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Growth Promoting Potential of Native *Bacillus megaterium* Strain EXB-53 in Some Vegetable Crops in Commercial Nursery

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In the present study, we have investigated the ability of bacterial antagonist, *Bacillus megaterium* strain EXB-53 to promote plant growth in different vegetable crops (chilli, capsicum, egg plant, tomato, cabbage and cauliflower) in commercial nursery. Our results suggested that seed treatment with talc based formulation of *B. megaterium* strain EXB-53 significantly increased vigour index up to 245% in different vegetable crops over untreated control plants. In addition, fresh plant weight increased up to 121% and dry weight up to 70%. Furthermore, the strain showed extensive root colonization ability and positive to multiple plant growth promoting traits such as indole-3-acetic acid (IAA) production, phosphate solubilization and production of volatile compounds. The fermentation process of EXB-53 was successfully scaled-up to 10L using a molasses urea medium and higher biomass ($14.41 Log_{10}$cfu ml^{-1}) was obtained at the end of 72hr after inoculation. Hence, in the current investigation, the possibility of exploiting *B. megaterium* EXB-53 for development of commercial plant growth promoting product of vegetable crops is discussed.

Key words: *Bacillus megaterium*, Growth promotion, Vegetable crops, Pilot scale production.

India is the second largest producer of vegetable crops and contributes about 13% of total world's production. Over the years, chemical fungicides have been used extensively for seed treatment of vegetable crops and application of chemical fertilizers has been practiced in cultivation of vegetable crops. In recent years, the extensive use of chemical fungicides and fertilizers is becoming unattractive in developed and developing countries because of increased consumer demand for residue free food commodities, environmental/health hazards associated with chemicals, resistance development in pathogens etc. Hence, sustainable and eco-friendly strategies are gaining wide spread acceptance in modern agricultural practices (Reddy, 2011). Use of plant growth promoting bacteria (PGPB) especially *Bacillus* species have gained widespread acceptance as biocontrol agents as well as plant growth promoters in sustainable agriculture. Among different species of *Bacillus*, *B. subtilis* have been studied well with respect to its biocontrol and PGP properties. Most of the commercial formulations like Companion, Kodiak, Serenade etc. used for biocontrol and growth promotion contain different strains of *Bacillus subtilis* as active ingredient (Coway *et al*., 2011; Kumar *et al*., 2011). However, the potential of other *Bacillus* species including *B. megaterium* have not been explored much for developing commercial growth promotion product(s). *B. megaterium* is a gram-positive, spore forming aerobic bacterium found associated with many types of plants and also present in diverse natural habitats.This bacterium is generally referred as "big beast"

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because of its large size approximately 100 times that of *Escherichia coli* and has been used from decades for production of several industrially important enzymes (De Bary, 1884; Vary *et al*., 2007). Few earlier attempts suggests that strains of *B. megaterium* have ability to promote plant growth through indole-3-acetic acid (IAA) production, phosphate solubilization, cytokinine signalling and production of volatile compounds as major mechanisms (Trivedi and Pandey, 2008; Ortiz-Castro *et al*., 2008; Zou, 2010; Hu *et al*., 2013). However, the mechanisms by which bacteria promote plant growth may differ from species to species and also from strain to strain (Chakraborty *et al*., 2012).

In our earlier study, two hundred and fifty eight phylloplane/pomoplane/endophytic bacterial isolates obtained from different vegetable/fruit crops were evaluated for their biocontrol efficacy against chilli anthracnose pathogen *Colletotrichum capsici* in post-harvest fruit bioassay under laboratory condition. Among the isolates tested, *B. megaterium* EXB-53 showed 54.33% reduction in lesion length of *C. capsici* (Ramanujam *et al*., 2012a). In the present investigation, EXB-53 was evaluated for its ability (i) to promote plant growth in some vegetable crops in commercial nursery (ii) to determine the root colonization ability (iii) to assess important growth promoting traits (IAA production, phosphate solubilization and production of volatile compounds) and (iv) to scale-up the biomass production at pilot scale levels.

MATERIALS AND METHODS

Microbial antagonist and seed material

Bacillus megaterium EXB-53 (Gen. Acc. JN167995) used in the study was originally obtained from the surface washings of green and ripe chilli fruits and identification was carried out based on 16S rDNA amplification (Ramanujam *et al*., 2012a). The pure culture was maintained in 0.5% glycerol stock stored at –20ºC and sub cultured on nutrient agar (NA) slants for further use.

Seeds of popular varieties of vegetable crops without any fungicide treatment were obtained from Regional Horticulture Research Station, University of Horticultural Sciences (UHS), Devihosur, Karnataka, India and Syngenta (India) Private limited, India. The details of each crop with respective variety and source are given in table 1.

