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Effect of inoculation of *Bacillus megaterium* isolates on growth, biomass and nutrient content of *Peppermint*

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Abstract

The investigation was carried out to study the effect of *Bacillus megaterium* isolates on growth, biomass and nutrient content of *Pepper mint*. *Pepper mint* was used as a host plant to study the growth response, biomass and nutrient content. *Bacillus megaterium* strains from different soil types of various agroclimatic zones were isolated, identified and confirmed using standard synaptic keys. A pot culture experiment was carried out to find out the influence of *Bacillus megaterium* isolates on seedlings of *Pepper mint* under greenhouse conditions. *Pepper mint* plants inoculated with *Bacillus megaterium* isolates manifested increase in plant height, number of leaves, number of branches, biomass and nutrient content compared to uninoculated plants. Among the ten isolates inoculated, the *Bacillus megaterium* isolates from zone 3 showed significantly high values in almost all growth parameters chosen for the study. Similarly biochemical parameters of the *Pepper mint* inoculated with ten isolates of *Bacillus megaterium* was studied. In the inoculated plants the biochemical parameters like chlorophyll content, nitrogen content and phosphorus content was higher as compared to uninoculated plants. The results suggests that plants inoculated with *Bacillus megaterium* isolates showed better growth response, biomass yield and nutrient content when compared to uninoculated plants. Also the studies showed that Zone 3 isolate was more effective among other isolates.

Keywords: *Bacillus megaterium*, *Pepper mint,* growth response, phosphorous content and chlorophyll content.

INTRODUCTION

Phosphorus is one of the 17 chemical elements required for plant growth and reproduction and is often referred to as the "energizer" since it helps store and transfer energy during photosynthesis. It is applied to the soil in the form of phosphatic fertilizers. Kucy and coworkers showed that the occurrence and distribution of phosphate solubilizing microorganisms (PSM) have been found in almost all the soils tested, although their populations vary with different soils, climate and cropping history [1].

Phosphorous solubilizing micro organisms are ubiquitous in soils and could play an important role in supplying P to the plants where plant available P content in soil is less. Approximately 95–99% of the soil phosphorus is present in the form of insoluble phosphates and hence cannot be utilized by the plants. According to Bryan, in order to increase the availability of phosphorus for plants, large amounts of fertilizer are used on a regular basis [2]. Soils having high pH have the problem of phosphorous availability for plants. In such a situation phosphate solubilizing microorganisms can be useful to reverse the process. Plant growth promoting rhizobacteria (PGPR) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms [3]. Microorganisms are involved in a range of processes that affect the transformation of soil P and thus are integral part of the soil P cycle. In particular soils, microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization [4]. Azcon and coworkers showed that the phosphate solubilizing microorganisms had got considerable synergistic effect on growth and development of crop plants [5].

Use of Phoshate Solubilizing Bacteria (PSBs) as bioinoculants will increase the available P in soil, helps to minimize the P-fertilizer application, reduces environmental pollution and promotes sustainable agriculture. PSM is a group of heterotrophic microorganism capable of solubilizing the inorganic P from insoluble sources. Application of Phosphate Solubilizing Microorganism (PSMs) in the field has been reported to increase crop yield. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by PSB [6]. Phosphate Solubilizing Bacteria are useful for all the crops i.e. Cereals, cash crops, leguminous crops, horticultural crops and vegetables etc.

Bacillus Megaterium is a Gram Positive, Rod Shaped Endospore-Forming Bacteria. It is a P solubilizing bacteria which has got PGPR activity also. The plant growth promoting rhizobacteria have capability of solubilising the insoluble phosphate in the soil and make them available to the plants.

Peppermint is a hybrid mint, a cross between the watermint (*Mentha aquatica*) and spearmint (*Mentha spicata*). Peppermint typically occurs in moist habitats, including stream sides and drainage ditches. Being a hybrid, it is usually sterile, producing no seeds and reproducing only vegetatively, spreading by its rhizomes.

