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PLANT-BENEFICIAL RHIZOBACTERIA FOR SUSTAINABLE INCREASED YIELD OF COTTON WITH REDUCED LEVEL OF CHEMICAL FERTILIZERS

SUMERA YASMIN[1], FAUZIA Y. HAFEEZ[1, 2*], MICHAEL SCHMID[3] AND ANTON HARTMANN[3]

[1]*National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad 3800, Pakistan*

[1, 2]*Department of Biosciences, COMSATS Institute of Information Technology Chak Shahzad Campus, Park road, Islamabad, Pakistan*

[3]*Helmholtz Zentrum Munchen, German Research Center for Environmental Health (GmbH), Department Microbe-Plant Interactions, Neuherberg/ Munich, Germany*

[*]*Corresponding author e-mail: fauzia_y@yahoo.com; fauzia@comsats.edu.pk*

Abstract

Traditional use of chemical fertilizers in agricultural production can not be over-emphasized, but with fertilizer costs going up, these need to be supplemented or substituted with biofertilizers. Twenty-two plant growth promoting bacteria (PGPB) isolated from cotton grown in Pakistani soils were selected to assess the range of growth promoting properties. Some important capabilities of practical utility shown by these strains were nitrogenase activity, indole acetic acid (IAA) production, P and Zn mobilization, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and siderophores production. Growth and yield of cotton plant was significantly increased by these bacterial inoculations with different reduced levels of nitrogen (N) and phosphorus (P) fertilizers under controlled conditions as well as in field trials of two years. Co-inoculation of *Pseudomonas aeruginosa* Z5 and *Bacillus fusiformis* S10 with half and 1/4th of the recommended N and P fertilizers improved the boll mass, lint and seed yield compared to un-inoculated controls. Fluorescence *in situ* hybridization (FISH) using domain, division and subdivision-level probes was employed in combination with confocal laser scanning microscopy for identification of cotton rhizosphere associated bacteria. The results of FISH were found to be in accordance with 16S rRNA sequence analysis. Here we demonstrate that the seed treatment of cotton plants with *Pseudomonas aeruginosa* Z5 (AY548952) and *Bacillus fusiformis* S10 (AY548956) can improve growth and yield parameters in cotton fields with reduced levels of chemical fertilizers. *Pseudomonas aeruginosa* Z5 was deposited to DSMZ German Culture Collection with accession no. DSM16519.

Introduction

Pakistan is the fourth largest producer of cotton in the world after China, United States and India with an average output of 6 million tones of cotton seed grown since 2000 (http://unctad.org/infocomm/anglais/cotton/crop.htm). Conventional use of chemical fertilizers to enhance the cotton production can not be ruled out, but as the costs of fertilizers are increasing at quite a high rate therefore, these have to be partially or fully replaced with some cost effective strategy (Adesemoye *et al.,* 2009). Application of bioinoculants or biofertilizers can be introduced as an integrated plant nutrient system for sustainable crop production (Dion *et al.,* 2010; Iftikhar *et al*., 2010; Babar *et al*., 2011). Among the bacterial inoculants, *Bacillus* and *Pseudomonas* are known to increase yield in different crops including cotton (Narula *et al.,* 2005; Yao *et al.,* 2006), besides protecting roots from the attack of pathogens due to the production of diverse microbial metabolites like siderophore, plant growth enhancement through indole acetic acid (IAA) production, nitrogen (N) fixation, uptake of phosphorus (P) and other minerals (Haq *et al.,* 2012; Kang *et al.,* 2012; Kang *et al.,* 2010).

It is very important to identify and detect the target bacteria from a set of organisms accurately before its use as an inoculant. Reliable identification of the introduced bacteria is needed to understand the rhizosphere interaction and colonization before a selection of strains. Fluorescence *in situ* hybridization (FISH) with 16S rRNA targeted fluorescently labeled oligonucleotide probes allows the identification of a specific microorganism in a mixture with other bacteria (Schonhuber *et al.,* 2001). FISH has been performed to investigate microorganisms in an aquatic ecosystems, biofilms, and sediments and in the soil habitat (Bhavanath *et al*., 2009; Oliveira *et al*., 2009; Schmid *et al*., 2009).

This research was directed at basic and applied aspects of using beneficial bacteria as microbial inoculants to promote growth and yield of cotton. The plausible mechanisms adopted by these rhizobacteria in growth promotion have been studied. This work has also emphasized the identification tools for a set of bacterial isolations using fluorescently labeled rRNA probes in combination with confocal laser scanning microscope. Net house and field evaluations were conducted to provide biological support for more basic investigations.

Materials and Methods

Source of microorganisms and cultural conditions: Twenty-two bacterial strains i.e., Z1 to Z12 and S1 to S10 (Table 1) were obtained from BIRCEN Culture Collection, NIBGE, Faisalabad. All the bacterial strains were stored on LB slants. Gram staining, colony and cell morphology of strains were studied using light microscopy. Reference strains i.e. *Azospirillum lipoferum* GSF 191, *A. brasilense* sp245, *E. coli* 18 Per Gpm, *Herbaspirillum seropedicare* Z67, Lm EGDE, *A. amazonense* (DSM2787), *A. irakense* KBC1 (DSM 11586) were used for FISH analysis and *Burkholderia vietnamiensis* JA-9 and *Burkholderia cepecia* LA-8 were used as positive controls for siderophores production. All the used reference strains were obtained from Department of Microbe-Plant Interactions, Helmholtz Zentrum Munchen, German Research Center for Environment Health (GmbH), Neuherberg/ Munich, Germany.