Initial evaluation with bacterial cell suspension under glass house condition

The initial evaluation of *B. megaterium* EXB-53 for PGP activity was carried out under glass house conditions at National Bureau of Agriculturally Important Insects (NBAII), Bangalore, Karnataka, India. The experiment was performed according to Siddiqui and Meon (2009) with some modifications. A loop full of pure culture was inoculated to 100ml molasses urea broth (MUB) containing cane molasses ($20g^{-L}$) and urea ($2g^{-L}$) and incubated on an orbital shaker for 48hr at 28ºC at 150 rotations per minute (RPM). Cell pellet was obtained by centrifuging the culture broth at 12,000 g for 20min and resuspended in 10ml of 0.5% carboxymethyl cellulose (CMC) in sterile double distilled water and the resulting suspension was used for chilli seed treatment. The population of bacterial cells in CMC solution was estimated by serial dilution and plating on NA.

Chilli seeds of Byadgi dabbi variety were surface-disinfected for 3 min in 1% (v/v) sodium hypochlorite followed by 70% (v/v) ethanol for 3 min and then rinsed thrice in sterile distilled water. Seeds were then treated with bacterial cell suspension (obtained as above) at the rate of 10ml kg^{-1} seeds for 10 min with constant shaking to allow the attachment of cells to the seed coat. Seeds treated with CMC solution alone served as control. Treated seeds were then surface dried in air flow chamber for 2 hr. The treated seeds were then sown in plastic trays (45 cm× 25 cm × 5 cm) filled with double sterilized coco-peat (Commercial grade - available as Bio-peat -SG compost) at the depth of 1 cm and no supplementary fertilizer was added during this study. The experiment was carried out under glass house conditions with daily temperature ranging from 21-32°C and 43-78% relative humidity (RH) during the study period. Each treatment had three replications with 100 seeds per replication. Observations on germination percentage, shoot length, root length and fresh/ dry weights were recorded after four weeks of sowing. Vigor index was calculated using the following formula

Vigor index = percent germination X seedling length (shoot length + root length) (Baki and Anderson, 1973).

Evaluation for growth promotion in commercial nursery

Preparation of talc formulation

Biomass of *B. megaterium* EXB-53 was obtained in MUB as described in section 2.2. For preparation of talc formulation, the broth culture was mixed with sterilized talc powder (1:2 ratio v/w) in laminar air flow chamber and dried until the moisture comes down to approx.12%. After drying, the formulation was stored in sterile polythene bags at room temperature until further use. The bacterial cell counts in talc formulation was estimated by serial dilution and plating on NA (Vidhyasekaran and Muthuamilan, 1995).

Nursery experiment

Plant growth promoting ability of *B. megaterium* EXB-53 in different vegetable crops under nursery conditions was assessed at local commercial nursery (Ekalavya nursery, Bangalore) that supplies planting material of different agricultural and horticultural crops to farmers. Talc formulation of *B. megaterium* EXB-53 was used for seed treatment at the rate of $10gkg^{-1}$ of seeds. Slurry was prepared by mixing formulation and water (2:1 ratio w/v) in a plastic container. Seeds were mixed with slurry thoroughly and air dried for two hours. The treated seeds were then sown in plastic trays filled coco-peat at the depth of 1 cm without any supplementary fertilizer. Seeds without any treatment served as control. Day temperatures ranging from 23-31°C and 45-80% relative humidity (RH) were observed during the study period under nursery conditions. Each treatment includes three replications with 100 seeds per replication. Observations on growth promoting parameters were recorded after four weeks of sowing and vigor index was calculated as described in section 2.2.

Root colonization assay

Root colonization ability of *B. megaterium* EXB-53 was assessed in treated and untreated plants from nursery experiment. Five plants from each treatment were randomly selected and brought to laboratory. The shoot part was exercised using a sterile scalpel and coco peat adhered to the roots was removed by gentle tapping. Ten root cuttings (~1cm) were cut using sterile scalpel in laminar chamber from each plant, a total of 50 root bits from five plants were placed on NA plates and incubated at 28°C for 48h. Observations were recorded for number of root bits showing bacterial growth around them and percent root colonization was calculated.

Assessment for plant growth promoting traits

Indole-3-acetic acid (IAA) production

IAA production by *B. megaterium* EXB-53 was evaluated in NB medium amended with or without 0.1 % (w/v) L-tryptophan (Sigma-Aldrich). Bacterial suspension (100µl - 2×10^5 cells ml^{-1}) was inoculated to 25ml NB in triplicates and incubated at 28°C in the dark for 7 days on an orbital shaker at 150 rpm. After incubation, the culture broth was centrifuged at 12,000 g for 30 min and supernatant was filtered through sterile Millipore membranes (pore size 0.22 ¼m) and used for quantitative estimation of IAA using UV-visible spectrophotometer (UV-1601, Shimadzu Corporation, Japan) according to Gordon and Weber (1951). The concentration of IAA in sample was determined using IAA standard curve at 530 nm and expressed as IAA equivalents (µg ml^{-1}).

