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CO-CULTIVATION OF TOMATO WITH TWO *BACILLUS* STRAINS: EFFECTS ON GROWTH AND YIELD

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) have gained worldwide importance and acceptance for agricultural benefits. By virtue of their rapid rhizosphere colonization and stimulation of plant growth, there is currently considerable interest in exploiting these rhizosphere bacteria to improve crop production. In current investigation, two bacillus strains viz: *Bacillus fortis* IAGS162 and *B. subtilis* IAGS174 were evaluated for their ability to promote growth of three tomato varieties under greenhouse and field conditions. All the three tomato varieties were individually treated with both test bacterial strains in both greenhouse and field experiments. Experimentation was performed in completely randomized block design. *In vitro* biochemical assays indicated the ability of these bacteria to produce indole acetic acid, siderophores and phosphate solubilization. Greenhouse experiments indicated the ability of both strains to significantly increase shoot length, root length, and total biomass in all three tomato varieties. Seemly, significant increases in total chlorophyll, carotenoid and sugar concentrations were found in tomato plants co-cultivated with the bacterial strains. Two consecutive field experiments also supported the outcomes of the greenhouse assays. Tomato plants receiving bacterial inoculum had significantly greater shoot growth and fruit yield. *B. subtilis* IAGS174 was superior to *B. fortis* IAGS162 in promoting most traits studied in the laboratory or greenhouse. This study provided strong evidence for the potential use of these both bacterial strains in agriculture.

Keywords: Plant growth promoting bacteria (PGPB), *Bacillu*s, Tomato, Indoleacetic acid, Phosphate solubilisation, Siderophores.

INTRODUCTION

Plant growth promoting (PGP) microbes exert beneficial effects on growth and development of plants (Bashan, 2005). These include mainly bacteria and some fungi (De Silva *et al.,* 2000). These microbes occupy specific microbial ecological niches in plant rhizosphere, interact with plant roots (Appuhn and Joergensen, 2006) via specific molecular signals, and play a vital role in biogeochemical cycling of nutrients and maintenance of plant health and soil fertility (Barea *et al.,* 2004). Plant roots secrete diverse organic nutrients and signalling molecules those attract microbial populations (Pinton *et al.,* 2007). This microbial community associated with plants roots is called the rhizo-microbiome (Chaparro *et al.,* 2013) and affects rooting morphology and the supply of available nutrients, thus improving plant health (Barea, 2000). Bacteria capable of colonizing plants roots and stimulating plant growth and health are called plant growth promoting bacteria (PGPB) (Esitken *et al.,* 2005). PGPB can interact with wide range of host plant species and encompass a large taxonomic diversity (Gomes *et al.,* 2010).

PGPB use in place of fertilizers is capable of improving plant yield and can help to sustain soil productivity and environmental health (Esitken *et al.,* 2005). PGPB increase plant growth by facilitating nutrients uptake via pathway including nitrogen fixation, phosphorus solubilization and siderophore production. They also improve root development and growth by producing phytohormones such as auxins (Egamberdiyeva, 2005), cytokinins (Garcia de Salamone *et al.,* 2001) and gibberellins (Gutierrez-Manero *et al.,* 2001). PGPB have been identified in many genera but most are *Bacillus* spp. (Esitken *et al.,* 2002) which form endospores to ensure prolonged life and give them stability in different formulations. The efficacy of PGPB has been demonstrated in field experiments.

Tomato is an economically important food crop cultivated on a large scale in many areas of the world. Its fruit plays an important role in the human diet and provides health benefits as a source of vitamins, minerals and antioxidants including phenolics, folate, lycopene and β-carotene) (Fraser *et al.,* 2009). In previous research, the authors screened native bacillus strains capable of inducing resistance against *Fusarium* wilt in tomato plants (Akram *et al.,* 2013). The participation of PGPB in the induction of systemic resistance, is related to the production of so called elicitors (inducers, determinants), activating the defense responses of plant

cells (Kloepper and Ryu 2006). These defense responses include production of phytoalexins and re-enforcement of plant physical defense barriers by increased deposition of lignin and tannins (Kloepper *et al.,* 2006). All these together produce conditions non-favorable for pathogen infection and invasion. In this way these bacterial microbes indirectly promote plant growth by inhibiting phytopathogenic microbes living in the same ecosystem (Vessey, 2003).