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If placed, it can grow anywhere, with a few exceptions. The aroma of peppermint has been found to enhance memory. As such, it can be administered by instructors to their students before examinations, to aid recall. It is a herbaceous rhizomatous perennial plant growing to 30–90 cm (12–35 in) tall, with smooth stems, square in cross section. The rhizomes are wide-spreading, fleshy, and bare fibrous roots. The leaves are from 4–9 cm (1.6–3.5 in) long and 1.5–4 cm (0.59–1.6 in) cm broad, dark green with reddish veins, and with an acute apex and coarsely toothed margins. The leaves and stems are usually slightly hairy. Flowering is from mid summer to late summer.

In the present study isolation and identification of *Bacillus Megaterium* from different soil types of various agroclimatic zones of Karnataka was carried out. Growth response, biomass and nutrient content of *Peppermint* inoculated with *Bacillus Megaterium* isolated from different soil types of various agroclimatic zones of Karnataka was studied under green house conditions.

MATERIALS AND METHODS

The experiments to study "Effect of inoculation of *Bacillus megaterium* isolates on growth, biomass and nutrient content of *Peppermint*" were conducted at the Department of Plant Biotechnology, University of Agricultural Sciences, G.K.V.K. Campus, Bangalore, Karnataka, India. The material used and methods followed are described below.

Collection of soil samples from different agro climatic zones of Karnataka

Karnataka state is divided into ten agro climatic zones on the basis of annual rainfall, soil type, cropping pattern and other climatic conditions.

Soil sampling

Four soil samples of 500 grams each were collected randomly from top six-inch layer of soil from each agro climatic zone and packed in polyethylene bag. They were transferred to Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, for further studies.

Processing of soil samples

Soil samples collected from each zone were dried inside the laboratory at 28^{o} C. Four soil samples collected from each zone were mixed well to get a pooled soil sample for a zone. Totally ten soil samples was obtained for the study. Each soil sample was sieved through 1000µ mesh to remove the bigger soil particles and debris. The sieved soil samples were used for the spore isolation.

Isolation of *Bacillus megaterium* from different zones

Bacillus megaterium was isolated from the soils collected from different zones, by growing in glucose mineral agar media. For isolation, cell material was checked microscopically for the presence of typical cells of *Bacillus megaterium* and purified on nutrient agar. *Bacillus megaterium* was isolated by heating the soil sample to kill non spore forming mesophiles, then the dilutions were made upto 10^{-3} and 10^{-4}. It was then plated on glucose mineral base agar medium. It was incubated at 30^{0}C for 2 days. Then all the isolates were subjected to various tests for confirming their identity.

Identification of *Bacillus megaterium*

The different tests for identification were carried as per Bergy's manual of Determinative Bacteriology.

Colony morphology and microscopic examination

All the check isolates and standard strains formed completely white, round, smooth and shiny colonies. During microscopic examination all the isolates were found to be gram positive rods. Presence of endospores was confirmed by endospore staining.

Physiological tests for *Bacillus megaterium*

All the physiological tests mentioned were conducted in duplicate for each isolate.

Gelatin liquefaction

Gelatin liquefaction test was performed according to the method described by Blazevic and Ederer [7]. Petriplates containing gelatin agar were spotted with overnight grown bacterial culture at 30^{0}C and incubated for 3 days. The plates were then flooded with 12% $HgCl_2$ solution and allowed to stand for 20 minutes and observed for clear zones around the growth of bacterium to indicate gelatin liquefaction.

Hydrolysis of starch

Hydrolysis of starch was performed according to the method described by Eekford [8]. Starch agar was prepared by spending 1gm of starch powder in 10 ml cold distilled water mixed with 90 ml of nutrient agar and autoclaved at 121^{0}C for 20 minutes. Petriplates containing starch agar were inoculated with test cultures and incubated at 30^{0}C for 3 days. After incubation the plates were flooded with Lugol's iodine, allowed to stand for 15 – 30 minutes and observed for clear zones around the colony to indicate hydrolysis of starch.