Table 1. Growth promoting properties of cotton rhizosphere associated bacteria.

S. No.	[a] Tentative identification	Strain	Source	[b] IAA (µg/ mL)	[c] ARA (nmol C_2H_2/ h/vial)	[d] P (µg/mL)	[e] Zn (mm)	[f] ACC deaminase
1.	*Bacillus* sp.	Z1	Soil	6.0 ± 0.3	18 ± 2.1	-	-	-
2.	*Pseudomonas* sp.	Z2	Soil	1.2 ± 1.1	1523 ± 8.97	6.7 ± 2.3	-	-
3.	*Bacillus* sp.	Z3	Soil	0.2 ± 0.1	404 ± 4.2	-	-	-
4.	Unidentified	Z4	Soil	-	1388 ± 5.1	-	-	-
5.	*Pseudomonas* sp.	Z5	Soil	1.2 ± 0.2	1625 ± 4.3	95 ± 0.1	2.0 ± 0.2	+
6.	*Azoarcus* sp.	Z6	Soil	-	1005 ± 7.4	-	-	-
7.	*Bacillus* sp.	Z7	Soil	-	158 ± 2.3	-	-	-
8.	Unidentified	Z8	Soil	-	355 ± 4.6	-	-	-
9.	Unidentified	Z9	Soil	-	458 ± 5	-	-	-
10.	*Azospirillum* sp.	Z10	Soil	-	20 ± 1.2	-	-	-
11.	*Pseudomonas* sp.	Z11	Soil	0.3 ± 0.1	135 ± 7.6	75 ± 0.2	1.2 ± 0.3	+
12.	Unidentified	Z12	Soil	-	843 ± 3.5	-	-	-
13.	*Enterobacter* sp.	S1	Washed root	5.1 ± 1.2	121.4 ± 8.1	-	-	-
14.	*Enterobacter* sp.	S2	Washed root	6.5 ± 1.4	29.3 ± 4.3	-	-	-
15.	*Bacillus* sp.	S3	Washed root	16.9 ± 1.6	235.3 ± 5.3	77 ± 1.2	1.2 ± 0.1	-
16.	*Bacillus* sp.	S4	Soil	0.2 ± 0.1	40.1 ± 2.7	-	-	-
17.	*Azoarcus* sp.	S5	Soil	1.6 ± 0.9	148.3 ± 4.8	-	-	-
18.	Unidentified	S6	Unwashed root	3.6 ± 0.7	17.3 ± 1.5	-	1.4 ± 0.2	-
19.	*Azoarcus* sp.	S7	Soil	1.6 ± 0.3	13.5 ± 2.1	-	-	-
20.	*Azotobacter* sp.	S8	Unwashed root	0.9 ± 0.2	252.8 ± 8.4	-	-	-
21.	*Bacillus* sp.	S9	Unwashed root	1.2 ± 0.5	93.3 ± 3.1	-	-	-
22.	*Bacillus* sp.	S10	Unwashed root	41.2 ± 0.9	67.1 ± 3.7	-	-	-

[a] Tentative identification based on morphological features observed under light microscope. [b] Indole acetic acid detected and quantified using HPLC [c] Acetylene reduction assay was carried out using Gas chromatograph. [d] Phosphate solubilization was measured using spectrophotometer, [e] Zinc mobilization was measured as ratio of zone diameter/ colony diameter

[f] ACC deaminase activity was qualitatively studied by using ACC as a sloe nitrogen source in DF salt minimal medium

The results of IAA, ARA, P and Zn are an average of three replicates. ± Standard deviation, - no IAA production/ P solubilization/ Zn mobilization/ ACC deaminase activity

Detection of growth promoting properties of bacterial strains: Nitrogen fixation by the bacterial strains was measured using acetylene reduction assay (ARA) as described by Hardy *et al*., (1968). Quantification of available phosphorus solubilized by the bacterial isolate was qualitatively detected on Pikovskaia's medium containing tricalcium phosphate and quantified by Phospho-molybdate blue color method (Murphy & Riley 1962). For the detection and quantification of indole acetic acid (IAA) production by the bacterial isolates, cultures were grown in Okon's malate medium. Tryptophan (100 mg/ L) was added as the precursor of IAA. After one week of growth of growth, qualitative estimations of IAA were performed by Fe-$HClO_4$ and Fe-H_2SO_4 reagents (Gordon & Weber, 1951). The ethyl acetate oxidation method was used for a quantitative estimation of IAA by HPLC using Turbochom software (Perkin Elmer USA) (Tien *et al*., 1979). Bacterial isolates were screened for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity qualitatively using vials containing ACC as sole nitrogen source (Penrose & Glick, 2003). Resistance of the selected isolates against different antibiotics was carried out on LB agar plates as described by Yasmin *et al*., (2004). Zn solubilization was studied on Tris-minimal salt medium containing zinc sulphate/ zinc oxide following the method of Sayer *et al*., (1995).