It was hypothesized that bacterial strains capable of inducing resistance in plants can also promote growth. The current research was designed to explore plants growth promoting ability of two previously studied bacillus strains under both greenhouse and field conditions along with characterization of these strains for presence of plant growth promoting biochemicals.

MATERIALS AND METHODS

Microorganisms: Two bacillus strains, *B. fortis* IAGS162 and *B. subtilis* IAGS174 were used. These were rhizospheric in nature and were obtained from the bacterial conservatories of the Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. Bacteria were grown on LB agar media and stocks kept in 80% glycerol at -20^{o}C.

In-vivo characterization of bacillus strains for plant growth promoting properties: Strains were first characterized for siderophores production, IAA production and phosphate solubilization. Siderophore production was tested qualitatively by the chrome azural S (CAS) assay as described by (Schwyn and Neilands, 1987). Bacteria were grown overnight in Luria Broth (LB) media and supernatant was prepared by centrifuging at 1900g. Upper clear supernatant was poured into wells made in plates containing CAS dye as indicator. Qualitative assay for siderophore production was performed by mixing bacterial supernatant with same volume of CAS assay solution (0.6 mM cetyltrimethylammonium bromide, 15 mM $FeCl_3$, 150 mM chrome azurol S, 0.5 M anhydrous piperazine, 0.75 M HCl) and after 4 h of incubation, absorption was recorded at 630nm.

IAA production was assayed by the method of Patten and Glick (1996). Bacteria were grown in LB broth in the presence and absence of L-tryptophan. Supernatant was reacted with double volume of Salkowski reagent (0.5 % $FeCl_3$ in 50 ml of 35% $HClO_4$) in the dark for 30 min and absorption was recorded at 535 nm. Quantities of IAA produced were determined by comparing with a standard curve.

Primary screening for phosphate solubilization was achieved by observing development of clear zones on Pikovskaya medium containing tricalcium phosphate as substrate. Quantitive assay was performed by estimating available phosphorus in Pikovskaya broth provided with known amounts of tricalcium phosphate. Bacteria were grown for 1 week in this media and amounts of soluble phosphate were determined in supernatant by a standard method (Watanabe and Olsen, 1965).

Effect of bacterial strains on tomato growth under greenhouse conditions: Plastic pots of 30cm diameter were filled with sterilized sandy loamy soil as growth media. Three different tomato varieties, Fine Star, Rio Grande and Red Power, were used. Ten seeds of a single tomato variety were sown in each pot. Bacterial inocula were prepared by in LB broth overnight. Bacterial cells were collected by centrifugation and resuspended in sterile distilled water at concentration of 1 x 10^8 cfu ml^{-1}, measured by taking OD at 600 nm. One hundred millilitres of bacterial inoculum was added to each pot. The control treatment was provided with 100 ml of sterile distilled water. Pots were arranged in completely randomized block design with five replications and the experiment was performed twice. After 40 days in a greenhouse environment with temperature of 25+2^{o}C and natural daylight conditions. Plants were provided with distilled sterilized water whenever needed. Plants were harvested and shoot and root length, and total fresh and dry biomass were recorded.

Effect of bacterial strains on production of plant growth related biochemicals: One gram leaf samples from young shoots were taken from greenhouse grown plants at final harvest. Leaf samples were extracted with methanol. For total chlorophyll estimations, absorbance was taken at 645 and 663 nm and carotenoids contents were quantified by taking absorbance at 450 nm according to the methods of Witham *et al.* (1971). Total sugars were estimated by the phenol sulphuric method as described by (Dubois *et al.* 1956).