Casein hydrolysis

Casein hydrolysis was performed according to the method described by Seely and Vandemark [9]. Petriplates of skim milk agar was streaked with test cultures and incubated at 30^{0}C for observing clear zone against black background.

Acid gas production

Acid gas production test was performed according to the method described by Seely and Vandemark [10]. Bacterial isolates were tested for acid and gas production by inoculating 5 ml of the sterile glucose broth with bromocresol purple (15 ml /l of 0.04% solution as pH indicator) in test tubes containing Durham's tube. The tubes were incubated for seven days at 30^{0}C. Accumulation of gas in these Durham's tube was taken positive for gas production and change in colour of the medium to yellow was taken as positive for acid production.

Catalase test

Catalase test was performed according to the method described by Blazevic and Ederer [11]. Nutrient slants were incubated at 30^0C for 24 hrs. After incubation these tubes were flooded with 1ml of 3% H_2O_2 and observed for gas bubbles. Occurrence of gas bubbles was scored positive for catalase test.

V-P Reaction

Sterilized V-P broth was dispensed in test tubes tested for acetyl methyl carbinol production after incubation for 3,5,7 days by mixing 3 ml of 40%(w/v) NaOH with cultures and adding 0.5 mg of creatine.The tubes were observed for the production of red colour after 30 minutes at room temperature.

Solubilization of insoluble phosphate by *Bacillus megaterium* in Sperber's medium

The overnight cultures of the *Bacillus megaterium* isolates were spotted on sperber's medium to observe the zone of solubilization by the isolates. The plates were incubated at 30^0C for 36 hrs, observed and measured the zone of solubilization produced by these isolates.

Inoculum preparation

The isolated colonies of *Bacillus megaterium* maintained on the Nutrient agar slants was inoculated in 250 ml conical flask containing 100 ml Nutrient broth and incubated at 30^0C under shaking at 100 rpm for six days. The grown cultures were homogenized and 25ml each culture ($10.1x10^8$cfu/ml) inoculated to each pot.

Pot Experiment

Bacillus megaterium isolates were grown separately in a 250 ml flask containing 100ml nutrient agar for 2 days. The grown cultures were homogenized and 25 ml of each of the solution was given to each pot. The geographical area of Karnataka is classified into ten agro-climatic zones *viz.,* North eastern transition zone (NETA-1), North eastern dry zone (NEDA-1), Northern dry zone (NDA-1), Central dry zone (CDA-1), Eastern dry zone (EDA-1), Southern dry zone (SDA-1), Southern transition zone (STA-1), Northern transition zone (NTA-1), Hilly zone (HA-1) and Coastal zone (CA-1). C - Control (uninoculated plant), T_1– *Bacillus megaterium* isolate from Zone 1, T_2 - *Bacillus megaterium* isolate from Zone 2, T_3 - *Bacillus megaterium* isolate from Zone 3, T_4 - *Bacillus megaterium* isolate from Zone 4, T_5 - *Bacillus megaterium* isolate from Zone 5, T_6 - *Bacillus megaterium* isolate from Zone 6, T_7 - *Bacillus megaterium* isolate from Zone 7, T_8 - *Bacillus megaterium* isolate from Zone 8, T_9- *Bacillus megaterium* isolate from Zone 9, T_{10}- *Bacillus megaterium* isolate from Zone 10.

Effect of *Bacillus megaterium* on growth of *Pepper mint*
Plant growth parameters

The observations with respect to the growth parameters including plant height, number of leaves, number of branches, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, and Nitrogen, phosphorus and chlorophyll content were recorded at different periodical intervals till vegetative stage. However the data presented here is only 60 days after treatment (DAT).

The plant height was measured from the soil surface to the tip of the growing point at 60 DAT. The numbers of fully opened leaves were recorded at 60 DAT. The harvested plants were weighed and then the root fresh weight was recorded and expressed as grams per plant. The harvested roots were dried in an oven at 60^0C for 2 days to attain constant weight and then the root dry weight was recorded and expressed as grams per plant. The harvested plants were weighed and then the shoot fresh weight was recorded and expressed as grams per plant. The harvested plants were dried in an oven at 60^0C for 4 days to attain constant weight and then the dry weight was recorded and expressed as grams per plant.