Phosphate solublizing ability

Qualitative detection of primary phosphate solublizing ability of *B. megaterium* EXB-53 was carried out by streaking on Pikovskaya's agar (Pikovskaya, 1948) plates amended with phosphate (PO_4^{3-}) as tri-calcium phosphate $Ca_3 (PO_4)_2$ (3g L^{-1}). The plates were incubated for 48h at 28°C and observed for appearance of a transparent halo zone around the streaked region indicating the phosphate solublizing ability.

Identification of volatiles compounds

B. megaterium EXB-53 was grown in ½ strength Murashige and Skoog (MS) medium containing 1.5% (w/v) agar, 1.5% (w/v) sucrose for 3 days at 28°C for extraction of volatile compounds (Zou *et al*., 2010). Volatile compounds from cell free culture filtrate was extracted using GC grade hexane and acetone as solvents at 1:1 ratio.The organic phase was separated and concentated in an rotary evoporator, the resulting residue was redissolved in 1 ml of respective solvent and used for volatile characterization. One µl of each sample was injected into gas chromatographic system (HP6890; Agilent Technologies, USA) coupled with a mass spectrometer (MS 5975 CVL, Agilent Technologies, USA). The HP-5 MS phenyl methyl siloxane non polar capillary column (0.25 mm x 30 m x0.25 µ) was used to separate the fractions with helium gas (99.99% purity) as moblie phase. The

split inlet was used with split ratio of 50:1 and inlet temperature of 280°C. The oven temperature program was set at 70°C min^{-2} with 2 min hold and a ramp of 10°C min^{-1} till 260°C and held for 5 mins with column flow of 1ml min^{-1}. The mass spectral detector was maintained at a temperature of 280°C with the interface temperature of 230°C. The mass spectra created using the MS was compared with the Wiley mass spectral library (Wiley W9N11.L and NIST 2.0 version) for identification of volatiles.

Pilot scale biomass production

The experiment was performed in a 10 litre capacity fermentor (Scigenics India private limited, India) with working volume of 7.5L. Molasses urea broth (MUB) containing cane molasses (20g L^{-1}) and urea (2 g L^{-1}) was used as mass production medium. One day old starter culture (1 x 10^5 cells ml^{-1}) was inoculated to pre-sterilized MUB at the rate of 10mlL^{-1} under aseptic condition. The operating conditions, temperature (28°C ± 0.5), aeration (0.8-1vvm) and rotations (250 rpm) were set at the start of the process. Samples were drawn in a sterilized beaker at every 24hr interval up to 72hr under aseptic conditions. Biomass of EXB-53 was estimated by serial dilution and plating on NA and expressed in terms of $\log_{10}$ CFU ml^{-1}.

Statistical analysis

All the experiments were repeated once and similar results were obtained. The data obtained from all experiments were analyzed in completely randomized block design with analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to compare means (Gomez and Gomez, 1984). The percentage values of root colonization were arc-sin transformed and CFUs values in mass production studies were log transformed (base 10) before statistical analysis. All statistical analyses were performed using SPSS version 16.0 for Windows (SPSS Inc. USA).

RESULTS

Growth promotion effect of B. megaterium EXB-53 under glass house conditions

Population of *B. megaterium* EXB-53 in CMC solution used for seed treatment was 8. 66 x 10^8 CFU ml^{-1}. Seed treatment with *B. megaterium* EXB-53 showed significantly higher germination percentage, shoot and root length in chilli var. Byadgi dabbi. As a result, vigor index in *B. megaterium* EXB-53 treatment (2615.31) increased significantly compared to untreated control (1605.52). Furthermore, fresh and dry weight of treated chilli plants increased by 122.22% and 84.04% respectively over untreated control under glasshouse conditions (Table 2 and Fig. 1).

Growth promotion effect of *B. megaterium* EXB-53 under nursery conditions

Population of *B. megaterium* EXB-53 in talc based formulation used for seed treatment in nursery experiment was 8 x 10^8 CFU g^{-1}. Seed treatment with *B. megaterium* EXB-53 significantly increased seed germination of all three chilli varieties, capsicum, egg plant, cabbage and cauliflower compared to respective untreated control. However, no significant effect was observed in case of tomato with respect to germination. Significant increase in root length was observed in chilli (HPH-12), capsicum, tomato, egg plant, cabbage and cauliflower over untreated control plants. Seed treatment significantly increased shoot length of all the tested varieties of vegetable crops except for tomato. As a result of increase in germination and root/shoot length,