Effect of inoculation with PGPR strains on cotton plant: Acid de-linted cotton seeds maintained at the pH of 6 by washing with water were surface sterilized by immersion in 0.1% $HgCl_2$ solution for three minutes followed by repeated washing with sterile distilled water.

Pot experiment: A pot experiment was conducted to study the beneficial effects of bacterial inoculants on cotton variety IR-FH-901. Seven bacterial strains namely Z1, Z2, Z5, Z11, S3, S9 and S10 isolated from cotton were inoculated as a single strain. Three similar sized seeds were sown in each plastic pot (7 cm diameter x 10 cm height) having sterilized sand and then inoculated with suspension of each bacterial strain in a proportion of 0.5 mL inoculum/ seed containing a pre-determined number of bacterial cells (10^6-10^7 cells/ mL) counted by plate count. The un-inoculated plants with and without N were used as controls. For N containing un-inoculated plants, KNO_3 (0.05%) was added giving an N concentration of 70 µL/ mL. These pots were then kept in a growth room maintaining a day/ night temperature of 30±2/ 25±2°C/ 25±2°C and a day length of 16 h. light intensity during the day was 20,000 Lux. The seedlings were thinned to 1 plant per pot after 5 days of germination. The plants were watered with an equal volume of 1/4th strength of nutrient solution (Hoagland & Arnon, 1950) with an interval of 3 days. Each treatment was replicated four times in a complete randomized design (CRD). At the time of harvesting after 60 days, root area and root length of plants were measured with "Root Image Analysis" program. The dry weight of plant biomass was recorded. Total N and P contents of the plants were determined by Kjeldahl's method (Yoshida *et al*., 1976) and a method as described by Ashraf *et al*., (1992), respectively.

Field experiment-I: The field trials were conducted at experimental fields of NIBGE. The effect of seven PGPR

strains, already tested in pot experiment, was evaluated on cotton variety IR-FH-901. The treatments were same as tested in pot experiment. Seven bacterial strains namely Z1, Z2, Z5, Z11, S3, S9 and S10 isolated from cotton were inoculated as a single strain with 1/4th of the recommended N& P using seed pelleting technique. Treatments with recommended N& P and with 1/4th of the recommended N & P dose without inoculation were selected as positive and negative controls, respectively. These treatments were distributed in a complete randomized design with four replicates. The plot size was 2 m x 4.5 m. Plots were initially over seeded and then hand thinned at first leaf stage. The plants were harvested at maturity stage after six months. The yield components were boll number, boll mass, lint yield and seed yield per plot. The dry weight of leaves, stem and bud case of the plants was recorded. Total N and P content in leaves, stem and bud case of the plants from different treatments was determined as mentioned earlier.

Field experiment-II: Another trial was conducted at experimental fields of NIBGE. The effects of two selected bacterial strains i.e., *Pseudomonas* sp. *Z5* and *Bacillus* sp. S10, already tested in pot and field experiments, were evaluated on cotton variety NIAB 846. The seeds of cotton variety NIAB 846 were obtained from Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The soil samples were collected from ten different sites of field (0–200 mm depth) before sowing and fertilization. The soil samples were mixed thoroughly and studied for viable cell count, pH, EC, N, P and organic matter. Total N and P of soil sample were measured by the method described by Bremner (1965) and Ashraf *et al*., (1992), respectively. Two bacterial strains namely Z5 and S10 were inoculated as a single strain as well as in combination with different levels of fertilization using seed pelleting technique. Treatments with recommended N & P and with half of the recommended N & P dose without inoculation were selected as positive and negative controls, respectively. The plot size was 3 m x 6 m and the treatments were distributed in a randomized design with four replicates. The plants were harvested at maturity after six months and studied for yield components.

Statistical analysis: Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the 0.05% level was tested to detect differences among treatments by using "MSTATC" program. Mean values and the standard deviation were calculated. The pot and field data was analyzed by CRD and RCBD, respectively.

Fluorescence *in situ* hybridization (FISH) for the identification of bacteria: FISH with fluorochrome labeled oligonucleotide probes was performed according to previously described protocols (Amann *et al.,* 1995; Stoffels *et al.,* 2001). The probes used were oligonucleotide synthesized with Cy3 fluorochrome at the 5′ end (Interactiva Biotechnologie GmbH, Ulm, Germany). All probe sequences, target positions, hybridization conditions and specificities are given in Table 2. Epifluorescence microscopy was performed with an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with filter sets 01, 09 and 15. The results were considered as positive that gave good signals with target cells.

Table 2. rRNA-targeted oligonucleotide probes, rRNA target positions (*E. coli* numbering), specificity and optimal hybridization conditions.

