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Effect of Plant Growth-Promoting Bacteria *Bacillus amyloliquefaciens* Y1 on Soil Properties, Pepper Seedling Growth, Rhizosphere Bacterial Flora and Soil Enzymes

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Abstract

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The *Bacillus amyloliquefaciens* Y1 strain was evaluated for its effects on soil properties, pepper seedling growth, rhizosphere bacterial flora and soil enzyme activities. Y1 solubilised insoluble phosphate, produced chitinase, and released siderophores in plate detection assay. In order to evaluate the plant growth promotion potential *in vivo*, strain Y1 was grown in media containing chitin powder and complex fertiliser. The pot experiment was conducted by treating pepper seedlings with C1/1 (Y1 culture, 50 ml), C2/3 (Y1 culture, 33 ml), C1/2 (Y1 culture, 25 ml), F1/1 (complex fertiliser, 50 ml), F1/2 (complex fertiliser, 25 ml), and W (water) at 10, 20, 30, 40, and 50 days after transplantation (DAT). Plants receiving Y1 had 52% (C1/2) and 68% (C1/1) more root and shoot biomass than W, and 14% (C1/1) and 18% (C2/3) more compared to F1/1 at 80 DAT. Total numbers of flowers per plant at 80 DAT were found significantly higher with the application of Y1 having 34 (C1/1), 35 (C2/3), and 22 (C1/2) compared to 4 (W), 12 (F1/1) and 10 (F1/2). In addition, chlorophyll content in pepper leaves was found to improve with the application of Y1. Furthermore, Y1 has significantly improved nutritional assimilation of total NPK, population of total culturable bacteria and chitinase producing bacteria and activities of chitinase and dehydrogenase in soil. At 60 and 80 DAT, the number of *B. amyloliquefaciens* at C1/1, C2/3, and C1/2 ranged from 2.3×10^4 to 4.6×10^4 CFU/g of soil. Our results concluded that *B. amyloliquefaciens* Y1 has positive effects on soil properties and can be suggested as a bio-fertiliser to minimise fertiliser application in modern agriculture.

Keywords: siderophore; chitin powder; chitinase; PGPR

The intensive agriculture entails the risk of excessive application of fertilisers to supply essential nutrients for plant growth and development. The rhizosphere plays an important role in transferring essential soil nutrients to plants. Physico-chemical properties and biological composition of rhizosphere are important for affecting the plant growth. Root exudates directly influence the nutrient availability or have indirect effects through interaction with rhizosphere microorganisms, to promote the circulation of plant nutrients, and reduce the need of chemical fertilisers as much as possible.

The rhizobacteria are a group of bacteria existing in the rhizosphere of plants. They are well known for their ability to colonise the plant root surface. Screening of plant growth promoting rhizobacteria (PGPR) involved biochemical estimations for phosphate solubilisation, production of hydrocyanic acid (HCN), siderophores, chitinase, ammonia, and indole-3-acetic acid (IAA) (Park *et al.* 2005; Ahmad *et al.* 2008).

PGPR may induce the plant growth by direct or indirect modes of action (Beauchamp *et al.* 1993;

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Kapulnik *et al.* 1996). Plant growth is directly stimulated by PGPR when colonised on root surfaces (Kloepper & Schroth 1981), by solubilising nutrients, fixing nitrogen, secreting growth regulators (de-Bashan & Bashan 2010), inducing systemic resistance (Dalisay & Kuć 1995), and competing for an ecological niche or substrate (Compant *et al.* 2005). Similarly, plant growth may be improved indirectly by bacteria secreting fungal cell wall degrading enzymes (Harman *et al.* 2004), producing antimicrobial compounds (Sopheareth *et al.* 2013), and affecting microbial diversity in the rhizosphere (Kloepper & Schroth 1981). Bacterial chitinase, siderophores, HCN, etc. produced in the rhizosphere can indirectly support the plant growth by suppressing hazardous effects of biotic stresses (Aeron *et al.* 2011). Based on these activities, Somers *et al.* (2004) classified PGPR as bio-fertilisers, phytostimulators, rhizoremediators, and biopesticides.

The genus *Bacillus* is widely studied as PGPR due to their best characterised root colonisation ability and sporulation ability. Inoculation of *Bacillus* sp. into soil as PGPR can solubilise fixed soil P and increase inorganic P availability to plants (Kumar & Narula 1999) resulting in higher crop yields (Yadav & Dadarwal 1997; Puente *et al.* 2004). Many strains of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus cereus* have been found to interact with plants and produce beneficial effects including disease suppression (Choudhary & Johri 2009). Plant-associated *B. amyloliquefaciens* has been shown to produce a variety of secondary metabolites (Chen *et al.* 2009) and enzymes such as chitinase involved in microbial antagonism (Niazi *et al.* 2014), thus supporting the plant growth indirectly by disease suppression. In addition, *B. amyloliquefaciens* FZB24 enhances development of plant resistance against competitive pathogenic fungi and bacteria (Kilian *et al.* 2000).