Development of talc based formulation: Talc based formulations of each strain were developed for application under field conditions. The bacterial strains were grown separately overnight in LB broth at 35^{o}C. Bacterial cells were collected by centrifugation at 1900 g for 15 min. Bacterial cell pellets were re-suspended in sterile distilled water at 10^4 cfu ml^{-1}. Fifty millilitres of the bacterial inoculum was mixed with 100 g of sterilized talc.

Field trials: Field trials were performed from February to April during 2011 and 2012 at the research station of the Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan. Temperature range was 13-38 ^{o}C and 09-31^{o}C in during experiment months of 2011 and 2012 respectively whereas relative humidity was recorded 36-64% and 46-82% in the same regard. Field was divided into sub-plots of size 3x6 meters. In each sub-plot, six raised beds were made ranging 2 meter in

length. No fertilizer or chemical was applied at any experimental stage. Tomato seedlings of the three varieties were raised in sterilized commercial seedling development medium. Two weeks after emergence, roots of seedlings were primed by dipping them in talc based bacterial inoculum and transplanted in on raised beds. Here three treatments were made viz: 1=plants roots primed with inoculum of *B. fortis* IAGS162, 2=plants roots primed with inoculum of *B. subtilis* IAGS174, 3= plants roots primed with sterilized talc alone to serve as control. Treatments were arranged in a completely randomized block design with five replications. For control treatments, seedlings were primed with sterilized talc. Seedlings were transplanted in plots with rows of 6 m length and 60 cm between rows. Plots were provided with water whenever needed. No fertilizers and any agrochemicals were applied in whole field experiment. Fifty seedlings were transplanted in each replicate. Sixty days after transplanting shoot development and yield were assessed. For data analysis, five plants were randomly selected from each sub-plot.

Statistical analysis: Data was subjected to analysis of variance (ANOVA) and Duncan's new multiple range test (DNMRT) using DSASTAT statistical package (Onofri, Italy).

RESULTS

Production of plant growth related substances: The potential plant growth promoting functionality of both bacillus strains was initially determined by production of some plant growth promoting substances as siderophores production, phosphate solubilization and IAA production (Table 1). *B. subtilis* IAGS174 was positive for siderophores production in contrast to *B. fortis* IAGS 162 (Table 1). Both strains produced IAA but *B. subtilis* IAGS174 showed significantly higher quantities (Table 1). Similarly, *B. subtilis* IAGS174 exhibited greater phosphate solubilization potential as compared to *B. fortis* IAGS 162 (Table 1).

Effect of bacterial strain on growth of tomato plants in greenhouse: Bacterial treatments of tomato plants showed significantly higher values in most of the variables measured in this study as compared to control plants (Table 2; Figure 1). However, the magnitude of growth promotion varied among the both strains. Symbiotically grown plants with *B. subtillis* IAGS174 conferred 59.7% increase in shoot length across average basis of all three varieties (Table 2). For *B. fortis* IAGS162 same type of increase in shoot lengths was 42.2%. In treated plants, root length significantly increased from 37.67 to 61.85% for *B. fortis* IAGS162 and 23.58 to 57.72% for *B. subtilis* IAGS174 for all three tomato varieties. Treated plants exhibited dense root network upon uprooting as compared to control (Figure 1). Analysis of %age increase in total biomass showed an average increase of 32.54 and 57.09% in fresh biomass under influence of *B. fortis* IAGS162 and *B. subtillis* IAGS174 respectively in all three tomato varieties under observations (Table 2). Notably similar types of significant changes were observed in dry biomasses of tomato plants under influence of both bacterial strains (Table 2).

Effect of bacterial strains on plant growth related biochemicals: Calorimetric assay detected 49.72 % more total chlorophyll contents across all three tomato varieties receiving *B. subtillis* IAGS174. For *B. fortis* IAGS162 same types of increase was 26.08 %. In the same way, plants receiving bacterial strains showed significant increases in carotenoids and total sugar contents (Figure 2). However, like total chlorophyll, more pronounced increases were observed in plants receiving *B. subtillis* IAGS174 as compared to control (Figure 2). Here carotenoids contents were increased from 23.15 to 49.52 % in plants co-cultivated with *B. subtillis* IAGS174. In the same way, total sugar contents, increased from 19.42 to 37.68% in all three tomato varieties when provided with *B. subtillis* IAGS174 in comparison to untreated control (Figure 2).

