Journal of Ecobiotechnology 2012, 4(1): 35-42
ISSN: 2077-0464
Available Online: http://journal-ecobiotechnology.com/

In vivo and *In vitro* studies of *Bacillus megaterium* var. *phosphaticum* on nutrient mobilization, antagonism and plant growth promoting traits

E. Leo Daniel Amalraj, S. Maiyappan and A. John Peter*

Research and Development Wing, Varsha Bioscience and Technology INDIA Private Limited, M.N.Reddy Colony, Balaji Nagar, Saidabad, Hyderabad-500 059, Andhra Pradesh, India

Abstract

Nutrient solubilisation efficiency, plant growth promoting traits and antagonistic effects of *Bacillus megaterium* var. *phosphaticum* were studied in the laboratory and screen house during 2010-11 at Hyderabad, India. Plate agar assay indicated varied solubilisation level when the media was blended with zinc carbonate (35.6%), zinc oxide (31.1%), k-bentonite (23%), rock phosphate (19.8%), tricalcium phosphate (3.1%) and zinc sulphide (0.0%). The release of available zinc is high (17.4 ppm) on day-12 when the test organism was grown in liquid media blended with zinc carbonate. The media pH was inversely proportional to the amount of nutrients solubilised on day-12. *B. megaterium* var. *phosphaticum* is found to produce plant growth promoting substances like biofilm and chitinase enzymes (strong), giberrellic acids and siderophores (moderate) and indole acetic acid (weak). Confrontation assay confirmed it's strong antagonistic activity against *Rhizoctonia solani* (41%), *Macrophomina phaseolina* (42%), *Sclerotium rolfsii* (27%) and *Fusarium oxysporum* (40%). The production of siderophores and chitinase enzymes justifies the strong antagonistic activity against these fungal pathogens. Potted plant assay using sunflower, *Helianthus annuus* as the test crop indicated superior plant growth and photosynthetic activities in treatment with *B. megaterium* var. *phosphaticum* + 75% of recommended chemical fertilizer dose as compared to treatment with 100% chemical fertilizers. It also improved significantly the uptake of nitrogen (7.97mg/100g dry mass), phosphate (3.41mg/100g dry mass), potash (38.12mg/100g dry mass), zinc (184mg/100g dry mass), iron (743mg/100g dry mass) and manganese (138mg/100g dry mass) as compared to treatments with 100% *B. megaterium* var. *phosphaticum*, 100% chemical fertilizers and untreated control. The findings of current study suggest reduction of 25% recommended dose of chemical fertilizers in combination with *B. megaterium* var. *phosphaticum* as seed dresser and soil application.

Keywords: *B. megaterium* var. *phosphaticum*, nutrients solubilisation, antagonistic activity, sun flower, PGPR and PSB.

INTRODUCTION

Bacillus megaterium var. *phosphaticum* is a large rod shaped Gram's positive bacterium commonly called as phosphobacterium [1,2]. Presence of *Bacillus* spp in agricultural fields reported to enhance plant growth directly and/or indirectly [3]. Increase in crop yield is reported on various crops including sugar beet [4], barley [5], alfalfa [6], clover, wheatgrass, perennial ryegrass [7] and cicer [8]. Production of amino acids, vitamins, indole acetic acid (IAA), gibberellic acids (GA_3) [9, 10] antibiotics [11,12] induced systemic resistance to plant pathogens, production of siderophore and inhibition of plant ethylene synthesis [13-16] are reported to be the possible reasons for crop yield increase in addition to nutrient solubilisation.

The role of *Bacillus megaterium* var. *phosphaticum* for enhancing mineral phosphorus (P) solubilisation is well documented [17, 18]. Several mechanisms have been proposed to explain the P solubilisation as they are associated with the release of organic and inorganic acids and the excretion of protons that accompanies to the NH_4^+ assimilation [19-24]. In addition, it could also be due to the release of phosphatase enzymes that mineralize organic P compounds [25]. Due to the P solubilisation capacity, *Bacillus megaterium* var. *phosphaticum* could be used along with natural phosphorus minerals.

Voluminous research is carried out on the role of *B. megaterium* var. *phosphaticum* as phosphate solubilizer. Han et al. [26] and Supanjani et al. [27] in addition have reported that pepper and cucumber are capable of absorbing phosphate and potash rocks when *B. megaterium* var. *phosphaticum* and *B. mucilaginosus* are inoculated in nutrient limited soil. However, its role against other nutrient solubilisation is not fully explored. Therefore, we have investigated the role of *B. megaterium* var. *phosphaticum* on potash, phosphate and zinc solubilisation, its antagonism against select phytopathogens *viz., Rhizoctonia solani, Macrophomina phaseolina, Sclerotium rolfsii* and *Fusarium oxysporum* and its capability of producing various plant growth promoting substances in the laboratory.

To substantiate the results obtained in the laboratory, we have carried out the potted plant bioassay using Sunflower (*Helianthus annus* L.) as a test crop treated with *B. megaterium* var. *phosphaticum* alone and in combination with chemical fertilizers. We have tabulated and discussed the results obtained in this article.

MATERIAL AND METHODS

Bacterial strain and period of study

Received: Nov 13, 2011; Revised: Dec 24, 2011; Accepted: Jan 18, 2012.