Regarding the plant growth promotion, much of PGPR research focuses on increasing nutrient availability to plants. Most of *in vivo* studies in this area of research have been conducted in sterile soil, and when done in non-sterile soil plant responses are less promising. Moreover, there have been considerable efforts to find PGPR to minimise the application of chemical fertilisers in non-sterile soil conditions. The objective of our study was to screen out PGPR traits of *B. amyloliquefaciens* Y1 using *in vitro* methods and *in vivo* pot study under non-sterile soil conditions. The pot experiment was carried out on pepper seedlings, as it is an important crop worldwide. Plant biomass was evaluated under different inoculum sizes to minimise the current trend of applying large amounts of fertilisers.

This study further included characterisation of soil by studying the effect of Y1 on soil composition, bacterial population, and enzymes like chitinase and dehydrogenase activities.

MATERIAL AND METHODS

Microorganisms. The *B. amyloliquefaciens* Y1 strain was isolated in our previous study from the field soil of Chonnam National University, South Korea (35.1764°N, 126.9081°E) and deposited with accession number KJ616752 in GenBank (Jamal *et al.* 2015). It was kept in a 25% glycerol solution at –70°C for further use.

Plant growth promoting traits of *B. amyloliquefaciens* Y1. For the qualitative estimation of hydrogen cyanide production, a picrate assay was used (Castric 1975). For phosphate solubilisation, Y1 isolate was streaked on Pikovskaya's agar medium (Pikovskaya 1948) and incubated at 28°C for 6 days. Plates were observed for clearing zones around the bacterial colony which indicate phosphate solubilising activity (Gaur 1990). For the qualitative estimation of chitinase, chitin agar plate amended with 2% phenol red was prepared and isolates were spot inoculated and incubated at 27 ± 2°C for 120 hours. Clear zones around the spot inoculants indicate the presence of chitinase (Wang *et al.* 2008). Qualitative production of siderophores was detected using CAS agar plate detection assay (Schwyn & Neilands 1987). For quantitative detection of siderophores, CAS shuttle assay was used (Payne 1994). The Y1 culture was grown in iron-free Fiss minimal medium at 30°C for 15 days. After every 24 h, a sample was withdrawn and centrifuged at 2700 *g* for 15 minutes. The CAS assay solution was added to the culture supernatant in equal proportion, mixed and allowed to stand for 20 minutes. Siderophores were detected by reduction in the intensity of blue colour at 630 nm. For the measurements, minimal medium was used as blank and % siderophore units were calculated by the following formula: $[(Ar - As)/Ar] \times 100 =$ % siderophore units; where: Ar – absorbance of reference (minimal media + CAS assay solution), As – absorbance of sample. All experiments were performed with three replications.

Effect of B. amyloliquefaciens Y1 on soil characteristics. The soil used in a pot experiment was

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a mixture of soil, sand, vermiculite, and compost at a ratio of 2 : 1 : 1 : 0.04 (v/v/v/v), respectively. The soil was analysed before transplantation and after harvesting plants. The rhizosphere soil was used after harvesting pepper seedlings at 80 days after transplantation. The soil was air-dried ground to pass through a 2-mm sieve and mixed thoroughly. Soil pH was checked on an electrode pH meter as 5 g of soil sample suspended in 50 ml of distilled water (1 : 10 w/v). Electrical conductivity of aqueous extract was measured (1 : 5). Total organic matter, N, P, K, Ca, Mg, and Mn were calculated before transplantation and after plant harvesting from Mehlich-1 extract using inductively coupled argon plasma spectrophotometry. Soil characterisations were performed three times with three replications per treatment.

Treatment preparations. For a pot experiment, two types of qualitative and five types of quantitative treatments were performed. In one qualitative type, Y1 strain was grown for seven days at 30°C in media containing 0.95 g crab shell powder, 0.5 g K_2SO_4, 0.5 g CaMg, 0.1 g yeast, 0.05 g micronutrients, 0.60 g sugar, and 0.6 g complex fertiliser NPK (17 : 17 : 21) per 100 ml for 7 days. In Y1 treatment, culture broth containing 2×10^6 per ml of Y1 was added to pot soil with three various volumes as C1/1 (Y1 culture, 50 ml), C2/3 (Y1 culture, 33 ml), and C1/2 (Y1 culture, 25 ml). The other type of qualitative treatment is complex fertiliser treatment (F) that contained 0.5 g K_2SO_4, 0.5 g CaMg, 0.05 g micronutrients, and 0.6 g NPK complex fertiliser (17 : 17 : 21) per 100 ml. Five types of quantitative treatments were based on concentration variations of Y1 and F. On the basis of volumes two types of fertiliser treatments were performed as F1/1 (complex fertiliser, 50 ml), F1/2 (complex fertiliser, 25 ml). Water (W, 50 ml) was performed as control. Total of five treatments was added at 10, 20, 30, 40, and 50 DAT.