Field experiment: Beneficial effects of bacterial strains were observed on shoot length, total number of fruits and yield of tomato plants during two field sessions. Likewise in green house experiments, *B. subtilis* IAGS174 strongly promoted growth and yield of tomato plants in all three tomato varieties, during both the seasons (Table 3,4). In case of tomato plants of variety Fine Star, shoot length significantly increased up to 41 and 34% under influence of *B. subtillis* IAGS174 for year 2011 and 12 respectively. Whereas for *B. fortis* IAGS162, same type of increase was 23 and 26% respectively. In the same way, *B. subtillis* IAGS174 increased shoot length up to 29 and 38% respectively in plants of variety Rio Grande, during the year 2011 and 2012. Tomato plants of variety Red Power showed increase of 29 and 37% when co-cultivated with *B. subtillis* IAGS174, whereas for *B. fortis* IAGS162, same type of increase was 22 and 27% during the year 2011 and 2012 respectively. In case of variety 'Red Power' *B. subtillis* IAGS174 induced plants for 31% increased shoot length whereas for *B. fortis* IAGS162, this increase was 26% on average basis for both year experiments.

On the whole, *B. subtilis* IAGS174 performed best and increased shoot length significantly up to 31.6% for year 2011 in tomato plants across average basis of all three varieties (Table 3). During year 2012, same strain provided significant increase of 41.3% in shoot length (Table 4).

Interestingly, inoculated plants got flowers and fruits early as compared to untreated control plants. *B. fortis* IAGS162 and *B. subtilis* IAGS174 provided up to

1.43 and 1.89 fold increase respectively in fruit set as compared to control across average basis of all three

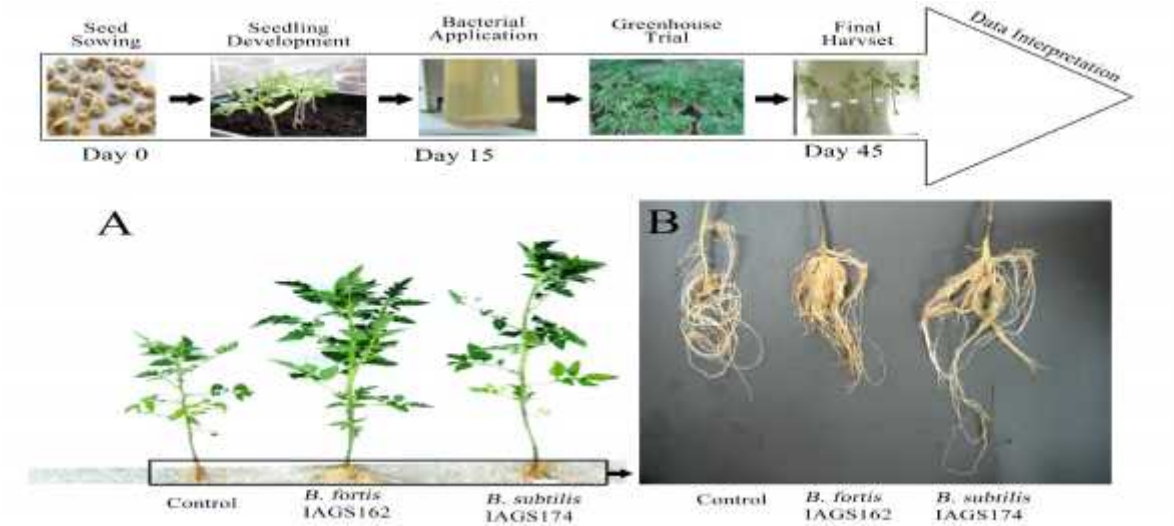

Figure 1. Effect of bacillus strains on growth of tomato plants under green house conditions. Values followed by different letters in each column are significantly different where $p \leq 0.05$.as governed by ANOVA and DNMRT. Vertical bars represent standard errors between replicates of single treatment.