*Corresponding Author
A. John Peter
Research and Development Wing, Varsha Bioscience and Technology INDIA Private Limited, M.N.Reddy Colony, Balaji Nagar, Saidabad, Hyderabad-500 059, Andhra Pradesh, India

Tel: +91-040-24074427; Fax: +91-040-2471787
Email: varsshabioscience@yahoo.com

The strain of *Bacillus megaterium* var. *phosphaticum* used throughout this study was obtained from the Regional Centre for Organic Farming (RCOF), Nagpur, India. The study was carried out at Hyderabad, Andhra Pradesh, India from March 2010 to August 2011.

Phosphate, potash and zinc (P, K & Zn) solubilisation- Qualitative assay

Phosphate solubilization was evaluated using Pikovskaya's agar media blended with 5.0 g^{-l} of tricalcium or rock phosphate [28]. Potash solubilization was assessed by the modified Aleksandrov medium amended with 2.0 g^{-l} of potassium bentonite (montmorillonite) as sole K source [29]. The zinc solubilization was assessed with 1.0 g^{-l} each of zinc oxide, zinc carbonate and zinc sulphide in modified mineral salts agar medium [30]. The media were autoclaved at 121°C for 20 minutes and poured into sterilized Petri plates. Using drop plate method, 5µl of culture was inoculated and the plates were incubated at 30 ± 0.5 °C. The diameter of clear zone surrounding the bacterial growth and the diameter of colony were measured after 5, 10 and 15 days of incubation.

Phosphate (P), potash (K) and zinc (Zn) solubilisation- Quantitative assay

Quantitative solubilisation assay was carried out using Pikovskaya [28], Aleksandrov [29] and Bunt [30] mineral salt liquid medium for P, K and Zn, respectively. About 100 ml of each media containing (250 µg/ml) of complex substrate was prepared, sterilized, cooled and inoculated with 1% *B. megaterium* var. *phosphaticum* inoculum (10^7 cfu ml^{-1}). The flasks were incubated at 28 ± 0.5°C for 12 days in a shaking incubator at 180 RPM. The amount of nutrient released and media pH were assessed at every 4 day interval. Soluble phosphates in culture supernatant was determined by sulphomolybdic method [31] and expressed as equivalent P. The available K and Zn were estimated using flame photometry and atomic adsorption spectrophotometer (AAS), respectively.

Detection of siderophore and *in vitro* biofilm production

The production of siderophore and biofilm were detected by the methods described by Schwyn and Neilands [32] and Christensen et al. [33], respectively.

Detection of Indole acetic acid (IAA) and gibberlic acid (GA_3) by thin layer chromatography (TLC)

Bacterial strain was inoculated and incubated in medium containing 0.1% tryptophan for 48 h at 28 ± 0.5°C. After incubation, the medium was centrifuged at 10000 RPM for 15 minutes. The supernatant was tested for IAA [34] and GA_3 [35] production using TLC plates.

Detection of chitinase production by dot blot assay

B. megaterium var. *phosphaticum* was grown in typtone soya broth at 28 ± 0.5°C, 180 RPM for 28 h. The culture was centrifuged at 10,000 RPM for 10 minutes to remove the bacterial cells. Supernatant was dried in liquid nitrogen and the samples were freeze dried under vacuum in a lyophilizer at -60°C and 0.08 MPa pressure till the samples turned into powder form. Polyacrylamide gel (5 x 5 cm) was prepared with the composition *viz*., 30% Acrylamide-bis acrylamide mix: 1.3ml; 1% glycol chitin: 0.1 ml; sodium acetate buffer (50mM; pH 5.2): 2.6 ml; TEMED: 4 µl; 40% ammonium per sulphate: 4 µl. Freeze dried sample was dissolved in 50 mM sodium acetate buffer at a final concentration of 10mg.ml^{-1} and was spotted @ 3 µl on the prepared gels. The gels were incubated at 37± 0.5°C overnight in a moist chamber. After incubation, the gels were stained with calcoflour white for 5 min and washed with water for 20 min twice and observed in a gel doc (Vilber Lourmat) under ultra violet light for chitin solubilization.

Antagonistic activity against select phytopathogens

The *in vitro* bio-efficacy of *B. megaterium* var. *phosphaticum* was evaluated by the method described by Kaur [36] against four select phytopathogens *viz., Sclerotium rolfsii, Rhizoctonia solani, Fusarium oxysporum* and *Macrophomina phaseolina*.

Pot culture assay

Short term pot culture assay was carried out to study the bio-efficacy of *B. megaterium* var. *phosphaticum* using sunflower as test crop. The soil was air-dried, sieved (<5 mm mesh), and sterilized at 121° C for 1 hour for 3 consecutive days. The soil was filled in the plastic pots of 5 kg holding capacity. The following treatments were made to study the bio-efficacy of the test organism:

T1- Untreated control with no chemical/ biological inputs, T2 - Sunflower seeds treated with *B. megaterium* var. *phosphaticum* @ 5ml/kg seed containing $2x10^{10}$ cfu ml^{-1}, T3 - Seeds were soaked overnight in 1% chemical fertilizers with diammonium phosphate, murate of potash and zinc sulphate at 65: 25: 10 ratio, followed by soil application (100%) @ 4.55:2.27:2.27:0.76 g/pot of N, P, K and Zn, respectively.

T4 - Seeds were treated with bacterial inoculant @ 5ml/kg and sown in the pots amended with chemical fertilizer @ 3.41: 1.70: 1.70: 0.57 g/pot of N, P, K and Zn, respectively (75% of recommended dose). Six replications were maintained for each treatment. The growth parameters like percent germination, shoot, root length, dry biomass and photosynthetic activity were recorded at 30 and 60 days after sowing (DAS). The macro (NPK) and micronutrient uptake by the plants were assessed by the method specified by Tandon [37]. Photosynthetic activity was estimated by infra red gas analyzer instrument.