Plant growth promotion assay. In order to investigate the effect of Y1 on pepper plant growth, the pot experiment was performed in a greenhouse, Chonnam National University, South Korea. The pepper (*Capsicum annuum* L. Chungok) seeds were sown in a 3 × 3 cm plastic cell plug tray filled with commercial grade bedding soil. Four-week pepper seedlings were transferred to 96 pots in total containing 600–700 g of soil mixture as described above. The experiments were conducted using a completely randomised design for six treatments with three replications per treatment. Pepper seedlings were treated with fertilisers as defined above at 10, 20, 30, 40, and 50 DAT. Pots were randomly distributed, with the position changed weekly to avoid any positional effects. After 40, 50, 60, and 80 days, the plants and soil were harvested for observations. Total chlorophyll content was measured using a SPAD 502 chlorophyll meter and reported as the mean of 30 leaves from three replications for each treatment. The plant samples were divided into shoots and roots, and then weighed. Root and shoot samples were oven-dried at 60°C for 48 h to calculate dry weight. Total numbers of flowers were counted and reported as the mean of three replications per treatment at 80 DAT. Soil samples were stored at 4°C for bacterial and enzyme activities like chitinase and dehydrogenase. Observations were made with the mean value of three replications per treatment. The experiment was performed twice.

Bacterial population. Total number of bacteria, thermophilic and chitinase producing bacteria were calculated in soil at 60 and 80 DAT. In order to find out the total number of bacteria, 10 g of soil was poured into the flask containing 100 ml of autoclaved distilled water, serially diluted and poured on TSA agar plate. For isolation of *B. amyloliquefaciens*, the flask containing soil was incubated at 60°C for 1 h, serially diluted and poured on TSA agar plate. For isolation of chitinase producing bacteria, chitin agar medium containing 0.5% swollen chitin (prepared from chitin 0.2% Na_2HPO_4, 0.1% K_2HPO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$, 0.01 yeast extract, and 1.8% agar; pH 7) was used. The plates were incubated at 30°C for 5–10 days and colonies with clear zones were counted. The population of bacteria is expressed as colony forming unit per gram dry weight of soil (CFU/g). Dry weight determinations were calculated after drying soil samples at 105°C for 24 hours. Each experiment was conducted with three replications and was repeated three times.

Enzyme activity assay in soil. To check the effect of Y1 on soil enzymes, chitinase and dehydrogenase activities in the rhizosphere soil samples were determined using a modified method of Trevors (1984) and Yedidia *et al.* (2000), respectively. Each experiment was conducted with three replications, and is expressed per gram dry weight of soil. Dry weight determinations were calculated after drying soil samples for 24 h at 105°C.

Statistical analysis. The data was subjected to analysis of variance using SAS v9.1 software (SAS Institute 2003). Mean values among treatments were compared by the least significant difference (*LSD*) test at 5% level ($P = 0.05$) of significance and presented as the mean values ± standard deviation (SD).

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RESULTS

Plant growth promoting traits of B. amyloliquefaciens Y1. In qualitative screening of HCN, *Pseudomonas florescens* was used as positive control and produced HCN. However, Y1 did not produce HCN. The Y1 strain showed a clear zone for phosphate solubilisation on Pikovskaya's agar plate amended with bromophenol blue and a clear zone for chitinase on chitin agar plate as well (Supplementary Figure S1A–C in EMS). The Y1 was found to produce siderophores when tested for qualitative (Supplementary Figure S1D) and quantitative estimation in iron-free Fiss minimal media. The Y1 produced a maximum of 50 units of siderophores during the period of 2-day incubation, and thereafter it decreased gradually until 15 days with the lowest value of 30 units in iron-free minimal media (Figure 1).

Effect of B. amyloliquefaciens Y1 on soil characteristics. The physical and chemical properties of soil before transplantation and after harvesting plants at 80 DAT were found to be different. Results in Table 1 indicate that the inoculation of Y1 improved soil properties. The pH, EC (electrical conductivity), and CEC (cation exchange capacity) were all affected by inoculation of Y1. The lowest values of pH and CEC (Cmol(+)/kg) were 5.5 (C1/1) and 8.36 (C2/3) among all treatments. Soil EC was found to relate positively with the bacterial inoculum size, as higher EC of 2.27 (dS/m) was recorded with C1/1 followed by C2/3, C1/2, F1/1, F1/2, and W. Total nitrogen and available phosphorus were found significantly higher in the soil receiving Y1 culture than in F1/1, F1/2, and W at 80 DAT. Based on this information, Y1 was found to affect the soil positively and it promoted the pepper plant growth.

