Lumyong, S. 1998. Indole-3-acetic acid production by Streptomyces sp.isolated from some Thai medicinal plant rhizosphere soils. *Eur. Asia J. Biosci*. 4: 31-34.

Noori, M. S. S. and Saud, H. M. 2012. Potential Plant Growth-Promoting Activity of Pseudomonas sp. Isolated from Paddy Soil in Malaysia as Biocontrol Agent. *J. Plant Pathol. Microbiol.*, 3:120.

Owen, A. and Zlor R. 2001. Effect of cyanogenic rhizobacteria on the growth of velvetleaf (Abutilon theophrasti) and Corn (*Zea mays*) in autoclaved soil and the influence of supplemented glycine. *Soil Biochem*., 33: 801-809.

Pidello, A. 2003. The effect of *Pseudomonas fluorescens* strains varying in pyoverdine production on the soil redox status. *Plant Soil*., 253: 373-379.

Prasad, M. P. 2014. Molecular characterization of Enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers. *Int. J. Pure App. Biosci.*, 2(4): 189-195.

Raaijmakers, J. M., Vlami, M. and de Souza, J. T. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie*

Van Leeuwenhoek., 81: 537-547.

Raval, A. A. and Desai, P. B. 2012. Rhizobacteria from rhizosphere of sunflower (*Helianthus annuus* L.) and their effect on plant growth. *Res. J. Rec. Sci*., 1(6): 58-61.

Ravikumar, P., Hutson, R. A. and Naresh, K. G. 2002. Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae*. *FEMS Microbiol. Lett*., 171: 223-229.

Reddy, B. P., Reddy, K. R. N., Rao, M. S. and Rao, K. S. 2008. Efficacy of antimicrobial metabolites of *Pseudomonas fluorescens* against rice fungal pathogens. *Current Trends Biotechnol. Pharm*., 2: 178-182.

Ronimus, R.S., Parker, L.E. and Morgan, H.W. 1997. The utilization of RAPD-PCR for identifying thermophilic and mesophilic *Bacillus* species. *FEMS Microbiol. Lett*., 147:75–79.

Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., Kersters, K., 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. *Syst, Appl. Microbiol*., 24, 554–560.

Zarrin, F., Saleemi, M., Zia, M., Sultan, T., Aslam, M., Rehman, R. and Chaudhary, M. F. 2009. Antifungal activity of plant growth-promoting rhizobacteria isolates against *Rhizoctonia solani* in wheat. *Afri. J. of Biotech*., 8: 219-225.

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