Statistical Analysis

The values presented are the means of two experiments each with six replicates performed at different occasions. Data obtained from all the experiments were subjected to two-way analysis of variance (ANOVA). Mean values between treatments were compared with Fisher's Least Significant Difference (L.S.D) test ($P<0.05$).

RESULTS AND DISCUSSION

Phosphate, potash and zinc solubilization

B. megaterium var. *phosphaticum* is so far known to solubilise the phosphate [18]. In the present study, we have observed that it

is capable of even solubilising zinc oxide, zinc carbonate and K bentonite in addition to TCP and rock phosphate (Figure 2). In plate assay, *B. megaterium* var. *phosphaticum* showed slender solubilization zone (2.8 mm) on Pikovaskaya agar amended with $Ca_3P_2O_5$ (Table 1 & Figure 1). However, it exhibited distinct clearing zone (17.8 mm) with 19.8% solubilisation where rock phosphate was used as substrate. About 20.7 mm zone size and 23.0% area of solubilisation was recorded in K-bentonite added media with 295.7 solubilisation efficiency (SE). Maximum zone size, solubilisation percent and efficiency were observed in zinc carbonate amended media (32.0 mm, 35.5% and 457 SE) followed by zinc oxide incorporated media (28.0 mm, 31.11% & 400 SE) (Table 1). Solubilisation zone was not observed where zinc sulphide was used as substrate (Figure 1).

Solubilisation of zinc and other test minerals can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion or production of chelating agents [38, 39]. In addition, production of inorganic acids such as sulphuric acid, nitric acid and carbonic acid could also facilitate the solubilisation [40, 41]. This is the first report based on literature survey that *B. megaterium* var. *phosphaticum* is capable of solubilising zinc and potash besides phosphate. So far, *Pseudomonas fluorescens* reported to solubilise zinc phosphate [42]. Mechanism behind the solubilisation is reported to be the production of gluconic and 2- keto gluconic acids in the solubilization of the zinc salts. Since culture broth of *B. megaterium* var. *phosphaticum* showed a shift in pH towards acidic range (Figure 2), it gives a clue that organic acid might be involved as reported by Nahas [39].

Table 1. Nutrient solubilisation by *B. megaterium* var. *phosphaticum* after 15 days of inoculation

Substrate	Solubilisation zone (mm)	Percent solubilisation	Solubilisation efficiency (E)
Tricalcium phosphate	2.8 (±0.12)	3.1	35.0
Rock phosphate	17.8 (±0.81)	19.77	254.2
Zinc oxide	28.0 (±1.27)	31.11	400.0
Zinc carbonate	32.0 (±1.46)	35.55	457.0
Zinc sulphide	ND*	ND	ND
K- bentonite	20.7 (±0.94)	23.0	295.7

*ND: Not detectable. Solubilization efficiency was calculated based on the formula; Solubilization diameter x colony diameter/100 as described by Nguyen et al. [43]. Values within parenthesis are standard error (SE).

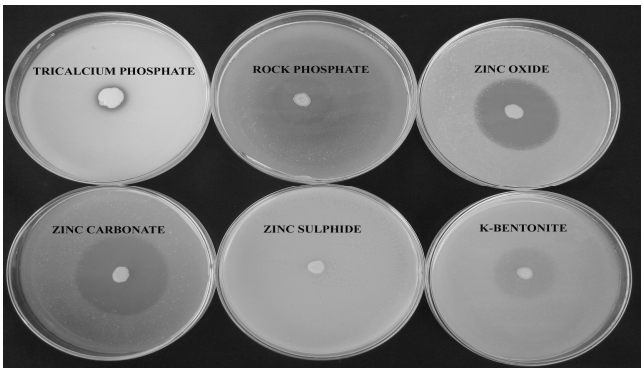


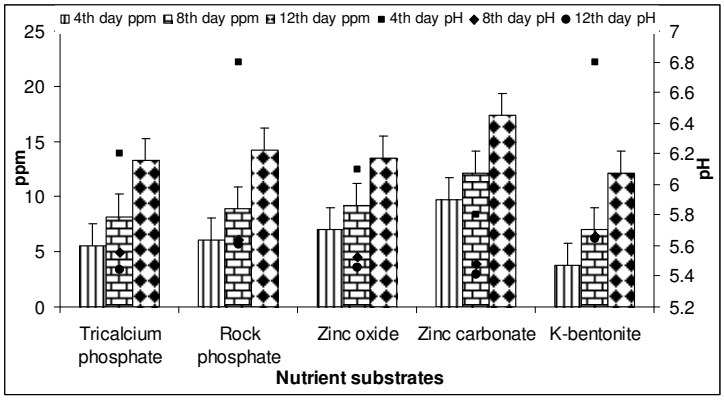
Fig 1. Solubilisation of complex nutrients by *B. megaterium* var. *phosphaticum*