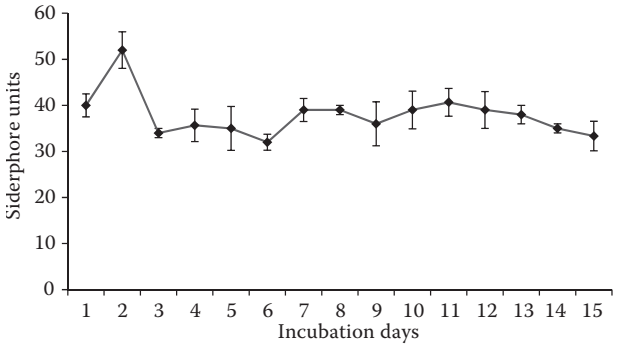

Figure 1. Change in siderophore production by *B. amyloliquefaciens* Y1 in iron-free Fiss minimal media during incubation for 15 days

Plant growth promotion assay. Shoot biomass of pepper plants was increased with the length of incubation time in all Y1 treatments. The plants treated with Y1 show higher root and shoot biomass by 52% (C1/2) and 68% (C1/1) compared with W, and 14% (C1/1) and 18% (C2/3) compared with F1/1 at 80 DAT (Table 2). Physical and chemical properties of soil changed with the application of bacterial culture and were found to have positive effects on plant growth as shoot biomass was significantly improved at 50, 60, and 80 DAT. Table 3 indicates that the Y1 inoculation promoted pepper plant growth as the total number of flowers per plant was higher than in fertiliser treatment and control at 80 DAT. Table 3 indicates that the Y1 inoculation promoted the pepper plant growth as the total number of flowers per plant was higher than in fertiliser treatment and control at 80 DAT. The treatments affected the plant growth above soil and underground.

Bacterial population. The total number of bacteria was found significantly higher in culture treat-

Table 1. Effect of *B. amyloliquefaciens* Y1 on soil characteristics

Sample	pH (1:5)	EC (dS/m)	MC (%)	CEC (Cmol(+)/kg)	OM (%)	TN	P	K	Ca	Mg
						(mg/kg)		(Cmol+/kg)		
BT	5.9^{b}	0.5^{c}	7.3^{d}	8.8^{c}	1.0^{c}	36.3^{bc}	42.4^{d}	0.09^{b}	6.3^{d}	1.97^{ab}
W	6.2^{a}	0.2^{d}	29^{a}	9.2^{a}	1.4^{ba}	16.5^{c}	272.2^{b}	0.05^{b}	13.54^{a}	2.9^{ab}
F1/2	6.0^{ba}	0.6^{c}	29^{a}	9.2^{a}	1.3^{ba}	32.9^{bc}	346.39^{ba}	0.04^{b}	11.70^{b}	2.4^{ab}
F1/1	5.9^{b}	1.0^{cb}	21^{b}	9.0^{b}	1.1^{bc}	35.33^{bc}	364.4^{ba}	0.02^{b}	10.06^{c}	2.6^{ab}
C1/2	5.6^{c}	1.3^{b}	18.5^{cb}	8.6^{d}	1.1^{c}	42.4^{ba}	402.3^{ba}	0.04^{b}	10.26^{c}	2.5^{ab}
C2/3	5.5^{c}	1.8a	19^{cb}	8.4^{e}	1.0^{c}	44.1^{ba}	526.2^{a}	0.04^{b}	10.69^{c}	2.1^{ab}
C1/1	5.5^{c}	2.3a	16^{c}	8.8^{c}	1.4^{ba}	44.2^{a}	473.8^{a}	0.06^{a}	9.50^{d}	3.3^{a}

EC – electrical conductivity; MC – moisture content; CEC – cation exchange capacity; OM – organic matter; TN – total nitrogen; BT– before transplantation; W – water; F1/2 – fertiliser (25 ml); F1/1 – fertiliser (50 ml); C1/2 – culture (25 ml); C2/3 – culture (33 ml); C1/1 – culture (50 ml); data represent means with different letters at the same sampling time being significantly different at $P = 0.05$ when compared by *LSD*

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Table 2. Effect of fertiliser and *B. amyloliquefaciens* Y1 inoculation on root weight, shoot weight, leaf chlorophyll, and shoot length of pepper seedlings in non-sterile soil