Biochemical studies of plants inoculated with *Bacillus megaterium*

The nitrogen estimation for root and shoot was carried out by Micro–Kjeldahl method [12]. Plant phosphorus concentration was estimated colorimetrically following the vanadomolybdate yellow colour method. Total chlorophyll content of the leaf was estimated following DMSO method [13]. The data obtained from the experiments were subjected to one-way analysis of variance for completely randomized design (CRD) using MSTAT-C software. The treatment means were separated by Duncan's Multiple Range test (DMRT) a 5% level of significance [14].

RESULTS AND DISCUSSION

The experiments were conducted at the Department of Plant Biotechnology, University of Agricultural Sciences, G.K.V.K. Campus, Bangalore. The material used and methods followed are described below

Isolation and identification of Bacillus megaterium

The bacterial cultures were isolated from ten agro climatic zones of Karnataka. The cell material was checked microscopically for the presence of typical cells of *Bacillus megaterium* and purified on nutrient agar plates. Then all the isolates were subjected to various tests for confirming their identity.

Colony morphology and microscopic examination

All the check isolates and standard strains formed completely white, round, smooth and shiny colonies. During microscopic observation all the isolates were found to be gram positive rods. Presence of endospores was confirmed by endospore staining. Then all the isolates were subjected to various tests for confirming their identity [15].

Physiological tests

All the physiological tests performed are presented in the table 1.

Phosphate solubilising efficiency of different isolates of Bacillius megaterium

Phosphate solubilizing microorganisms are ubiquitous in nature, their number vary with type of soil, climate, vegetation etc

[16]. In the present investigation an attempt has been made to isolate *Bacillus megaterium,* a phosphate solubilizer and also a PGPR, from different soil types present in various agroclimatic soils of Karnataka. The efficiency of P solubilization by *Bacillus magaterium* was studied in green house experiment using all *Bacillus megaterium* isolates to make a preliminary selection of efficient isolate using *Pepper mint* as indicator plant and the strains were designated as better based on the zone of solubilization. Good P solubilizing zone is an indication of utilization of insoluble Phosphorus and also PGPR activity there by improving the plant growth.

Table 1. Physiological Tests for *Bacillus megaterium* isolates

Zones	VP Test	Anaerobic Growth	Acid from Glucose	Gas from glucose	Hydrolysis of casein	Hydrolysis of starch	Hydrolysis of gelatin	Production of solubilising zones in sperbers media
C	-	-	+	-	-	-	+	+
T_1	-	-	+	-	-	-	+	+
T_2	-	-	+	-	-	-	+	+
T_3	-	-	+	-	-	-	+	+
T_4	-	-	+	-	-	-	+	+
T_5	-	-	+	-	-	-	+	+
T_6	-	-	+	-	-	-	+	+
T_7	-	-	+	-	-	-	+	+
T_8	-	-	+	-	-	-	+	+
T_9	-	-	+	-	-	-	+	+
T_{10}	-	-	+	-	-	-	+	+

+ = Positive and **-** = Negative

The Phosphate solubilising efficiency of different isolates of *Bacillius megaterium* was tested on modified Sperber's medium. All the isolates found to have good solubilising ability. Zone 7, Zone 3 and Zone 4 showed very good solubilising ability in Sperber's media. A study showed that the mechanism of P solubilization is due to production of several organic acids and also several workers isolated and enumerated P solubilizers based on P solubilizing zone with different sources of insoluble Phosphorous [17].

Response of *Pepper mint* to inoculation of Bacillus megaterium isolates Sand: soil mixture in the ratio of 1:1 v/v was filled into pots of uniform size. Planting holes were made at the centre of the pots to enable the inoculation of *Bacillius megaterium* isolates and 25ml inoculum representing each zone *Bacillius megaterium* isolate was separately added to the pot as per the treatment allocation. The plant height, number of leaves and number of branches is presented in table 2.