S.No.	Probe	Sequence (5′-3′)	Position	% FA[a]	Specificity	Reference
1.	EUB338	GCTGCCTCCCGTAGGAGT	16S, 338-355	0	Bacteria	Amann *et al.,* 1990
2.	ALF1b	CGTTCGCTCTGAGCCAG	16S, 19-35	20	alpha *Proteobacteria*	Manz *et al.*,, 1992
3.	BET42a	GCCTTCCCACTTCGTTT	23S, 1027-1043	35	beta *Proteobacteria*	Manz *et al.*,, 1992
4.	GAM42a	GCCTTCCCACATCGTTT	16S, 1250-1267	35	gamma *Proteobacteria*	Manz *et al.*,, 1992
5.	AZO440a	GTCATCATCGTCGCGTGC	16S, 440-45	50	*Azospirillum* spp.	Stoffels *et al.*,, 2001
6.	Aama1250	CACGAGGTCGCTGCCCAC	16S, 1250-1267	50	*A.amazonense*	Stoffels *et al.*,, 2001
7.	Abras1429	CCACCTTCGGGTAAAGCCA	16S, 1420-1438	40	*A.brasilense*	Stoffels *et al.*,, 2001
8.	Airak1423	CACCGGCTCAGGTAAAG	16S, 1423-1440	10	*A. irakense* cluster	Stoffels *et al.*,, 2001
9.	Airak 985	TCAAGGCATGCAAGGGTT	16S, 985-1003	35	*A. irakense* cluster	Stoffels *et al.*,, 2001
10.	Alila 1113	ATGGCAACTGACGGTAGG	16S, 1113-1130	35	*A. lipoferum*	Stoffels *et al.*,, 2001
11.	Sino1421	CTACCTTCGGGTAGAACC	16S, 421	35	*Sino rhizobium meliloti*	Ludwig *et al.*,, 1998
12.	Smel 585	CCCTCACTTAACAATCCG	16S, 585	35	*Sino rhizobium meliloti*	Ludwig *et al.*,, 1998
13.	Smel 729	CCAGACCAGTGAGCCGCC	16S, 729	35	*Sino rhizobium meliloti*	Ludwig *et al.*,, 1998
14.	Rhi 247	TCGCTGCCCACTGTC	16S, 247	35	*Rhizobium* spp.	Ludwig *et al.*,, 1998
15.	SUBU1237	CCCTCTGTTCCGACCATT	16S, 1237-1254	60	*Berkholderia* spp. & *Sutturella* spp.	Stoffels *et al.,,* 1998
16.	CF319a	TGGTCCGTGTCTCAGTAC	16S, 319-336	35	Cytophaga-*Flexibacter*	Manz *et al.*,, 1996
17.	HGC	TATAGTTACCACCGCCGT	23S, 1901-1918	35	Gram positive bacteria with high G+C	Roller *et al.*,, 1994
18.	LGC	TGGAAGATTCCCTACTGC	16S, 354-371	20	Gram positive bacteria with low G+C	Stoffels *et al.*,, 2001
19.	ACA 23	ATCCTCTCCCATACTC	16S, 23	35	*Acinobacter*	Wagner *et al.*,, 1994
20.	LGC 354a	TGGAAGATTCCCTACTGC	16S, 354-371	35	Low G+C, *Bacillus sphaericus*	Stoffels *et al.*,, 2001
21.	LGC 354b	CGGAAGATTCCCTACTGC	16S, 354-371	35	Low G+C, *Bacillus* spp.	Stoffels *et al.*,, 2001
22.	LGC 354c	CCGAAGATTCCCTACTGC	16S, 354-371	35	Low G+C, *Streptococcus* spp.	Stoffels *et al.*,, 2001

[a] Amount of Foramide (%, v/v) in hybridization buffer

Analysis of 16S rRNA gene sequence: The same single colony used for FISH analysis of the selected 7 bacterial isolates i.e. Z1, Z2, Z5, Z11, S3, S9 & S10 were used to identify these bacteria by 16S rRNA gene sequencing (Hafeez *et al*., 2006). 16S rRNA sequences of the isolates were determined commercially by DSMZ, Braunschweig, Germany. The new sequences were added to an alignment of approximately 6000 published homologous sequences from bacteria, using the alignment tool of the software package ARB (http://www.arb-home.de). The resulting 16S rDNA sequences were analyzed for homologies to sequences deposited in the GenBank databases using BLAST (www.ncbi.nlm.nih.gov).

Results and Discussion

Morphological and physiological characteristics of 22 bacterial strains from cotton rhizosphere are given in Table 1. Following morphological characterization, motility and Gram staining, the strains were compared with those of standard species using Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Seven strains i.e., Z1, Z3, Z7, S3, S4, S9 and S10 were Gram positive while all the others were Gram negative. These strains were belong to different genera i.e., *Azospirillum, Azotobacter*, *Azoarcus, Bacillus, Enterobacter and Pseudomonas* (Table 1).