Nutrient released in liquid culture

The degree of solubilization of various complex nutrients differed in solid and liquid media. The correlations between the size of clear zones on the plates of precipitated phosphate agar and quantitative data of P solubilization in the liquid media varies [44]. We have observed decrease in pH of the media over a period of 12 days when *B. megaterium* var. *phosphaticum* was grown in complex nutrient substrate (Figure 2). The solubilization of TCP and rock phosphate in the liquid medium was observed with a significant drop in pH 3.35 and 5.6, respectively after 12 days from the set neutral pH. The soluble-P concentration ranged from 13.3 ppm in TCP and 14.2 ppm in rock phosphate amended media. The mechanism of solubilisation is inferred due to the production of organic acids such as monocarboxylic acid (acetic, formic), monocarboxylic hydroxy (lactic, glucenic, glycolic), monocarboxylic, ketoglucenic, decarboxylic (oxalic, succinic), dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids [45]. The pH of the broth was acidic in all the media after 12 days of inoculation. The pH reduced from 7.0-7.3 to 2.9-6.22 and lowest pH was recorded in $ZnCO_2$ amended media (2.97). Increasing trend of solubilization was observed in all the treatments except in zinc sulphide (ZnS) incorporated media. The dissolution of ZnS (sphalerite) by the bacteria was through their ability to oxidize ferrous ions [46]. Non-oxidization of ferrous ions could be the possible reason for not solubilising ZnS in both media.

Estimation of soluble zinc using AAS indicated increased zinc release *viz.,* 17.4 ppm and 13.5 ppm in $ZnCO_3$ and ZnO amended media, respectively. Results obtained indicated that available zinc levels increased with the increase in incubation period. The findings of Da Costa and Duta [47] pertaining to bioaccumulation of Cu, Zn Cd and Pb in the cells of *Bacillus* sp, *B. cereus*, *B. sphaericus* and *B. subtilis* substantiate the present results. The microbes absorb required quantity of available zinc into the cells and leaves the excess remain in the solution. The available K was recorded as 12.1ppm in the media amended with K-bentonite (Figure 2) with no drastic decline in the pH as compared to other treatment on 12th day after inoculation (Figure 2). However, pH of the nutrient amended media on day 12 was observed to be inversely proportional to the amount of nutrients solubilised.

Pepper and cucumber are reported to absorb rock material (P and K rocks) when *B. megaterium* var. *phosphaticum* and *B. mucilaginosus* inoculated in nutrient limited soil [26,27]. The present study proved that *B. megaterium* var. *phosphaticum* could also be used for solubilising phosphate, potash and zinc when the nutrients are available in the form of TCP, rock phosphate, $ZnCO_3$, ZnO and K-bentonite.



Data are means of six replicates; error bars indicate standard deviations.

Fig 2. Nutrients released by *B. megaterium* var. *phosphaticum* in broth culture amended with the complex substrates

Plant growth promoting traits

B. megaterium var. *phosphaticum* was found to be strong producer of biofilm and chitinase enzyme (Table 2). The development of biofilms is a process that involves both a quorum of cells and multicellular behaviour [48]. It helps in better colonization in the rhizospheric area of the plants. About 9 mm of clearing zone was recorded in colloidal chitin amended media (data not shown) observed under gel documentation system equipped with ultra violet light. Previous reports have shown that species of *Bacillus* are known to produce chitinolytic enzymes [49, 50].

Moderate siderophore activity was observed in chrome azurol S (CAS) media (Figure 3). The role of siderophore production in plant growth promotion is described by two mechanisms: one is direct supply of iron to plants [51] and the other is indirectly depriving Fe for fungal pathogens [52]. The TLC plates observed under UV light revealed that *B. megaterium* var. *phosphaticum* was a moderate and weak producer of GA_3 and IAA, respectively. IAA functions as an important signal molecule in the regulation of plant development including organogenesis, tropic and cellular responses such as cell expansion, division, differentiation and gene regulation [53].

Table 2. Plant growth promoting traits of *B. megaterium* var. *phosphaticum*

Strain	PGP traits				
	Siderophore	Biofilm	IAA	GA_3	Chitinase
B. megaterium var. *phosphaticum*	++	+++	+	++	+++

+ weak, ++ moderate, +++ strong.

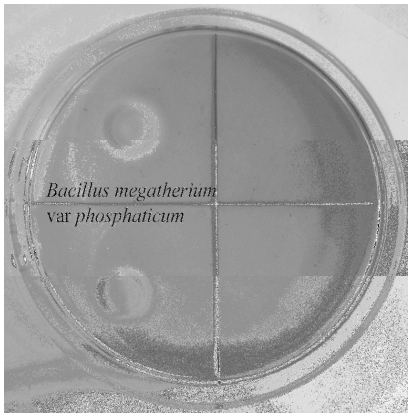
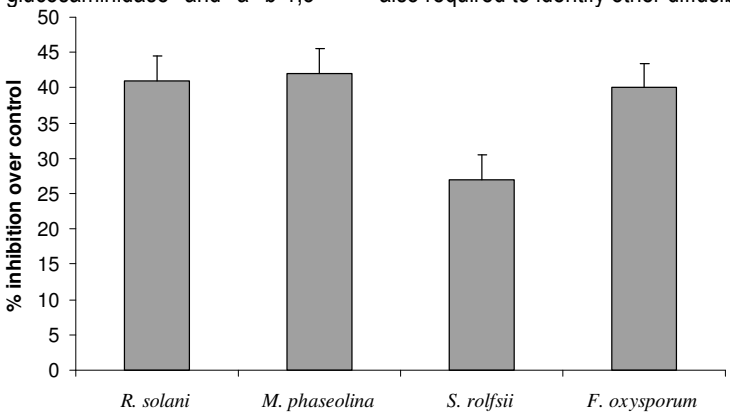