	Treatment	FSW (g)	DSW (g)	FRW (g)	DRW (g)	CC (μg/cm^2)	Plant height (cm)
40 DAT	W	9.3 ± 1.15^{ab}	0.9 ± 0.08^{ab}	3.5 ± 0.05^{a}	0.3 ± 0.06^{a}	37.0 ± 1.2^{ab}	31.3 ± 3.21^{ab}
	F1/2	19.3 ± 1.15^{a}	1.7 ± 0.35^{b}	1.8 ± 0.2^{ab}	0.1 ± 0.05^{b}	40.63 ± 0.5^{a}	40 ± 2^{a}
	F1/1	20.6 ± 1.15^{a}	3.2 ± 0.14^{a}	1.4 ± 0.41^{ab}	0.1 ± 0.01^{b}	40.4 ± 1.76^{a}	43.6 ± 0.58^{a}
	C1/2	19.3 ± 1.15^{a}	1.8 ± 0.23^{b}	1.2 ± 0.31^{b}	0.1 ± 0.03^{b}	41.8 ± 4.37^{a}	43 ± 2.65^{a}
	C2/3	18 ± 2^{a}	1.5 ± 0.3^{b}	1.5 ± 0.16^{ab}	0.1 ± 0.03^{b}	41.2 ± 2.72^{a}	42 ± 2.65^{a}
	C1/1	17.3 ± 2.31^{a}	1.6 ± 0.27^{b}	1.0 ± 0.43^{b}	0.11 ± 0.04^{b}	40.9 ± 1.5^{b}	39 ± 2.0^{a}
50 DAT	W	15.3 ± 3.06^{ab}	1.8 ± 0.03^{ab}	2.9 ± 0.94^{a}	0.3 ± 0.11^{a}	37.7 ± 3.13^{ab}	44.6 ± 1.15^{ab}
	F1/2	24 ± 1.63^{b}	2.4 ± 0.25^{a}	2.2 ± 0.32^{b}	0.2 ± 0.03^{ab}	43.4 ± 0.53^{a}	49.3 ± 3.06^{a}
	F1/1	27.5 ± 1.91^{a}	2.5 ± 0.23^{a}	1.8 ± 0.32^{ab}	0.1 ± 0.03^{ab}	42.2 ± 2.11^{a}	50.3 ± 2.08^{ab}
	C1/2	28 ± 1.63^{a}	2.8 ± 0.13^{a}	2.3 ± 0.27^{b}	0.2 ± 0.04^{ab}	41.9 ± 1.23^{a}	52.6 ± 1.15^{ab}
	C2/3	28 ± 1.63^{a}	2.7 ± 0.27^{a}	2.1 ± 0.37^{b}	0.2 ± 0.02^{ab}	40.2 ± 0.94^{a}	56.7 ± 2.62^{a}
	C1/1	26 ± 2^{a}	2.4 ± 0.8^{a}	1.5 ± 0.2^{ab}	0.2 ± 0.03^{b}	41.7 ± 0.83^{a}	53.3 ± 4.93^{a}
60 DAT	W	15 ± 2^{ab}	1.2 ± 0.64^{ab}	2.3 ± 0.2^{ab}	0.4 ± 0.0^{a}	36.3 ± 1.96^{b}	35 ± 5^{ab}
	F1/2	32.7 ± 4.6^{b}	4.01 ± 0.36^{a}	2.8 ± 0.39^{b}	0.4 ± 0.09^{a}	45.8 ± 1.76^{a}	59.3 ± 3.06^{a}
	F1/1	30.6 ± 6.1^{b}	3.3 ± 0.26b	2 ± 0.57^{ab}	0.1 ± 0.04^{ab}	46.2 ± 1.27^{a}	55.3 ± 1.15^{b}
	C1/2	36 ± 0.86^{a}	4.2 ± 0.06a	3.7 ± 0.1^{a}	0.5 ± 0.03^{a}	47.7 ± 0.28^{a}	58.6 ± 1.1^{a}
	C2/3	38.6 ± 0.94^{a}	4.3 ± 0.26a	1.6 ± 0.06^{c}	0.2 ± 0.04^{ab}	47 ± 0.53^{a}	60.7 ± 0.47^{a}
	C1/1	36 ± 1.63^{a}	4 ± 0.63^{a}	2.0 ± 0.34^{ab}	0.3 ± 0.07^{ab}	46 ± 1.04^{a}	61.3 ± 3.7^{a}
80 DAT	W	18 ± 1.41^{ab}	1.9 ± 0.02^{ab}	4.7 ± 0.52^{a}	0.3 ± 0.15^{ab}	37.8 ± 3.32^{ab}	56 ± 6^{ab}
	F1/2	38.3 ± 7.23^{b}	5.6 ± 0.3.29^{b}	7.9 ± 3.29^{b}	0.9 ± 0.18^{a}	43.6 ± 0.51^{b}	68.3 ± 9.86^{b}
	F1/1	46 ± 4^{ba}	6.7 ± 3.22^{b}	6.7 ± 3.22^{b}	0.9 ± 0.38^{a}	46.2 ± 0.9^{a}	69.3 ± 5.68^{b}
	C1/2	56.3 ± 3.21^{a}	8.5 ± 1.85^{a}	9.9 ± 1.85^{a}	1.1 ± 0.32^{a}	48.3 ± 2.36^{a}	70.6 ± 3.51^{b}
	C2/3	59 ± 0.81^{a}	8.4 ± 1.59^{a}	8.9 ± 0.59^{a}	0.9 ± 0.13^{a}	49.6 ± 1.51^{a}	73 ± 2.94^{a}
	C1/1	59.3 ± 3.77^{a}	8.3 ± 1.02^{a}	9 ± 1.02^{a}	0.9 ± 0.0.17^{b}	49.6 ± 1.08^{a}	69.6 ± 1.52^{a}