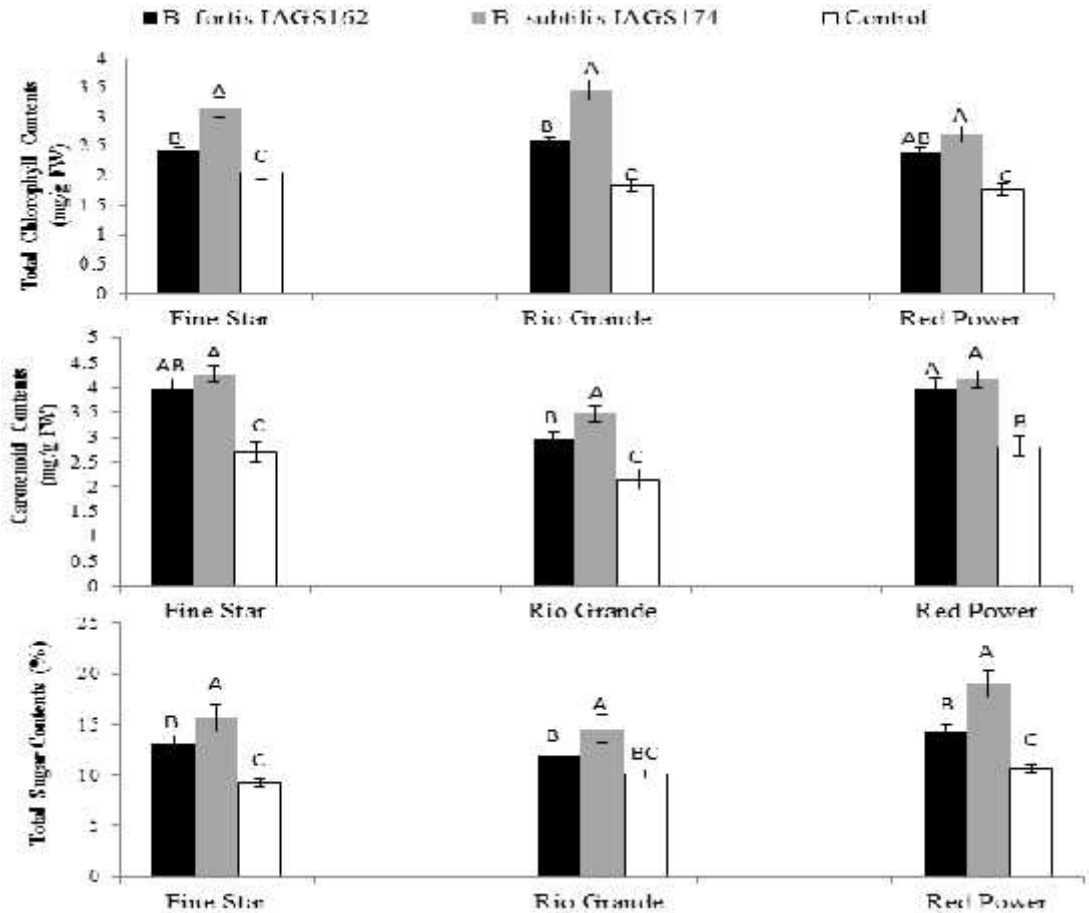

Figure 2. Effect of bacillus strains on plant's growth related biochemicals. Values followed by different letters in each column are significantly different where $p \leq 0.05$.as governed by ANOVA and DNMRT. Vertical bars represent standard errors between replicates of single treatment.

varieties and both seasons (Table 3). Increase in fruit yield per plant across both season and all three varieties was 1.49 and 1.73 folds for *B. fortis* IAGS162 and *B. subtilis* IAGS174 respectively (Table 3,4).