Fig 3. Siderophore production by *B. megaterium* var. *phosphaticum*

***In vitro* antagonistic activity against selected pathogens**

B. megaterium var. *phosphaticum* showed better antagonistic activity ranging from 27 to 43% against select phytopathogens (Figure. 4). It's *in vitro* antagonistic activities were recorded as 41, 42, 27 & 40% against *Rhizoctonia solani, Macrophomina phaseolina,*

Sclerotium rolfsii and *Fusarium oxysporum,* respectively. There was a distinct clearing zone between the bacterial and pathogenic fungal colony (Figure. 5), that indicates the inhibition was due to the production of diffusible antifungal compounds. Production of antibiotics, siderphores, HCN, and hydrolytic enzymes like chitinases, proteases, lipases etc., are reported as few diffusible antifungal compounds. *Bacillus subtilis* AF1 has antifungal properties through the secretion of b-1, 4-*N*-acetyl glucosaminidase and a b-1,3-glucanase [54]. It also exhibited moderate production of siderophore and chitinase enzyme (Table 2). As chitinase production is reported to act as good antagonistic agent in the case of *Streptomyces griseus, B. chitinolyticus* and *B. ehimensis* [55], we infer that siderophore and chitinase production by *B. megaterium var. phosphaticum* could also be responsible for its antagonistic activity against select pathogens under *in vitro* condition. Further studies are also required to identify other diffusible antifungal compounds.



Data are means of four replicates; error bars indicate standard deviations.

Fig 4. *In vitro* antagonistic of activity of *B. megaterium* var.*phosphaticum* against select phytopathogens.

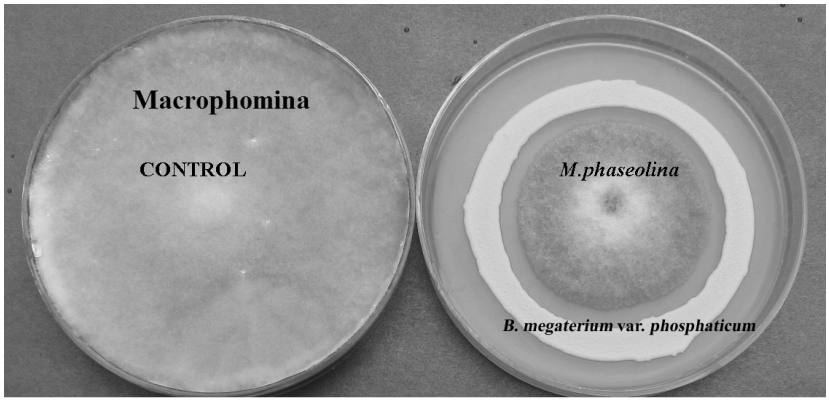


Fig 5. *In vitro* bio-efficacy of *B. megaterium* var. *phosphaticum* against *Macrophomina phaseolina*

Role of *B. megaterium* var. *phosphaticum* in plant growth promotion and nutrient mobilization

The physiochemical properties of the alfisol soil used in this study were pH 7.75; EC: 0.156; organic carbon (C): 0.9%; nitrogen (N) 92.8 kg/ha; phosphorous (P): 23.1 kg/ha; potassium (K): 175.72 kg/ha. It is evident from the table 5 that among all the treatments, T4 (chemical fertilizer 75% + bioinoculant) significantly ($P< 0.05$) improved the plant growth. The T4 treatment showed significant shoot length (32.0 cm), root length (31.66 cm), total dry biomass (0.82 g) and photosynthetic activity (0.64) at 60 DAS when compared with the untreated check. It also improved the macronutrient uptake in plants (Table 4) where N, P and K were recorded as 7.97, 3.41 and 38.12 mg/100 mg of the plant material, respectively. The uptake of micronutrient such as Zn (184ppm), Fe (743 ppm), and Mn (138ppm) were significant in T4 treatment as compared to T3, T2 and T1.

T3 treatment (100% RDF) initially showed significant impact on the growth and development at 30 DAS with 15.0 cm shoot length, 9.16 cm root length and 0.27 g total dry biomass as compared to T4 (chmical fertilizer 75% RDF + Bioinoculant). However, over the period of next 30 days, the plants growth, macro (N 6.57, P 1.88 and K 18.68 mg/100mg) and micro (Zn 149 ppm, Fe 448 ppm and Mn 84 ppm) nutrient uptake was statistically declined as compared to T4. It is evident that the application of bacterial inoculant in combination with chemical fertilizer (75% RDF) resulted better nutritional values in sunflower tissues as compared to sunflower that met its entire nutrient requirement chemical fertilizers (100% RDF).

Zinc plays an important role in many biochemical reactions within the plants. Plants show reduced photosynthetic carbon metabolism due to zinc deficiency. It helps in the formation of chlorophyll and carbohydrates and biosynthesis of cytochrome and synthesis of leaf cuticle. In this study, the zinc solubilising potential of *B. megaterium* var. *phosphaticum* also correlated with the significant increase in photosynthetic activity (0.64) in T4, T3 (0.68) and T2 (0.59) as compared to T1 (0.55) (Table 3).