Some important capabilities of practical utility shown by these strains were nitrogenase activity, IAA production, P-solubilization, siderophore production and ACC deaminase activity. Out of 22 bacterial strains, fifteen were found to produce IAA ranged 0.2-41.2 µg/ mL. *Bacillus* sp., S10 produced highest amount of IAA i.e. 41.2 µg/ mL. Relatively higher amount of IAA produced by bacterial strains would indicate that these strains may contribute to plant growth as IAA induces better root growth, which can explore more soil volume for nutrients (Tyler *et al.,* 2008). All bacterial strains fixed N ranged from 8-1625 nmoles C_2H_4 produced/ h/ vial. *Pseudomonas* sp. Z5 showed maximum nirtogenase activity. High nitrogenase activity is assumed to facilitate the plant with more fixation of atmospheric nitrogen and thus availability of more N to cotton plants. Only four strains, *Pseudomonas* spp. Z2*,* Z5, Z11 and *Bacillus* sp. S3 were capable of phosphate solubilization ranged 75-95 µg/ mL. The strain Z5 exhibited highest P solubilization i.e. 95 µg/ mL (Table 1). Among plant growth promoting rhizobacteria, phosphate-solubilizing bacteria are already used as commercial biofertilizers for agricultural improvement (Hafeez *et al.,* 2006; Zaidi *et al.,* 2009). ACC deaminase-containing strains Z5 and Z11 may lower the level of ACC in the stressed cotton plants infected with fungal pathogen therefore, reducing the level of stress caused by ethylene and hence the injury to the host plant (Tyler *et al.,* 2008). The bacterial strains S3, S6, Z5 and Z11 were able to mobilize both the insoluble zinc compounds i.e., zinc oxide and zinc sulphate. Zn mobilization by these bacterial strains measured as ratio of zone diameter/ colony diameter ranged from 1.2-2 mm. *Pseudomonas* sp. Z5 showed maximum clear zone diameter from all the tested strains (Table 1). Previous studies also showed the presence of Zn mobilizing PGPR (Intorne *et al.,* 2009) in rice rhizosphere (Tariq *et al.,* 2007). The resistance of all bacterial strains against different antibiotics was carried out to study their potential competitiveness. Strain Z2 showed higher level of resistance to all the six tested antibiotics followed by strain Z11. The tested strains showed relatively more sensitivity to kanamycin and streptomycin. *Bacillus* sp. S10 was resistant to chloramphenicol, kanamycin, tetracycline, spectinomycin but showed susceptibility to streptomycin at all concentrations and ampicillin, only at higher concentration. Strains Z2, Z11 and S10 may show better competitiveness under natural soil conditions but more field study will confirm these findings. Antibiotic resistance possessed by rhizobacteria confers an ecological benefit for their survival and viability in the rhizosphere (Dobereiner & Baldani, 1997).

rRNA-targeted probes and FISH was evaluated to establish a quick screening test for a set of new isolates from any crop. A total number of 11 bacterial strains were tested with 16S rRNA directed phylogenetic oligonucleotide probes for the characterization of their phylogenetic affiliation in a top to bottom approach. All these strains gave positive hybridization with the probe Eub-338. Using group-specific probes for the alpha-, beta- and gamma-proteobacteria, six bacterial strains gave positive hybridization signals with alpha-proteobacteria and four with gamma-proteobacteria specific probes while non of the tested bacteria gave positive signal with beta-proteobacteria specific probe (Fig. 1). These strains were further hybridized with probes specific for *Azospirillum* spp. i.e., AZO44a, Airak985, Airak985, Alila1113, Abras1420, Aama1250 but none of them gave positive signal with these probes except the reference strains of *Azospirillum* used as positive control. These strains were not hybridized with probes specific for *Rhizobium* spp. Therefore, these strains were further hybridized with genus-cluster probes i.e., LGCa 354, LGCb 354, LGCc 354. Strain specific 16S rRNA targeted oligonucleotide probes were also developed to be used for identification of *Pseudomonas* sp. Z5 and *Bacillus* sp. S10 (data not shown). Using nested application of phylogenetic oligonucleotide probes, bacterial strains could be identified without applying other chemical testing. The results indicated the potential of this technique as a rapid screening method for bacterial identification. In the conventional identification of bacteria, time consuming series of physiological and biochemical tests are necessary, the identification of isolated bacteria using FISH and molecular phylogenetic probes targeting 16S- or 23S-rRNA is rapid (Bhavanath *et al.,* 2009).

The complete 16S rRNA sequence analysis of selected strains Z1, Z2, Z5, Z11, S3, S9 and S10 was carried out. A fragment of 1500 bp was obtained after amplification. 16S rRNA sequences of selected strains were determined commercially by DSMZ, Braunschweig, Germany. The sequences of *Bacillus fusiformis* Z1, *Pseudomonas putida* Z2, *P. aeruginosa* Z5 & Z11, *B. fusiformis* S3, *B. pumilus* S9 and *B. fusiformis* S10 were allocated with accession numbers of AY548950, AY548951, AY548952, AY548953, AY548954, AY548955 and AY548956, respectively (Table 3).

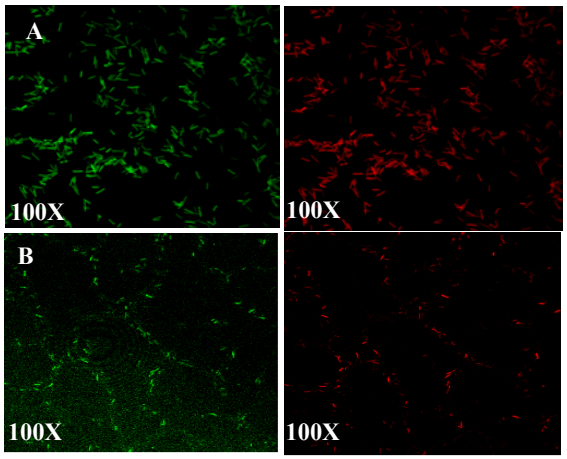

Fig. 1. Mutiple probe application in fluorescence *in situ* hybridization (FISH) and confocal scanning laser microscopy (CSLM).
In situ whole cell identification of *Pseudomonas aeruginosa* Z11 (A) and *P. aeruginosa* Z5 (B) using FLUOS-labeled EUB 338 (green) and Cy3-labeled GAM42a (red) probes