Table 1. Seeds of different vegetable crops used in the study

S. N.	Crop	Variety	Source
1	Chilli	Byadgi kaddi	UHS, Devihosur
2	Chilli	Byadgi dabbi	-do-
3	Chilli	HPH-12	Syngenta (India)
4	Capsicum	Indra	-do-
5	Tomato	Heem Sohna	-do-
6	Egg plant	Harit	-do-
7	Cauliflower	Tetris	-do-
8	Cabbage	Summer Queen	-do-

vigour index of treated plants increased significantly compared to untreated control plants. Seed treatment with EXB-53 enhanced vigour index from 25 to 245% over control depending on the variety tested. Among all the vegetables crops tested, *Capsicum* var. Indra showed better response to seed treatment with 245% increase in vigour index over untreated control. Among the three different chilli cultivars tested, chilli var. HPH-12 showed better response with 222% increase in vigour index after seed treatment whereas other two local chilli cultivars, Byadgi dabbi and Byadgi kaddi showed 32 and 54% increase in vigour index respectively. Egg plant var. Harit showed 59%, cabbage var. SummerQueen 81% and cauliflower var. Tetris 52% increase in vigour index over control under nursery conditions. Tomato var. HeemSohna showed least response to seed treatment with EXB-53 with only 25% increase in vigour index. Furthermore, seed treatment also increased fresh plant weight of all the tested varieties ranging from 11.50 to 121.70% increase over untreated control. In case of dry weight, significant increase was observed in all three chilli varieties, capsicum and tomato (16.60 to 70.26%) over control. However, no significant effect was observed in bacterized egg plant, cabbage and cauliflower plants with respect to increase in dry weight (Table 3 and Fig 2).

Root colonization ability

Root bits of treated plants showed significantly higher bacterial colonization around them and the presence of *B. megaterium* cells was confirmed by microscopic observations. Treated plants showed 50-100% bacterial root colonization compared to untreated control plants which showed 0-46% bacterial colonization (Fig 3).

Plant growth promoting traits

B. megaterium EXB-53 produced 6.60 µg ml^{-1} of IAA in absence L-tryptophan and levels got increased significantly (8.20µgml^{-1}) in presence of L-tryptophan. Furthermore, *B. megaterium* EXB-53 developed a clear zone around streaked region on Pikovskaya's agar, indicating the ability of the strain to solubilise inorganic phosphate (Table 4 and Fig. 4).

Volatile compounds showing >95% similarity in database searches were considered as probable compounds produced by EXB-53. Database search resulted in identifying 23

Table 2. Effect of *Bacillus megaterium* EXB-53 on plant growth promotion of chilli var. Byadagi dabbi under glasshouse conditions

S. N.	Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Vigour index	Fresh weight (gm)	Dry weight (gm)
1	*B. megaterium* EXB-53	77.33 ^{a} ±5.74	13.38 ^{a} ±1.70	20.42 ^{a} ±2.50	2615.31 ^{a} ±55.72	73.33^{a} ±1.72	8.30^{a} ±0.39
2	Control	54.67 ^{b} ±5.74	11.02 ^{b} ±4.14	18.26 ^{b} ±4.95	1605.52 ^{b} ±34.72	33.00^{b} ±2.48	4.51^{b} ±1.03

Values are means of three replications ± standard errors (SE). Values in columns followed by the same letter are non - significant with each other according to DMRT ($P < 0.05$).

Table 3. Effect of *Bacillus megaterium* EXB-53 on plant growth promotion of vegetable crops under nursery conditions

Treatment	Germination (cm)	Root length (cm)	Shoot length (cm)	Vigour index	Fresh weight (gm)	Dry weight (gm)
Chilli var. Byadagi dabbi						
EXB-53	96.67±8.29 a	8.22±0.63 a	15.08±0.56 a	2252.41±94.70 a	93.87±6.24 a	9.28±2.83 a
Control	70.67±11.47 b	8.75±0.26 a	11.94±0.25 b	1460.07±74.73 b	54.83±2.53 b	5.68±0.20 b
Chilli var. Byadagi kaddi						
EXB-53	80.00±7.21 a	8.46±0.96 a	9.63±1.18 a	1451.28±92.72 a	93.20±5.04 a	13.28±0.83 a
Control	69.33±5.18 b	8.02±1.12 a	7.82±0.65 b	1097.68±96.40 b	54.47±6.22 b	7.80±0.50 b
Chilli var.HPH-12						
EXB-53	90.67±5.74 a	8.64±2.04 a	10.87±2.23 a	1771.15±90.80a	126.93±4.35 a	19.48±0.67 a
Control	56.00±6.45 b	4.55±1.91 b	4.29±1.82 b	549.36±91.77 b	57.25±1.94 b	16.71±0.23 b
Capsicum var. Indra						
EXB-53	92.00±6.94 a	6.09±0.25 a	9.47±0.20 a	1433.09±50.56 a	119.73±13.53 a	15.53±6.20 a
Control	57.33±5.74 b	3.73±0.33 b	3.51±0.40 b	415.20±51.94 b	74.84±9.40 b	9.94±3.72 b
Tomato var. Heem Sohna						
EXB-53	96.00±0.94 a	10.05±0.49 a	15.03±1.76 a	2450.61±86.67 a	298.93± 6.26 a	38.13±1.19 a
Control	97.33±1.47 a	8.70±0.45 b	14.27±1.15 a	1956.45±71.99 b	243.47±12.02 b	28.83±4.00 b
Egg plant var. Harit						
EXB-53	100.00±0.00 a	11.24±0.39 a	12.19±3.31 a	2342.67±53.96 b	113.87±7.40 a	19.93±5.45 a
Control	84.00±1.21 b	8.13±0.81 b	7.30±3.32 b	1306.99±50.80 a	87.07±5.63 b	21.09±3.04 a
Cabbage var. Summer Queen						
EXB-53	93.33±5.74 a	9.98±2.50 a	11.51±2.34 a	2009.52±42.95 a	99.33±9.28 a	27.85±4.15 a
Control	84.00±4.42 b	7.88±2.10 b	4.96±2.65 b	1111.20±60.92 b	81.20±8.11 b	25.55±5.51 a
Cauliflower var. Tetris						
EXB-53	100.00± 0.00 a	10.57± 1.11a	12.09± 1.88 a	2266.00±66.79 a	159.07±6.62 a	19.56±1.33 a
Control	93.33±1.47 b	8.57±2.56 b	7.32±2.27 b	1489.01±52.33 b	142.67±12.38 b	17.83±2.38 a