Table 2- Growth parameters of *Peppermint* influenced by *Bacillus megaterium* isolates

Zones	Plant height(cm) 60 DAT	Number of Leaves/plant 60 DAT	Number of Branches (cm) 60 DAT	Fresh weight (g/plant) 60 DAT			Dry weight (g/plant) 60 DAT		
				Shoot	Root	Total	Shoot	Root	Total
C	20.22	38	20	3.70	3.10	6.80	0.20	0.30	0.50
T_1	33.23	79	32	6.20	5.77	11.97	0.69	0.53	1.22
T_2	37.45	74	33	6.23	5.85	12.08	0.77	0.79	1.56
T_3	46.32	99	36	6.77	5.80	12.57	0.98	0.91	1.89
T_4	44.24	96	39	6.66	5.66	12.32	0.75	0.77	1.52
T_5	30.33	69	28	5.98	5.14	11.12	0.63	0.55	1.18
T_6	33.12	71	29	6.28	5.33	11.61	0.72	0.70	1.42
T_7	34.56	73	31	6.10	5.32	11.42	0.66	0.60	1.26
T_8	29.41	59	30	5.87	5.20	11.07	0.71	0.66	1.37
T_9	39.12	72	29	6.10	5.30	11.40	0.75	0.54	1.29
T_{10}	37.11	68	31	6.40	5.50	11.90	0.80	0.71	1.51
SEM±	0.114	1.278	0.811	0.153	0.048	0.089	0.036	0.025	0.026
CD at 5%	0.336	3.771	2.392	0.453	0.142	0.263	0.107	0.076	0.080

DAT: Days after treatment T_1 to T_{10}: Treatments for isolates from zone 1 to 10

Screening studies were conducted using *Pepper mint* grown in pots under green house conditions. Studies revealed that in general the plants inoculated with *Bacillus megaterium* isolates showed higher growth when compared to uninoculated control. Zopade [18] reported that increase in plant growth by inoculation with biofertilizers might be due to micro-element and plant growth regulator content in the biofertilizers. Similar results were reported by [19] several workers. They tested the PSM and found a zone of clearance of tricalcium phosphate around the growth of the colony in Pikovaskya's solid medium. The height of the inoculated plants remained always greater than the uninoculated plants. However, the plants differed significantly in height in response to some isolates within the treatments but the highest was seen in zone 3 isolate. The least plant height was recorded in control. The *Pepper mint* at 60th DAT the maximum plant height (46.32 cm) was recorded in zone-3 isolate treated plants. The increased height in the inoculated plants

may be due to the increased nutrient uptake and PGPR activity [20]. The lowest plant height (20.22 cm) in *Pepper mint* was recorded at 60th DAT in control. The *Pepper mint* at 60th DAT the maximum number of leaves (99/plant) was recorded in zone-3 isolate treated plants. The lowest number of leaves (38/plant) in *Pepper mint* was recorded at 60th DAT in control. In *Pepper mint* at 60th DAT the maximum number of branches (36/plant) was recorded in zone-3 isolate treated plants and the lowest number of branches (20/plant) was recorded at 60th DAT in control plant. Similar results were obtained when combined inoculation of Rhizobium, a phosphate solubilizing *Bacillus megaterium sub sp. phospaticum* strain-PB and a biocontrol fungus *Trichoderma spp*. showed increased germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea compared to either individual inoculations or an uninoculated control in chickpea as per the studies conducted by Rudresh and coworkers [21] .

The fresh weight and dry weight of the plants harvested at 60 days after treatment are presented in table 2. The fresh weight and dry weight in the plants inoculated with *Bacillus megaterium* isolates were higher than uninoculated plants. The total biomass per plant was significantly influenced by the application of *Bacillus megaterium* isolates in *Pepper mint* which is presented in table 2. In *Pepper mint*, among different treatments, the zone 3 isolate treatment recorded significantly maximum total biomass. The highest fresh weight (12.57 g/plant) and (1.89 g/plant) dry weights was recorded in zone 3 isoate. The lowest total biomass was recorded in control. The lowest fresh weight (6.80 g/plant) and dry weight (0.50 g/plant) was recorded in control plants. Increased biomass may be due to enhanced plant growth, number of leaves and branches, increase in root growth which was influenced probably by greater availability of mineral nutrients, plant growth promoting substances and enzymes. Similar results were obtained on inoculations with *Bacillus spp.* which increased shoot fresh weight with *B. megaterium* [22].