FSW – fresh shoot weight; DSW – dry shoot weight; FRW – fresh root weight; DRW – dry root weight; CC – chlorophyll content; data represent means ± standard deviation from four replications; means with the same letters at the same sampling time in the same column are not significantly different at $P = 0.05$ when compared by *LSD*

ments compared to F1/2, F1/1, and W treatments, when the soil was sampled at 60 and 80 DAT (Figure 2A). The Y1 strain grown in crab shell powder showed to improve the total number of chitinase producing bacteria in soil compared to fertiliser and control (Figure 2B). Figures 2C and 2D showed a significant recovery of thermophilic and *B. amyloliquefaciens* population at 60 and 80 DAT from the soil receiving bacterial culture. Fertiliser addition to the pepper pot soil also had a positive impact on total number of bacteria, thermophilic bacteria, and chitinase producing bacteria when compared to the control.

Enzyme activities in soil. Enzyme activities in soil revealed a highly significant interaction with bacterial inoculation, as chitinase and dehydrogenase activities were found to increase with Y1 application (Figures 3A and 3B). The bacterial inoculation caused a significant increase in the bacterial population in soil which indirectly affected dehydrogenase and chitinase

Table 3. Number of flowers per plant after harvesting at 80 days after transplantation

Treatment	Number of flowers per plant
NT	4 ± 1^{c}
F1/2	10 ± 2.6^{c}
F1/1	12 ± 3.7^{c}
C1/2	22 ± 6.6^{b}
C2/3	35 ± 6.8^{a}
C1/1	34 ± 9.2^{a}

Data represent means ± standard deviation from four replications; means with the same letters at the same sampling time in the same column are not significantly different at $P = 0.05$ when compared by *LSD*

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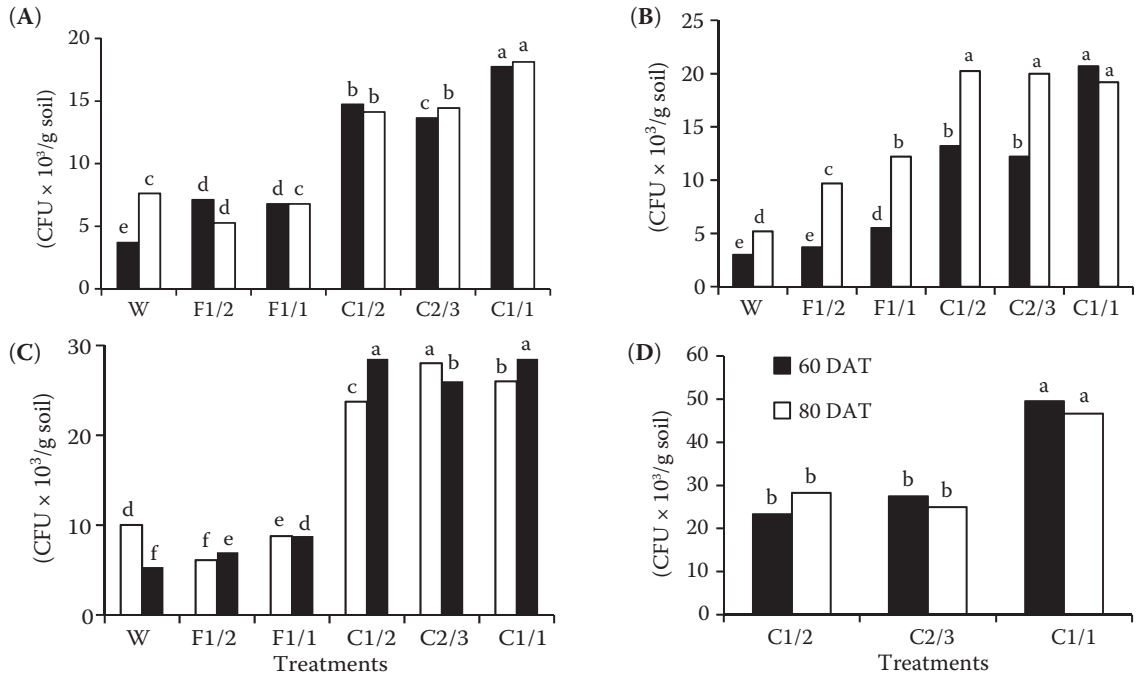