Values are means of three replications ± standard errors (SE). Values in columns followed by the same letter are non - significant with each other according to DMRT ($P < 0.05$).

Fig. 1. Effect of *Bacillus megaterium* EXB-53 on plant growth promotion of chilli var. Byadgi dabbi under glass house conditions. (a) Control (b) EXB-53 treated

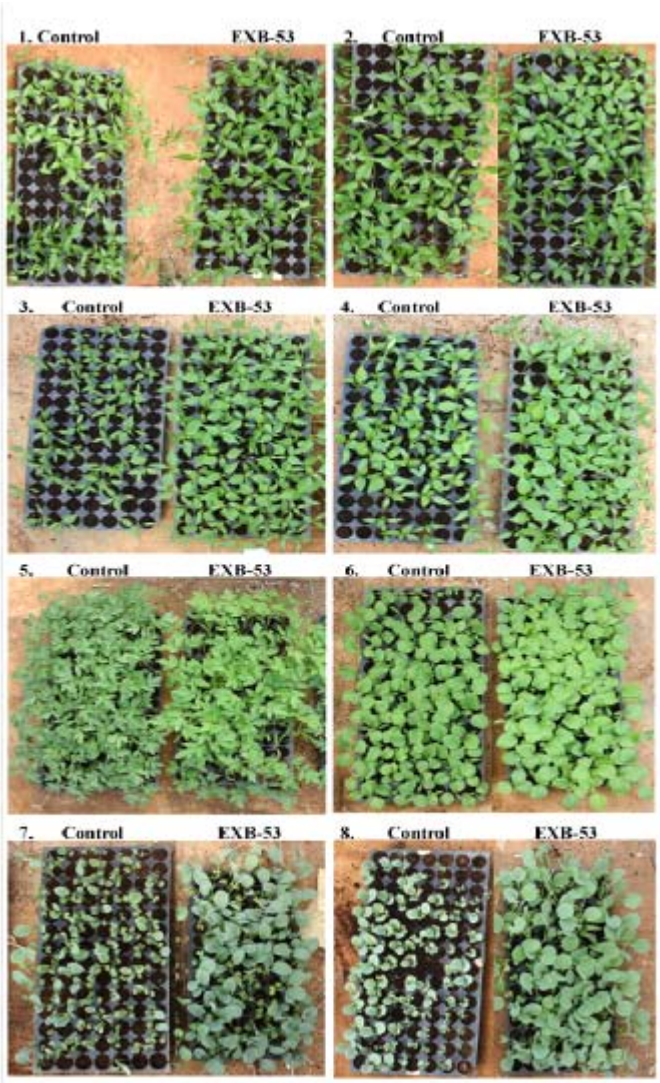

Fig. 2. Effect of *Bacillus megaterium* EXB-53 on plant growth promotion of vegetable crops under nursery conditions. 1. Byadgi kaddi; 2. Byadgi dabbi; 3. HPH-12; 4. Indra; 5. Heem Sohna; 6. Harit; 7. Summer-queen 8. Tetris

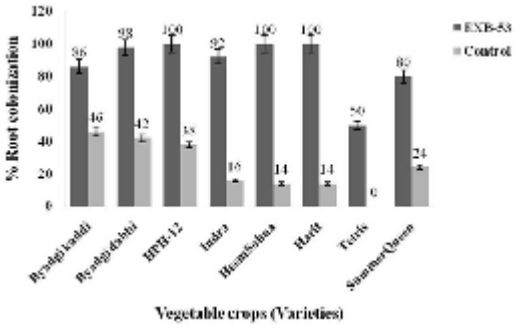

Fig. 3. Root colonization of ability of *Bacillus megaterium* EXB-53 in different vegetables. Values are the means of three replications and vertical bars represent the standard error (±SE) of between each replication

Fig. 4. Phosphate solubilization of *Bacillus megaterium* EXB-53 on Pikovskaya's agar amended with phosphate (PO_4^{3-}) as tri-calcium phosphate $Ca_3(PO_4)_2$. Transparent halo around the streaked region indicates the phosphate solubilization ability of the strain.