Biochemical studies of *Pepper mint* plants inoculated with *Bacillus megaterium* isolates

The earliest report of increasing P uptake and dry weight of plants through inoculation of phosphate solubilizing organisms was already made [23]. In *Pepper mint*, total phosphorous content of the plants inoculated with *Bacillus megaterium* isolates differed significantly among various isolates. In *Pepper mint* highest phosphorous content(5.99 mg/plant dry wt) was observed in zone 3 isolate and lowest in uninoculated control (2.04 mg/plant dry wt) plants.

Table 3- Biochemical parameters of *Peppermint* influenced by *Bacillus megaterium* isolates

Zones	*Peppermint*	
	Total Nitrogen Content (mg/plant dry wt)	Total Phosphorous Content (mg/plant dry wt)
C	11.08	2.04
T_1	16.98	3.78
T_2	17.07	4.99
T_3	19.70	5.99
T_4	18.66	4.18
T_5	16.67	4.11
T_6	18.00	5.17
T_7	17.90	4.89
T_8	16.09	3.98
T_9	18.54	4.99
T_{10}	15.78	4.84
SEM±	0.183	0.065
CD at 5%	0.541	0.194

T_1 to T_{10}: Treatments for isolates from zone 1 to 10

Table 4. Total Chlorophyll Content of *Peppermint* influenced by *Bacillus megaterium* isolates

Zones	Total Chlorophyll (mg/g fw) of peppermint
C	0.82
T_1	1.35
T_2	1.78
T_3	2.00
T_4	1.90
T_5	1.73
T_6	1.65
T_7	1.60
T_8	1.46
T_9	1.81
T_{10}	1.42
SEM±	0.048
CD at 5%	0.142

T_1 to T_{10}: Treatments for isolates from zone 1 to 10

However the total nitrogen content in *Pepper mint* differed significantly among the plants inoculated with various *B. megaterium* isolates which is presented in table 3. In *Pepper mint* plants inoculated with zone 3 isolate recorded the highest (19.70 mg/plant dry wt) nitrogen content. The lowest nitrogen content (11.08 mg/plant dry wt) recorded in uninoculated control plants. A similar result was

observed when wheat inoculated with mixed inocula of *B. megaterium* and *A. lipoferum* exhibited high shoot dry weight, total nitrogen (N) yield and the shoot phosphorus content increased by 37 and 53 % compared to the plants inoculated with *A. lipoferum* and uninoculated ones, used as control, respectively [24].

However the chlorophyll content in *Pepper mint* differed significantly among the plants inoculated with various *B. megaterium* isolates which is presented in table 4. In *Pepper mint* highest total chlorophyll content (2.00 mg/g fw) of chlorophyll was recorded in plants inoculated with zone 3 and lowest total chlorophyll content was recorded in uninoculated control plants (0.82 mg/g fw).

Results of the present study revealed enhanced plant growth, biomass and nutrient content of *Pepper mint* due to inoculation with *Bacillus megaterium* strains isolated from different soil types of various agro-climatic zones of Karnataka and the zone 3 isolate was found to be the most efficient strain compared to other strains of *Bacillus megaterium* isolates in promoting plant growth, biomass and nutrient uptake. However, the plants differed significantly in plant growth, biomass and nutrient content in response to some isolates within the treatments but the highest was seen in case of zone 3 isolate. Since, all the isolates belong to the same species of *Bacillus megaterium*, their effect on growth in terms of plant growth, biomass and nutrient content may not be as significant as those usually observed in the plants inoculated with different species or genera or in combination with other beneficial microorganisms.

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