In case of individual varieties, tomato plants of variety 'Fine Star' cultivated under influence of *B. fortis* IAGS162, showed 21.3% increase in yield whereas in case of *B. subtilis* IAGS174, this increment reached up to 32.5% across both season experiments. In the same way, *B. subtilis* IAGS174 showed significantly higher increase in yield aspects in rest of the tomato varieties. Plants of "Rio Grande" and 'Red Power' showed significant increase in yield up to 41.6 and 33.7% respectively on average basis for both season experiments.

Table 1. Characterization of bacillus strains for production of growth promotion related substances.

Bacterial species	IAA Production (µg/mL)		Siderophores production	Phosphorus solubilization (µg mL–1)
	Without L-Tryptophan	With L-Tryptophan		
B. fortis IAGS 162	0.83^{B}	09.79^{B}	-	182^{B}
B. subtilis IAGS174	1.39^{A}	18.07^{A}	2.23^{A}	329^{A}

Values followed by different letters in each column are significantly different where $p \leq 0.05$.as governed by ANOVA and DNMRT at *p=0.05*.

Table 2. Effect of bacillus strains on growth parameters of three tomato varieties under greenhouse conditions.

Treatments	Fine Star				Rio Grande				Red Power			
	Shoot Length (cm)	Root Length (cm)	Total biomass (g)		Shoot Length (cm)	Root Length (cm)	Total Biomass (g)		Shoot Length (cm)	Root Length (cm)	Total Biomass (g)	
			Fresh	Dry			Fresh	Dry			Fresh	Dry
T1	26.51 B	23.55 AB	46.47 A	05.89 AB	29.18 B	22.75 B	38.28 B	05.96 AB	31.05 B	28.20 B	52.42 B	7.26 B
T2	32.44 A	29.10 A	51.57 A	06.92 A	37.33 A	26.12 A	44.74 A	07.09 A	43.88 A	32.81 A	63.68 A	9.96 A
UC	17.57 C	18.17C	31.17 B	04.15 C	20.04 C	15.73 C	23.55 C	04.07 BC	22.55 C	24.11 BC	38.61 C	5.60 C

Values followed by different letters in each column are significantly different where $p \leq 0.05$.as governed by ANOVA and DNMRT at *p=0.05*.

T1= B. fortis IAGS162, T2= B. subtilis IAGS174, UC= Untreated control.

Table 3. Effect of bacillus strains on growth and yield of tomato plants under field conditions in year 2011.

Treatments	Fine Star			Rio Grande			Red Power		
	Shoot Length (cm)	Number of Fruits per plant	Yield per plant	Shoot Length (cm)	Number of Fruits per plant	Yield per plant (kg)	Shoot Length (cm)	Number of Fruits per plant	Yield Per plant (kg)
B. fortis IAGS162	52.54^{B}	16.71^{B}	02.23^{AB}	42.50^{A}	11.53^{AB}	01.45^{A}	51.25^{A}	19.24^{B}	02.16 AB
B. subtilis IAGS174	67.20^{A}	21.99^{A}	02.71^{A}	45.67^{A}	14.68^{A}	01.62^{A}	58.41^{A}	23.58^{A}	02.72 A
Untreated Control	39.82^{C}	11.62^{BC}	01.97^{C}	33.13^{B}	08.20^{BC}	00.97^{B}	40.29^{B}	14.69^{BC}	01.57 BC

Values followed by different letters in each column are significantly different where $p \leq 0.05$.as governed by ANOVA and DNMRT at *p=0.05*.

Table 4. Effect of bacillus strains on growth and yield of tomato plants under field conditions in year 2012.

Treatments	Fine Star			Rio Grande			Red Power		
	Shoot Length (cm)	Number of Fruits per plant	Yield per plant (kg)	Shoot Length (cm)	Number of Fruits per plant	Yield per plant (kg)	Shoot Length (cm)	Number of Fruits per plant	Yield per plant (kg)
B. fortis IAGS162	65.51^{AB}	21.56^{B}	02.16^{B}	49.61^{B}	19.85^{AB}	01.67^{B}	63.22^{B}	27.23^{A}	02.82^{AB}
B. subtilis IAGS174	73.52^{A}	27.86^{A}	02.86^{A}	57.67^{A}	22.63^{A}	02.04^{A}	72.41^{A}	29.25^{A}	03.06^{A}
Untreated Control	48.59^{C}	14.92^{C}	01.44^{C}	35.32^{C}	12.26^{C}	01.17^{C}	51.24^{BC}	21.99^{B}	02.07^{C}

Values followed by different letters in each column are significantly different where $p \leq 0.05$.as governed by ANOVA and DNMRT at *p=0.05*.