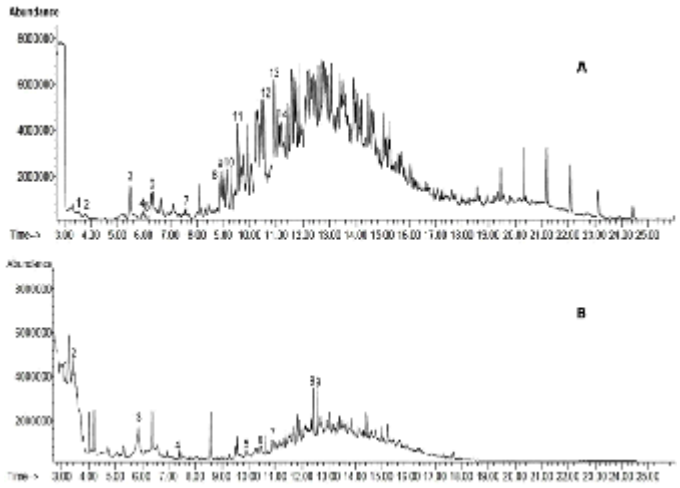

Fig. 5. Chromatograms of volatile compounds produced by *Bacillus megaterium* EXB- 53. (A). Hexane fraction. (B). Acetone fraction

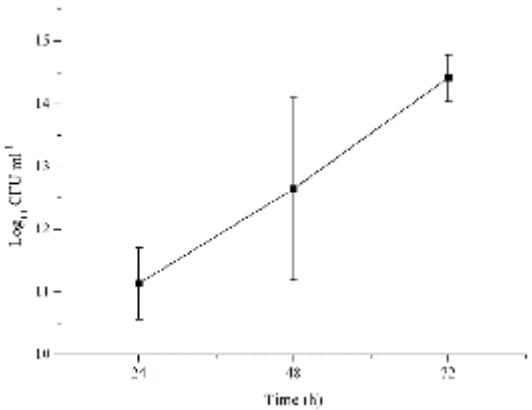

Fig. 6. Biomass production of *Bacillus megaterium* strain EXB-53 in pilot scale fermentor

compounds which included aldehydes, alkanes, alcohols, organic acids, ketones and aromatic components. The volatiles such as 2,5-Dimethylpyrazine, 2-Ethyl Hexanol, Tetradecane, Pentanoic acid, Benzeneacetaldehyde, 2-

Table 4. Important plant growth promoting traits of *Bacillus megaterium* EXB-53

IAA equivalents (µg ml^{-1})		Phosphate
With L-TRP	Without L-TRP	solubilization
8.20^{a} ±0.48	6.60^{b} ±0.25	Positive

Values are means of three replications ±standard errors (SE). Values in columns followed by the same letter are non - significant with each other according to DMRT ($P < 0.05$). L-TRP is L-Tryptophan (0.1%)

Table 5. Volatile compounds produced by *Bacillus megaterium* EXB-53 identified through GC-MS studies