Figure 2. An overview of changes in total number of bacteria (**A**), chitinase producing bacteria (**B**), thermophilic bacteria (**C**), and *B. amyloliquefaciens* (**D**) in the rhizosphere of pepper seedlings at 60 and 80 DAT after the application of W, F1/2, F1/1, C1/2, C2/3, and C1/1 for five times

The population of bacteria is expressed as per gram dry weight of soil; dry weight determinations were calculated after drying soil samples at 105°C for 24 h; means with the same letters at the same observation time are not significantly different at 0.05 level of *LSD* test; DAT – days after transplantation

concentrations in soil. In addition, all sizes of bacterial inoculums caused a significant increase with respect to the non-inoculated treatments. The activity of chitinase was found higher at 40, 50, 60, and 80 DAT in the soil receiving Y1 culture grown in crab shell powder. The number of bacteria increased with fertiliser application which resulted in higher dehydrogenase and chitinase activity in soil compared to the control (W).

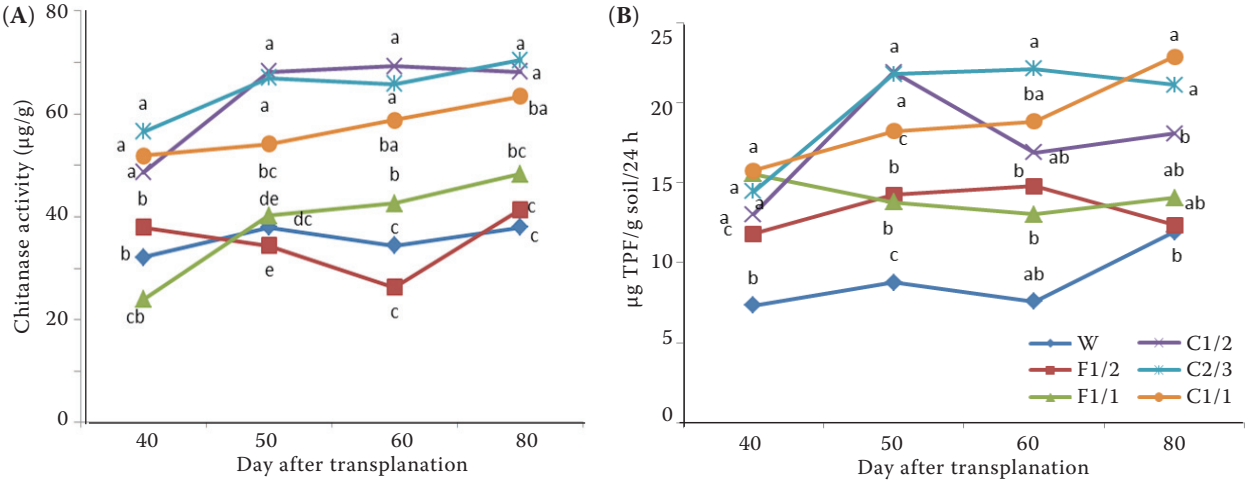

Figure 3. Changes in chitinase (**A**) and dehydrogenase (**B**) activity in the rhizosphere of pepper seedlings at 40, 50, 60, and 80 DAT after the application of W, F1/2, F1/1, C1/2, C2/3, and C1/1 for five times

Enzyme activities are expressed as per gram dry weight of soil; dry weight determinations were calculated after drying soil samples at 105°C for 24 h; means with the same letters at the same observation time are not significantly different at 0.05 level of *LSD* test

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DISCUSSION

In this study, *Bacillus amyloliquefaciens* Y1, isolated in our previous work (Jamal *et al.* 2015), was tested for plant growth promotion *in vitro* and *in vivo*. Y1 strain was found to solubilise insoluble phosphate, produce chitinase and release siderophores *in vitro*, making it to characterise as PGPR in an *in-vivo* pot experiment. Many strains of *Bacillus amyloliquefaciens*, *Bacillus cereus*, and *Bacillus subtilis* have been found to interact with plants and produce beneficial effects by induction of resistance in plants, protection against plant pathogens, plant growth promotion traits like siderophore production etc. (Choudhary & Johri 2009). Our pot study suggested that Y1 can be a PGPR candidate as pepper plant fresh weight and dry weight were found to increase with the application of Y1 (C1/2, C2/3, and C1/1) compared to the fertiliser (F1/1 and F1/2) treatment and control (W) at 60 and 80 DAT. In this experiment, one of the growth promotion mechanisms may be phosphate solubilisation in the soil by Y1. El-Azeem *et al*. (2007) reported that solubilisation of tricalcium phosphate (TCP) by bacterial isolates produced organic acids and lowered the pH value in solid and liquid media. Similarly, in our study, the pepper plant soil with bacterial inoculation was found to have low pH compared to F1/1, F1/2, and W at 80 DAT. Kloepper and Schroth (1989) reported that *Bacillus* inoculants increased wheat yield up to 43% and growth may be stimulated as a result of improvement in N and P nutrition by phosphate-solubilising and N_2-fixing bacteria.