Table 3. Plant growth promotion by *Bacillus megaterium* var. *phosphaticum* in sunflower

Treatments	Shoot length (cm)		Root length (cm)		Total dry biomass (g)		Photosynthetic activity on 60 DAS
	30th DAS	60th DAS	30th DAS	60th DAS	30th DAS	60th DAS	
Control (T1)	10.41^{c} (±1.22)	26.41^{ab} (±2.29)	7.58^{b}(±0.82)	18.58^{c} (±3.02)	0.23^{b} (±0.01)	0.53^{c} (±0.01)	0.55^{c} (±0.01)
Bioinoculant (T2)	12.83^{b} (±0.60)	24.5^{ab} (±1.89)	7.58^{b}(±1.01)	24.16^{ab} (±2.88)	0.24^{b} (±0.01)	0.54^{c} (±0.01)	0.59^{b} (±0.02)
100% Inorganic fertilizer (T3)	15.0^{a} (±0.88)	28.33^{a} (±3.33)	9.16^{a}(±0.40)	22.5^{b} (±1.96)	0.27^{a} (±0.01)	0.70^{b} (±0.01)	0.63^{a} (±0.02)
75% Inorganic fertilizer + Bioinoculant (T4)	15.41^{a} (±1.06)	32.0^{a} (±3.89)	11.0^{a}(±1.76)	31.66^{a} (±3.45)	0.27^{a} (±0.01)	0.82^{a} (±0.01)	0.64^{a} (±0.01)
LSD	2.12	4.52	2.23	6.48	0.02	0.02	0.04
CV%	18.7	21.07	29.72	30.58	15.38	18.42	7.84

Values superscripted by the same alphabet are not significantly different according to Fisher's least significance difference test (P< 0.05)
Parameters were recorded as 60 days after sowing

Table 4. Macro and micronutrient uptake pattern in plant at 60 DAS

Treatment	Macronutrients (mg)/ 100 mg of dry plant material			Micronutrients in PPM		
	Nitrogen	Phosphorus	Potash	Zn	Fe	Mn
Control	5.71^{c}(±0.103)	1.68^{c}(±0.083)	15.10^{c}(±0.108)	138^{b}(±13.4)	443^{c}(±12.97)	31^{c}(±3.32)
Bioinoculant	6.72^{b}(±0.083)	2.13 ^{b}(±0.082)	18.82^{b}(±0.092)	160^{b}(±12.8)	622^{b}(±19.02)	91^{b}(±8.6)
Inorganic	6.57^{b}(±0.071)	1.88^{c} (±0.071)	18.68^{b}(±0.087)	149^{b}(±12.1)	448^{c}(±13.2)	84^{b}(±5.4)
Inorganic + Bioinoculant	7.97^{a}(±0.092)	3.41^{a}(±0.097)	38.12^{a}(±0.116)	184^{a}(±13.9)	743^{a}(±26.4)	138^{a}(±11.75)
LSD	0.82	0.40	6.8	23.67	52.65	35.23
CV%	21.2	30.3	32.6	23.6	25.3	28.2

Values superscripted by the same alphabet are not significantly different according to Fisher's least significance difference test (P< 0.05)

It is evident that the enhancement in plant growth attributes to the application of NPK and Zn @ 75 % RDF along with bioinoculant, *B. megaterium* var. *phosphaticum*. Similar kind of growth and development was recorded on array of crops [56-58]. Dhale et al [59] also reported that *Azospirillum,* phosphate solubilising bacteria and pink coloured facultative methylotrops along with chemical fertilizers resulted significant increase in shoot length of cotton. Growth enhancement by *Bacillus* may be associated to its ability to produce hormone, especially IAA [60] and siderophore [61, 62]. It is also known that P availability in soils is important for the uptake of N from soils and its utilization in plant [63]. Hence, higher available P due to the solubilization with inoculated *B. megaterium* var. *phosphaticum* might cause an enhancement of N uptake (Table 4).

Combinations of effective microbes like *B. megaterium* var. *phosphaticum* with 25% reduction in chemical fertilizers give immense benefit to the test crop, *H. annus*. These results shall attract the policy makers across the globe for the integration of effective microbes with reduced fertilizer dose as the chemical fertilizers are made from non-renewable energy sources.

CONCLUSIONS

The *in vitro* and *in vivo* studies indicated that *B. megaterium* var. *phosphaticum* is capable of reducing 25% of chemical fertilizer input in *Helianthus annus* by improving phosphate, potash, Zn, Fe, Mn and nitrogen uptakes. The study concludes that *B. megaterium* var. *phosphaticum* is not only a phosphate solubilizer as it was reported by various researchers, but also proved to solubilise K, Zn, Fe and Mn and found to have antagonistic activity against select phytopathogens *viz*., *Sclerotium rolfsii, Rhizoctonia solani, Fusarium oxysporum* and *Macrophomina phaseolina*. These findings are unique and first of their kinds reported in this research article.

REFERENCES

[1] Cooper R, 1959. Bacterial fertilizers in Soviet Union, *Soil fert,* 22:327-333

[2] Menkina R.A. 1963. Bacterial fertilizers and their importance for agricultural plants, *microbiologia.* 33:352–358.

[3] Ankit Kumar., Anil Prakash., B.N. Johri. 2011. *Bacillus* as PGPR in Crop Ecosystem. Bacteria in Agrobiology: *Crop Ecosystems,* DOI 10.1007/978-3-642-18357-7-2.

[4] Cakmakci R., F. Kantar., O.F. Algur. 1999. Sugar beet barley yields in relation to *Bacillus polymyxa* and *Bacillus megaterium* var. *phospaticum* inoculation. *J. Plant Nutrition Soil Science.* 162: 437–442.

[5] Salantur A., A. Ozturk., S. Akten., F. Sahin., F. Donmez. 2005. Effect of inoculation with non-indigenous and indigenous rhizobacteria of Erzurum (Turkey) origin on growth and yield of spring barley. *Plant and Soil*. 275: 147-156.