S. No.	Retention time	Compound	Mass spectra (%)	Relative
Hexane Fraction				
1	3.531	Cinnamene	2,7,39,44,51,58,63,73,78,89,98,104	2.08
2	3.789	2,5-Dimethylpyrazine	18,28,42,52,58,67,73,81,94,108	1.73
3	5.481	2 Ethyl Hexanol	41,51,57,65,70,77,83,98,112	6.23
4	5.939	2-Propanone, 1-phenyl	27,37,43,51,60,65,77,86,91,98,105,115,122,128,134	1.73
5	6.012	1-Heptanol, 2-propyl-	29,39,43,57,71,85,97,111,126,140	1.73
6	6.268	(2E)-4-Methyl-2-undecene	29,35,41,50,56,63,69,79,84,97,111,125,140,152,168	5.19
7	7.667	3-Methylundecane	15,29,43,50,57,64,71,77,85,99,112,126,135,141,155,170	1.73
8	8.959	4-Methyldodecane	15,29,43,57,71,77,85,99,112,127,135,141,155,169,184	6.92
9	9.018	2-Methyldodecane	2,15,29,43,57,71,85,99,113,127,141,155,169,184	6.92
10	9.124	3-Methyldodecane	23,29,37,43,57,66,71,85,91,99,113,127,155,169	8.65
11	9.558	Tridecane	29,43,57,71,85,99,112,127,141,155,184	13.84
12	10.522	3-Methyltridecane	29,43,57,71,77,85,91,99,113,127,140,154,169,183,198	15.57
13	10.921	Tetradecane	15,29,43,51,57,65,71,85,99,113,127,141,155,169,198	17.30
14	11.344	3-Methyltetradecane	29,37,43,51,57,65,71,79,85,99,113,127,141,154,169,183,197,212	10.38
Acetone Fraction				
1	3.108	Pentanoic acid	18,27,37,43,53,60,68,74,81,87,93,102	8.52
2	3.519	2-Pentanone, 4-hydroxy-4-methyl	43,59,69,83,101	22.73
3	5.798	Benzeneacetaldehyde	29,39,51,65,77,91,120	11.36
4	7.385	Methyl N-hydroxybenzenecarboximidoate	31,42,55,73,82,91,105,121,133,151	3.41
5	9.993	2, 4 - Dimethoxy- Acetophenone	43,53,63,77,92,107,122,135,150,165,160	2.84
6	10. 428	2-Phenyloctane	27,41,51,65,77,91,105,119,133,147,161,175,190	8.52
7	10.874	Tetradecane	29,43,57,71,85,99,112,127,140,155,198	5.68
8	12.413	Benzoic acid, 4-ethoxy-, ethyl ester	15,29,39,50,65,65,76,90,109,121,138,149,166,179,194	18.47
9	12.554	Phenol, 2,6-bis (1,1-dimethylethyl)- 4-methyl	18,29,41,57,67,81,91,105,115,131,145,161,177,189,220	18.47

Pentanone, 4-hydroxy-4-methyl etc. were found in EXB-53 culture.The details on volatile compounds identified in EXB-53 along with their retention time (RT), mass spectra, relative percentage and chromatograms are mentioned in the table 5 and fig 5.

Biomass production at Pilot scale

The MUB medium supported rapid growth and high biomass of EXB-53 in pilot scale process. The biomass of 11.13 $\log_{10}$ cfu/ml was observed at 24hr after inoculation, which gradually increased to 14.41 $\log_{10}$ cfu/ml at the end of 72hr (Fig 6).

DISCUSSION

Use of plant growth promoting rhizobacteria (PGPR) and other microbial inoculants has been proved useful in plant growth promotion and disease management in sustainable agricultural practices (Lugtenberg and Kamilova, 2009; Saharan and Nehra, 2011). Beneficial interactions between plant and microbes can result in direct plant growth promotion through extensive root colonization, production of phytohormones and nutrient mobilization (Van-Loon, 2007; Hayath et al., 2010).The results of our current investigation clearly indicated the ability of *B. megaterium* EXB-53 to promote plant growth in different vegetable crops under nursery conditions. Furthermore, *B. megaterium* EXB-53 exhibited extensive root colonization ability and was positive to IAA production, phosphate solubilization and production of volatile compounds which are considered as important PGP traits of any microbial inoculant. The present study is agreement with earlier reports where *B. megaterium* was consistent in improving rooting performance, root length and dry matter content of root in mint (Kaymak *et al*., 2008). Inoculation of phosphate solublizing *B. megaterium* var. *phosphaticum* increased mineral availability, uptake and plant growth in pepper and cucumber (Han *et al*., 2006).

In the present study, seed treatment with *B. megaterium* EXB-53 showed 25-245% increase in vigour index of different vegetable crops over control plants under nursery conditions. A degree of positive variation was observed with respect to increase in vigour index of each variety tested. This can be attributed to the quality and quantity of root exudates(s) produced by each variety during different growth phases which might have influenced the colonization and growth promoting potential of *B. megaterium* EXB-53. Extensive literature is available on influence of root exudates on colonization of various PGPRs in rhizosphere and rhizoplane (Antoun and Prévost, 2005; Benizri et al., 2001; Somers et al., 2004). For example, the concentration and type of root exudates influenced the colonization *Pseudomonas fluorescens* WCS365 and its mutant's in tomato seedlings (De Weert *et al*., 2003). L-malate present in the root exudates of *Arabidopsis thaliana* acted as the major chemo attractant for *B. subtilis* FB17 for its activity in the rhizosphere (Rudrappa *et al*., 2008).

Root colonization is one of the important primary factors in successful plant–microbe interactions. The process of root colonization is a complex event influenced by many biotic and abiotic factors. Inability to colonize plant roots by PGPR often results in failure of desired effect (Lugtenberg et al., 2002; Kamilova et al., 2005; Antoun and Prévost, 2005). In the present study, root bits from plants treated with *B. megaterium* EXB-53 showed comparatively higher (50-100%) bacterial colonization compared to untreated control plants (0-46%). The additional increase in the bacterial colonization due to EXB-53 has resulted in the increased growth of treated plants. Although, root colonization alone cannot be attributed to beneficial effect but it is one of the important parameters considered for growth promotion of different crops. In addition to root colonization, about 80% of bacteria colonizing the rhizosphere have been reported to produce IAA with tryptophan as main precursor molecule for IAA biosynthesis (Kumar *et al*., 2011). IAA is a key substance responsible for shaping plant root architecture such as root vascular tissue differentiation, regulation of lateral root initiation, polar root hair positioning etc. (Hayath *et al*., 2010; Glick,2012). In the present study, *B. megaterium* EXB-53 was positive to IAA production and significantly higher levels of IAA were observed in presence of L-tryptophan precursor. Another best-studied mechanism of direct plant growth promotion by PGPBs includes phosphate solubilization. Although, the amount of phosphorus present in soil is quite high but most of it is present in insoluble form and thus not available readily for plant uptake. From several