Pot experiment results showed that the bacterial inoculation significantly improved the plant growth parameters when compared to un-inoculated control plants supplemented with 1/4th of the recommended doses of N and P but the maximum growth promotion was observed with un-inoculated control plants supplemented with full/ recommended doses of N and P. These results evaluated *Pseudomonas* sp. Z5 as a best growth promoting strain as it significantly increased root area, plant biomass and P. *Bacillus* sp. S10 showed better results regarding N and K uptake. Total accumulation of plant N was significantly higher in response to inoculation with *Bacillus* sp. S9. According to the results obtained, Z5 not only solubilized phosphates significantly *in vitro* but was also able to mobilize P efficiently in cotton under *In vivo* conditions (Table 4). It has been reported that the production of IAA by bacteria enhances the development of host plant root system and thus helps in growth of crop plants. Further, solubilization of iron by microbial siderophores, solubilization of P and mobilization of Zn were found to significantly increase crop yield (Intorne *et al.*, 2009).

Table 3. Identification of the bacterial strains used in the present study.

S. No.	Strains	Identification	
		16S rRNA	**Fish**
1.	Z1	*Bacillus fusiformis* AY548950	LGC Gram positive, *Bacillus* sp.
2.	Z2	*Pseudomonas putida* AY548951	Gamma *Proteobacteria*
3.	Z3	ND	LGC Gram positive, *Bacillus* sp.
4.	Z5	*Pseudomonas aeruginosa* AY548952	Gamma *Proteobacteria*
5.	Z7	ND	LGC Gram positive, *Bacillus* sp.
6.	Z10	ND	LGC Gram positive, *Bacillus* sp.
7.	Z11	*Pseudomonas aeruginosa* AY548953	Gamma *Proteobacteria*
8.	Z12	ND	Cytophaga-*Flexibacter*
9.	S3	*Bacillus fusiformis* AY548954	LGC Gram positive, *Bacillus* sp.
10.	S9	*Bacillus pumilus* AY548955	LGC Gram positive *Bacillus* sp.
11.	S10	*Bacillus fusiformis* AY548956	LGC Gram positive, *Bacillus* sp.

Table 4. Effect of inoculated cotton rhizosphere associated bacteria on root length, root area, plant biomass, P and N content of cotton var. IR-FH-901 (Pot experiment).

S. No.	Strains	Root length (m)	Root area $(mm)^2$	Plant biomass (g)	N (%)	P (µg/g)	K (µg/g)
1.	*Bacillus* sp. Z1	0.3 DE	55.5 D	0.6 BC	0.7 BC	0.3 C	0.09D
2.	*Pseudomonas* sp. Z2	0.7 B	74.9 C	0.6 BC	0.7 BC	0.4 BC	0.3 C
3.	*Pseudomonas* sp. Z5	0.6 BC	94.5 B	0.8 B	0.6 C	0.6 B	0.3 C
4.	*Pseudomonas* sp. Z11	0.7 B	1.9 DE	0.6 BC	0.7 BC	0.3 C	0.3 C
5.	*Bacillus* sp. S3	0.5 BCD	60.9 CD	0.6 BC	0.7 BC	0.3 C	0.5 B
6.	*Bacillus* sp. S9	0.4 CDE	64.3 CD	0.6 BC	1.0 A	0.3 C	0.08D
7.	*Bacillus* sp. S10	0.2 E	23.4 F	0.2 D	0.9 AB	0.3 C	2.0 A
8.	[a] C- (1/4th NP)	0.2 E	37.2 EF	0.4 CD	0.5 C	0.4 BC	0.01D
9.	[b] C+ (Full NP)	1.1 A	129.1A	1.2 A	0.7 BC	1.6 A	0.02D

[a] Negative control: without inoculation with 1/4th of the recommended doses of N and P
[b] Positive control: without inoculation with full/ recommended doses of N and P
Means are the average of four replicates. Means followed by the same letter differ non-significantly at p = 0.05 according to DMRT

Table 5. Effect of inoculated cotton rhizosphere associated bacteria on dry matter, P, N uptake and yield components of cotton var. IR-FH-901 (Field experiment I).

Strains	Leaves			Stem			Bud case			Boll no. / plant	Boll mass	Lint yield	Seed yield
	Dry wt.	N	P	Dry wt.	N	P	Dry wt.	N	P		(g/ plant)		
	(g/plant)	(%)	(µg/g)	(g/plant)	(%)	(µg/ g)	(g/plant)	(%)	(µg/ g)				
Bacillus sp. Z1	30.8E	2.9E	4.0D	55.8C	1.2A	0.3D	31.8E	1.4H	0.2G	44.13BC	88.4 H	36.2 D	49.4 F
Pseudomonas sp. Z2	37.1B	3.2B	2.2F	52.3H	1.1B	0.3D	28.1I	1.2I	0.3F	44.80BC	78.0 I	28.8 E	45.2 G
Pseudomonas sp. Z5	27.9H	3.1C	4.4B	58.1B	1.2A	0.7A	37.3B	1.9B	1.0A	48.20ABC	98.0 G	38.9 CD	57.1 E
Pseudomonas sp. Z11	33.0C	2.7G	4.7A	52.8G	1.2A	0.3D	34.2D	1.7C	0.3F	51.93ABC	109.2 E	40.5 BC	63.5 D
Bacillus sp. S3	39.2A	3.3A	2.6E	55.4D	1.2A	0.4CD	29.2H	1.6F	0.4E	55.33AB	120.8 C	48.1 A	69.3 C
Bacillus sp. S9	28.3G	2.8F	2.2F	54.9E	1.2A	0.6AB	30.9G	1.5G	0.8B	47.53ABC	113.1 D	43.0 B	65.2 D
Bacillus sp. S10	32.7D	2.8F	2.6E	78.4A	1.1B	0.3D	44.2A	2.0A	0.5D	59.13A	127.7 B	48.9 A	74.5 B
[a] C- (1/4[th] NP)	30.5E	2.6H	2.6E	51.1I	1.0C	0.3D	31.4F	1.68D	0.7C	39.20 C	106.9 F	41.2 BC	63.5 D
[b] C+ (Full NP)	29.3F	3.0D	4.3C	53.5F	1.2A	0.5BC	35.6C	1.65E	0.4E	51.27ABC	136.6 A	49.1 A	80.7 A