DISCUSSION

Natural agriculture ecosystems depend upon beneficial microorganisms to sustain higher crop productivity (Rosas *et al.,* 2009). The beneficial influences impaired by these microbes have been reported in terms of biofertilization, stimulation of root growth, rhizoremediation, plant stress management and biocontrol (Lugtenberg and Kamilova, 2009). In current study, tomato plants were co-cultivated with two strains of bacillus that were previously reported for inducing systemic resistance in tomato against Fusarium wilt (Akram *et al.,* 2013). Since these strains were previously approved for ISR properties, it was anticipated that these strains can also promote growth of tomato. The production of plant growth related traits of these bacterial strains like IAA production, siderophores production and phosphate solubilization evaluation depicted that strain *B. subtilis* IAGS174 was t superior in observed traits in as compared to *B. fotris* IAGS162.

Many plants have been shown to perform batter in the presence of PGP bacteria capable of releasing phytohormones (Egamberdiyeva, 2005). These phytohormones accelerate plant growth by modulating plant growth and developmental processes. Exogenous IAA produced by bacteria controls an array of processes of plant growth and development. IAA stimulates primary and lateral root growth and increase root hair formation (Remans *et al.,* 2008). Growth promotion of tomato plants can be concomitant with more than one plant growth promoting traits of bacteria like siderophores production, hydrogen cyanide production (HCN), AAC (1-aminocyclopropane-1-carboxylic acid) deaminase activity and phosphorus solubilization ability (Sundra *et al.,* 2002). AAC deaminase assimilates allocation arrays in plants and positively effects plants root growth. In the same way PGP ability of our bacterial strains could be attributed towards IAA production, siderophores production and phosphorus solubilization.

In greenhouse experiments root inoculation of bacterial strains significantly promoted growth attributes of tomato plants of all three tomato varieties. Greenhouse studies were further supported by field evaluations. These strains provided significant increases in shoot length and yield of tomato plants. Strain *B. subtilis* IAGS174 was superior in these traits. In addition, it was observed that inoculation of bacterial strains increased chlorophyll, carotenoid and sugar contents which provide additional evidence supporting the finding of previous studies. These biochemicals are considered as markers of plant growth. Application of PGP microbes induces plants for higher production of these biochemicals in plants. These plant mediated mechanism are proposed by researchers as driving force behind plant growth promotion by PGP bacterial strains (Silva *et al.,* 2003). Likewise our results indicated that bacterial strains induced tomato plants for increased production of total chlorophyll, carotenoid and sugar contents.

The use of PGPB promotes plant growth and development through a variety of mechanisms. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake can be attributed in plant growth promotion. PGPB have been reported to improve plant growth either through direct stimulation by the synthesis of phytohormones (Xie *et al.,* 1996) or by decreasing the effect of pathogens (Weller *et al*., 2002). Some rhizobacteria belonging to Bacillus spp. have been found to produce lipopeptides, surfactins, bacillomycin D, and fengycins, which are secondary metabolites mainly with inhabitant pathogen activity and ultimately lead to plant growth promotion (Chen *et al*., 2006). Also some species of bacteria were recorded as highly aggressive colonizers of the rhizosphere of various crop plants and has a broad spectrum antagonistic activity against plant pathogens (Li *et al*., 2002; Weller *et al*., 2002).

Conclusion: Considering all results, it is very likely that the growth promotion of tomato plants co-cultivated with

both selected Bacillus strains was improved significantly. Further evaluations of these bacterial strains for desirable traits proved them fit for field applications.

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