decades, farmers have depended on chemical fertilizers as source of phosphorus and other essential nutrients. The disadvantages associated with chemical fertilizers have been reported by many researchers worldwide and hence biological means of providing essential phosphorous to plants could substitute for chemical fertilizers (Richardson *et al*., 2001; Khan *et al*., 2007). In the present study, *B. megaterium* EXB53 was positive to the phosphate solubilization, which can act as essential mode of phosphorous uptake by plants. In addition to IAA production and phosphate solubilization ability, certain bacterial volatile compounds are also involved in stimulating plant growth (Kai *et al*., 2009). In the present study, an attempt was made to identify different volatile compounds produced by *B. megaterium* EXB-53 through GC-MS studies. Results indicated that EXB-53 had ability to produce different volatile compounds which are likely to play essential roles in plant growth promotion of vegetable crops. *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a have been reported to promote growth of *Arabidopsis* plants by releasing volatile compounds such as 2,3 -butanediol and acetoine (Ryu *et al*., 2003). Zou *et al*., (2010) demonstrated the ability of *B. megaterium* strain XTBG34 to produce volatile compound such as 2-Pentylfuran which significantly increased plant growth in *Arabidopsis thaliana.* Characterization of plant growth promoting volatiles from bacteria is an emerging are of area of research (Blom et al., 2011; Bhattacharyya and Jha, 2012). Production of volatiles is strain-specific and depends on cultural conditions; however, continuation of the present study would be necessary in order to determine the specific volatile compound(s) responsible for growth promotion.

After the evaluation of any microbial inoculants for various biocontrol properties including PGP traits under *in vitro* and *in vivo* conditions, the next essential requirement for product development for commercial application is suitable mass production and formulation technology (Köhl *et al*., 2011). In our previous study, five different media namely sucrose yeast extract broth (SYEB), molasses yeast extract broth (MYEB), molasses urea broth (MUB), nutrient broth (NB) and tryptic soya broth (TSB) were evaluated for biomass mass production of EXB-53 in shake flasks. In addition, talc and liquid formulation were also evaluated for shelf life of *B. megaterium* EXB-53 at ambient conditions. Among the different media tested, MUB supported maximum growth of *B. megaterium* EXB-53 (10.17 Log cfu ml^{-1}) after 48h of inoculation (Ramanujam *et al*., 2012b). In the present study, pilot-scale production of *B. megaterium* EXB-53 was performed with MUB in 10L fermentor as a continuation to our earlier shake flask studies. Considerably increase in biomass (up to 43%) of EXB-53 was observed under pilot-scale conditions compared to laboratory shake flask conditions. Scale-up of production process with low-cost medium is an essential pre-requisite for development of commercial product under industrial conditions (Köhl *et al*., 2011). Results of our previous investigation had demonstrated that talc and liquid formulations of *B. megaterium* EXB-53 had shelf-life of five months when stored at ambient conditions (Ramanujam *et al*., 2012b). Talc based formulations of PGPR and other microbial inoculants have been extensively used for seed treatment of various crops due to ease of production, application economic and low-cost (Nakkeeran *et al*., 2005). In the present study, seed treatment with talc formulation of *B. megaterium* EXB-53 promoted plant growth in different vegetables crops tested under nursery conditions.

In conclusion, seed treatment with bacterial antagonist, *B. megaterium* EXB-53 resulted in significant increase in seedling vigour, fresh/dry weight of different vegetable crops under nursery conditions. The potential of *B. megaterium* EXB-53 as plant growth promoter of vegetable crops may be attributed to extensive root colonization ability and to also to the presence of multiple PGP traits like IAA production, phosphate solubilization and production of volatile compounds. Further, the strain can be mass produced in molasses urea broth (MUB) and talc formulation can be prepared easily which can act as an effective delivery system of the *B. megaterium* EXB-53. Potential strain along with suitable mass production/formulation technology and insights into mechanism(s) of action will help in developing eco-friendly strategy(s) in modern agricultural practices. Overall, the study indicates that *B. megaterium* EXB-53 can be potentially exploited in development of commercial product

for seed treatment of vegetable crops. The approaches like this will be helpful in obtaining healthy and robust plants with high vigour and also minimize the usage of chemical fungicides for seed treatment.

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