[6] Comakli B., M. Dasci. 2009. Effects of biofertilizer, cowpat ash and phosphorus and seed yield of alfalfa. *Asian Journal of Chemistry.* 21: 689-696.

[7] Holl F.B., C.P. Chanway., R. Turkington., R.A. Radley. 1988. Response of crested wheatgrass (*Agropyron cristatum* L.), perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) to inoculation with *Bacillus polymyxa*. *Soil Biol. Biochem.* 20: 19-24.

[8] Elkoca E., F. Kantar., F. Ahin. 2008. Influence of nitrogen and phosphorus solubilizing bacteria on the nodulation, plant growth and yield of chickpea. *Journal of Plant Nutrition.* 31: 157–171.

[9] Bottini R., F. Cassan., P. Picolli. 2004. Gibberellin production by bacteria and its involvement in plant growth promotion. *Appl Microbiol Biotechnol.* 65:497–503.

[10] Bloemberg G.V., B.F.J. Lugtenberg. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr Opin Plant Biol.* 4:343–350.

[11] Weller DM. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu Rev Phytopathol.* 26:379–407.

[12] Podile A.R., H.C. Dube. 1998. Plant growth-promoting activity of *Bacillus subtilis* strain AF1. *Curr Sci.* 57:183–186.

[13] Richardson A.E., J.M. Barea., A.M. McNeill., C. Prigent-Combaret. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil.* 321:305–339.

[14] Idris E.E.S., D.J. Iglesias., M. Talon., R. Borriss. 2007. Tryptophan-dependent production of Indole-3- Acetic Acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol Plant Microbe Interact.,* 20:619–626.

[15] Gutierrez-Manero F.J., B. Ramos., A. Probanza., J. Mehouachi., M. Talon. 2001. The plant growth promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberelins. *Physiol Plant.* 111:206–211.

[16] Whipps J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot.* 52:487–512.

[17] Lach D., V.K. Sharma., P.S. Vary. 1990. Isolation and characterization of a unique division mutant of *Bacillus megaterium*. *J. Gen. Microbiol.* 3: 545-553.

[18] Vazquez P., G. Holguin., M.E. Puente., A. Lopez-Cortes., Y. Bashan. 2000. Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biology and Fertility of Soils.* 30:460-468.

[19] Kucey R. M. N. 1983. Phosphate-solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. *Can. J. Soil Sci*. 63: 671-678.

[20] Roos W., M. Luckner. 1984. Relationship between proton extrusion and fluxes of ammonium ions and organic acids in *Penicillium cyclopium*. *Journal of Genera Microbiology*. 130:1007-1014.

[21] Abd-Alla M.H. 1994. Use of organic phosphorus by *Rhizobium leguminosarum* biovar, *viceae* phosphatases. *Biol. Fertil. Soils.* 18:216-218.

[22] Illmer, P., A. Barbato and F. Schinner, 1995. Solubilization of hardly soluble AlPO4 with P– solubilizing microorganisms. *Soil Biol. Biochem.* 27: 260–70.

[23] Asea P. E. A., R. M. N. Kucey., J. W. B. Stewart. 1988. Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. *Soil Bid Biochem*. Vol. 20, No. 4,459-464.

[24] Whitelaw M. A. 2000. Growth promotion of plants inoculated with phosphate solubilizing fungi. *Adv. Agron.* 69:99-151.

[25] Stevenson F.J. 1986. Cycles of soil (carbon, nitrogen, phosphorus, sulfur, micronutrients). *John Wiley and Sons New York*: 231-284.

[26] Han H.S., K.D. Supanjani Lee. 2006. Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. *Plant soil and Environment.* 52 (Suppl 3): 130–136.

[27] Supanjani Han H.S., J.S. Jung., K.D. Lee. 2006. Rock phosphate-potassium and rock-solubilising bacteria as alternative, sustainable fertilisers. *Agronomy for Sustainable Development.,* 26 (Suppl 4): 233-240.

[28] Pikovskava R. 1948. Mobilization of P in soil in connection with vital activity by some microbial species. *Microbiologica.* 17: 362- 370.

[29] Xiufang Hu, Jishuang Chen., Jiangfeng Guo. 2006. Two phosphate- and potassium-solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. *World Journal of Microbiology & Biotechnology.* 22: 983–990.

[30] Bunt J.S., A.D. Rovira. 1955. Microbiological studies of some subantartic soils. *J. Soil Sci.* 6: 119-128.

[31] Olsen S.R., C.V. Cole., F. S. Watanabe., L. A. Dean. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. USDA Circular 939.

[32] Schwyn B., J.B. Neilands. 1987. Universal Chemical Assay for the Detection and Determination of Siderophores. *Analytical Biochemistry*. 160: 47-56.

[33] Christensen G.D., A.L. Bisno., W.A. Simpsom., E.H. Beachey. 1982. Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun*. 37:318-326.

[34] Bentley J.A. 1962. Analysis of plant hormones. Methods Biochem Anal. 9:75-124.

[35] MacMilan J., P. J. Suter. 1963. Thin layer chromatography of the gibberellins. *Nature* (London) 97: 790.

[36] Kaur R, Singh RS, Alabouvette C. 2007. Antagonistic activity of selected isolates of fluorescent *Pseudomonas* against *Fusarium oxysporum* f. sp. *ciceri*. *Asian J. of Plant Sci.* 6(3): 446-454.