[a] Negative control: without inoculation with 1/4[th] of the recommended doses of N and P

[b] Positive control: without inoculation with full/ recommended doses of N and P

Means are the average of four replicates. Means followed by the same letter differ non-significantly at p = 0.05 according to DMRT

The effect of 7 PGPR strains was evaluated on cotton variety IR-FH-901 under field conditions. All inoculated treatments increased yield of cotton as compared to control plants with 1/4[th] of the recommended NP. Boll mass, lint yield and seed yield of positive control plants with full/ recommended NP were found to be highest compared to the other inoculated treatments with 1/4[th] of the recommended NP. P content was significantly increased in cotton plants inoculated with *Pseudomonas* sp. Z5 (Table 5). *Bacillus* sp. S10 increased the yield (127.7 g boll mass/ plant, 48.9 g lint/ plant and 74.5 g seed/ plant) approximately equal to that of positive control plants with recommended doses of N and P (Table 5). Therefore, field experiment evaluated *Bacillus* sp. S10 as the most effective growth promoting strain. Although, S10 was N-fixing, IAA and siderophore producing strain but not as significantly as did *Pseudomonas* sp. Z5 suggesting that the growth promotion was probably either due to other mechanisms or hormone production as *Pseudomonas* sp. Z5 produced IAA less than that of the *Bacillus* sp. S10. Insoluble P dissolution by the *Pseudomonas* sp. Z5 may not necessarily be the primary mechanism of plant growth promotion in cotton. The functional traits such as IAA, siderophores and nitrogenase activity may enhance the potential of *Bacillus* sp. S10 as biofertilizer. *Bacillus* spp. were extensively applied in China as part of a complex called "yield increasing bacteria". *Bacillus subtilis* FZB 24 was reported to have a high ability to improve plant growth and yield based on increasing the capacity of roots to mobilize and take up nutrients and substances for overall reproductive plant fitness (Yao *et al.*, 2006).

Another field trial was conducted at NIBGE to evaluate the effects of antagonistic *Pseudomonas* sp. Z5 and *Bacillus* sp. S10 (already tested in pot and field experiments) on cotton variety NIAB 846. In first field experiment, the effects of bacterial inoculation was studied up to 1/4[th] of the recommended NP but in second field trial conducted in year 2009, the effects of bacterization were studied with half of the recommended doses and full/ recommended doses of N & P as well as bacterization without any fertilization. The soil sample collected from field had pH 8.5, EC 4.7 mS/ cm and organic matter 0.61%. Total N and available P were 0.9 mg/ g soil and 3.7 µg P/ g soil, respectively. The population density of indigenous bacteria was 8.5±0.9 Log_{10} CFU/ g soil. The yield parameters i.e. boll mass, lint and seed yield per plot were found to be highest at the recommended doses of N & P when inoculated with *Bacillus* sp. S10 followed by *Pseudomonas* sp. Z5. At half of the recommended doses of N & P, *Bacillus* sp. S10 performed well followed by mixed inoculation of S10 and Z5 as compared to un-inoculated control plants. Furthermore, the yield obtained with *Bacillus* sp. S10 inoculation supplied with half of the recommended NP was higher than that of the un-inoculated control plants grown with full/ recommended doses of N and P (Fig. 2). When the effects of bacterization without fertilization was compared with one other or with the control plants, all the bacterial inoculations increased the yield parameters compared to un-inoculated plants but *Bacillus* sp. S10 performed better relative to all other treatments. In general, the effects of chemical fertilizers on yield parameters were higher than those of bio-inoculants alone but these inoculants increased the yield of cotton at lower levels of fertilizers and were higher than those of un-inoculated plants supplied with the recommended doses of N and P. The use of these microbial inoculants may increase the cotton yield with saving chemical fertilizers. The use of microbial inoculants were reported to increase 20, 15 and 60-80% in yield of paddy, wheat and legumes, respectively and a saving of 50-100% of chemical fertilizer (Adesemoye *et al*., 2009). Hafeez (2009) reported that the use of "*BioPower*" (commercial biofertilizer product of NIBGE) increased the yield from 10-60% with the saving of chemical fertilizer from 30-90% for different crops. The benefit to the farmers in terms of cost per hectare is 47-426 US $.