Many studies regarding physical and chemical changes in soil properties by rhizobacterial inoculation have been reported earlier (Jonasson *et al.* 1996; Gong *et al.* 2009). N and P in soil are essential nutrients for plant growth and development. Our data showed that Y1 inoculation significantly affected total N and P in soil. The inoculated soil (C1/2, C2/3, and C1/1) with the higher concentration of total organic matter, nitrogen and phosphorus showed more plant biomass compared to F1/2, F/1, and W. Both seeds and soil, when inoculated with *Bacillus* spp. (PSB), showed the high crop yield due to solubilisation of fixed soil P and applied phosphates (Puente *et al*. 2004). Kim *et al*. (2007) reported in their soil plant experiment that increased soil microbial activity and plant growth may be due to the high total nitrogen content, organic matter, and available phosphorus concentrations.

The buildup of *B. amyloliquefaciens* in soil influences the growth of plants. From infested soil, a significant population of *B. amyloliquefaciens* was recovered which modulated plant growth promotion through direct and indirect PGPR traits. Moreover, the long-term survival of *B. amyloliquefaciens* in soil makes it very important inocula to ensure a consistent synergistic relationship as reported by Choudhary and Johri (2009). This may result from successful colonisation to compete in unsterilised soil with the autochthonous population. Our results show that mineral fertilisation and bacterial inoculation appeared to increase microbial biomass (total culturable bacteria, PSB and fungi) and changed consistently the community structure according to the treatments applied. This is similar to the Canbolalt *et al*. (2006) findings. An increased CFU of the bacterial population in the rhizosphere soil of tomato plants was caused by inoculation of *P. ehimensis* KWN38 in soil (Naing *et al.* 2015). The population of chitinase producing bacteria was increased by Y1 inoculation grown in media containing chitin, which is in accordance with a previous report of Manjula and Podile (2001). The application of *P. ehimensis* RS820 grown in crab shell powder has been reported to improve the number of gelatinolytic and chitinolytic bacteria. This minimised the disease caused by *M. incognita* along with plant growth promotion (Hong *et al.* 2013).

The type and size of bacterial population can affect soil enzyme activities. Dehydrogenase is known to be one of the important enzymes to predict the overall microbial population in soil (Gu *et al.* 2009; Lee *et al.* 2009). In our study, a high number of bacteria with C1/2, C2/3, and C1/1 was found to have a positive effect on dehydrogenase activity in soil, as high dehydrogenase activity was observed compared to fertiliser and control treatments. The chitinase activity was found to significantly increase by Y1 culture application. Chitinase in soil indicates that the soil has more organic matter which can degrade and provide more food to plants and beneficial microorganisms. Kim *et al*. (1997) reported that phosphatase activity in soil increased with the inoculation of phosphate solubilising bacteria. Higher chitinase activity in inoculated soil may be due to crab shell powder added in Y1 culture, providing more chitin as substrate for chitinase production in soil. Nagrajkumar *et al*. (2004) and Zhou *et al*. (2012) reported in their studies that PGPR indirectly promote the plant growth by producing the fungal cell wall degrading enzyme to protect plants against pathogens. Jung *et al*. (2005) found that cellulase activity was increased due to inoculation of *P. illinoisensis* to the root zone of pepper.

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This is similar to our work, which showed that Y1 addition increased the chitinase and dehydrogenase enzyme activity in soil.

In summary, the results show that the *Bacillus amyloliquefaciens* Y1 has a potential to increase the growth of pepper seedlings in non-sterile soil under controlled conditions. The inoculation study showed that Y1 can be formulated as PGPR at low and high concentrations. The chemical and biochemical properties of soil before transplantation and after harvesting revealed that the long-term survival of Y1 promoted the pepper plant growth directly or indirectly by improving NPK, total culturable bacteria and enzyme activities in soil. Moreover, our study suggests that the next step is to determine that Y1 is a potential bio-fertiliser under real world field conditions with benefits to agricultural production. The risk of environmental pollution and high-cost modern farming can be minimised and more sustainable and environmentally friendly agricultural production can be achieved by using Y1 in field conditions. *Bacillus amyloliquefaciens* Y1 has a potential to be used as PGPR under controlled conditions.

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