[37] Tandon H.L.S. 1993. Methods of analysis of soil, plants, waters and fertilizers. Fertilizer development and consultation organization, New Delhi, India, pp. 144.

[38] Sayer J.A., G.M. Gadd. 1997. Solubilization and transformation of insoluble metal compounds to insoluble metal oxalates by *Aspergillus niger*. *Mycol. Res.,* 101: 653-651.

[39] Nahas E. 1996. Factors determining rock phosphate solubilization by microorganisms isolated from soil. *World J. Microbiol. Biotechnol. 12*: 567-572.

[40] Rodriguez H., F. Reynaldo. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv*. 17: 319-339.

[41] Seshadre S., R. Muthukumarasamy., C. Lakshminarasimhan., S. Ignaacimuthu. 2002. Solubilization of inorganic phosphates by *Azospirillum halopraeferans*. *Curr. Sci.,* 79(5): 565-567.

[42] Simine C.D.D., J.A. Sayer., G.M. Gadd. 1998. Solubilization of zinc phosphate by a strain of *Pseudomonas fluorescens* isolated form a forest soil. *Biol. Fertil. Soils.* 28: 87-94.

[43] Nguyen C., W. Yan., F. Le Tacon., F. Lapayrc. 1992. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Larcaria bicolor* (Maire) PD. *Orton. Plant and Soil,* 143:193-199.

[44] Gupta R., R. Singal., A. Shankar., R.C Kuhad., R.K. Saxena. 1994. A modified plate assay for screening phosphate solubilizing microorganisms. *J. Gen. Appl. Microbiol.* 40: 255-260.

[45] Lal L. 2002. Phosphatic biofertilizers. Agrotech, Publ. Academy., Udaipur, India, 224.

[46] Fowler, T. A., and F. K. Crundwell. 1998. Leaching of zinc sulfide by *Thiobacillus ferrooxidans*: experiments with a controlled redox potential indicate no direct bacterial mechanism. *Appl. Environ. Microbiol.* 64:3570–3575.

[47] Da Costa A.C.A., F.P. Duta. 2001. Bioaccumulation of copper, zinc, cadmium and lead by *Bacillus* sp., *Bacillus cereus*, *Bacillus sphaericus* and *Bacillus subtilis*. *Braz. J. Microbiol.* 32: 1-5.

[48] Davey M. E., G.O. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64:847–867.

[49] Wen C.M., C.S. Tseng., C.Y. Cheng., Y.K. Li. 2002. Purification, characterization and cloning of a chitinases from *Bacillus* sp. NCTU2. *Biotechnol. Appl. Biochem*. 35: 213-219.

[50] Chang W.T., Y.C. Chen., C.L. Jao. 2007. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. *Bioresour. Technol.* 98: 1224-1230.

[51] Glick B.R., C.L. Patten., G. Holguin., D.M. Penrose. 1999. Biochemical and genetic mechanisms used by plant growth-promoting bacteria. Imperial College Press, London.

[52] Ahmad F., I. Ahmad., M.S. Khan 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol Res.* 1631:73–181.

[53] Ryu R., C.L. Patten. 2008. Aromatic amino acid-dependent expression of indole-3-pyruvate decarboxylase is regulated by 4 TyrR in *Enterobacter cloacae* UW5. *American Society for Microbiology*. 190 (Suppl 21): 1-35.

[54] Manjula K., A.R. Podile. 2005. Production of fungal cell wall degrading enzymes by a biocontrol strain of *Bacillus subtilis* AF 1. *Ind J Exp Biol*. 43: 892–896.

[55] Hoster F., J.E. Schmitz., R. Daniel. 2005. Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. *Appl Microbiol Biotechnol*. 66: 434–442.

[56] Jha D., R.S. Mathur. 1993. Combined effect of nitrogenous fertilizers and *Azospirillum brasilense* on the yield of nitrogen uptake by pearl millet. *International Journal of Tropical Agriculture.* 11: 31-35.

[57] Hedge D.M., B.S. Dwivedi., S.N. Bahu., Sudhakara. 1999. Bio-fertilizers for cereal production in India. *Indian Journal of agriculture Science*. 69: 73-83.

[58] Selvakumari G., M. Basker., D. Jayanthi., K. Mathan. 2000. Effect of integration of fly ash with fertilizers and organic manures on nutrient availability, yield and nutrient uptake of rice. *J. of Soil Sci. the Soc.* 48: 268-278.

[59] Dhale D. A., S. N. Chatte., V. T. Jadhav. 2011. Response of bioinoculants on growth, yield and fiber quality of cotton under irrigation. *Agric. Biol. J. N. Am.* 2(2): 376-386.

[60] Sheng X.F., W.Y. Huang. 2001. Physiological characteristics of strain NBT of silicate bacterium. *Acta Pedol. Sin.* 38: 569–574.

[61] Ito T. 1993. Enzymatic determination of itoic acid, a *Bacillus subtilis* siderophore and 2, 3-dihydroxybenzoic acid. *Appl. Environ. Microbiol. 59*: 2343– 2345.

[62] Hu X., G.L. Boyer. 1996. Siderophore-mediated aluminium uptake by *Bacillus megatherium* ATCC 19213. *Appl. Environ. Microbiol.* 62: 4044–4048.

[63] Kim T., W. Jung. B. Lee., T. Yoneyama., H. Kim., K. Kim. 2003. P effects on N uptake and remobilization during regrowth of Italian ryegrass (*Lolium mutiflorum*). *Environ. Exp. Bot.* 50: 233–242.