Present study resulted in the selection of *P. aeruginosa* Z5 (AY548952) and *Bacillus fusiformis* S10 (AY548956). *Bacillus fusiformis* S10 was found to be effective cotton growth promoting bacteria by showing its consistence performance in pot experiments as well as in field

experiments of two years. Because of nitrogen fixing ability, P solubilization, Zn mobilization, ACC-deaminase activity, the production of siderophores and growth hormone, *P. aeruginosa* Z5 strain can be regarded as a novel bioinoculant for cotton. *Pseudomonas aeruginosa* Z5 was deposited to "DSMZ German Culture Collection" with accession no. DSM16519. These bacterial strains i.e. *Pseudomonas aeruginosa* Z5 and *Bacillus fusiformis* S10 may be used as a premium quality supplemental inoculant for cotton, which may enhance nutritional availability and produce superior yields with reduced production cost. Further studies have been carried out to study the biocontrol potentials and colonization of these inoculants in the cotton rhizosphere using FISH and strain-specific antibodies. Moreover, partial identification and the rapid selection system by FISH may be a very useful tool for excluding similar bacterial isolates encountered during screening.

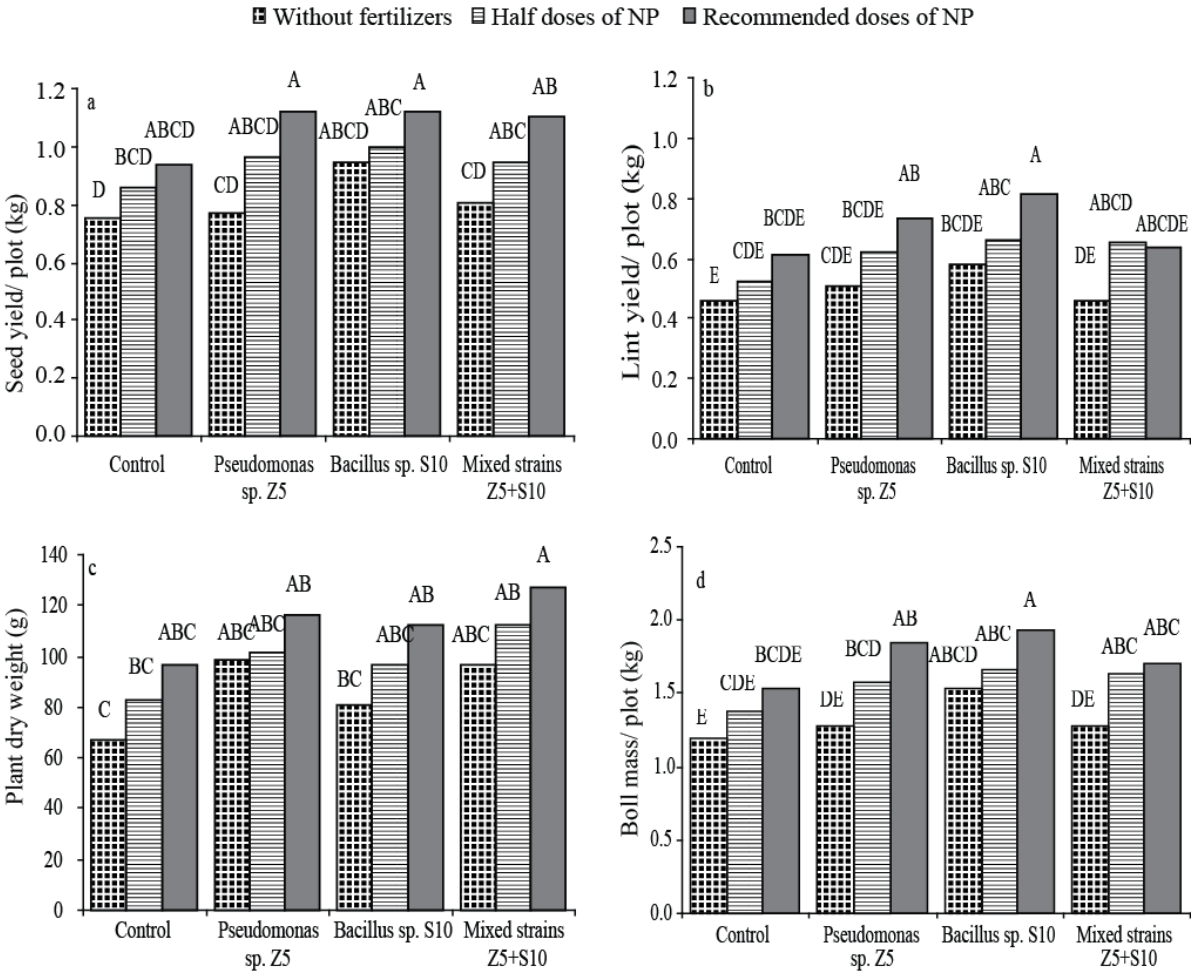

Fig. 2. Effect of inoculated cotton rhizosphere associated bacteria on yield components of cotton var. NIAB 846 (Field experiment II). (a) Effect on seed yield, (b) lint yield, (c) plant dry weight and (d) boll mass.
Means are the average of four replicates. Means followed by the same letter differ non-significantly at p = 0.05 according to DMRT. NP: Nitrogen and phosphorus, Half NP: Half of recommended nitrogen and phosphorus doses

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