

Survival and Phosphate Solubilizing Ability of *Bacillus megaterium* in Liquid Inoculants under High Temperature and Desiccation Stress

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ABSTRACT

Inoculation of phosphate-solubilizing microorganisms along with rock phosphate is known to enhance the available P from soil. The success of such solubilization is largely dependent on the ability of the inoculant strain to survive under adverse environmental conditions. In this context, liquid inoculants are gaining importance and are becoming popular with longer shelf-life. In the present investigation, a preliminary study was conducted to determine the survival of *Bacillus megaterium* in liquid formulations supplemented with osmo/cell-protectants under the influence of high temperature, desiccation stress and their subsequent influence on P-uptake by cowpea plants. Liquid inoculant-2 containing osmoprotectants viz., polyvinyl pyrrolidone (PVP), high quantity of glycerol (12 ml L⁻¹) and glucose supported higher viable population up to a storage period of four weeks at 48°C (log₁₀ 10.62 CFU ml⁻¹) and desiccation stress (log₁₀ 10.04 CFU ml⁻¹) as compared to liquid inoculant-1 containing osmoprotectants viz., PVP, low quantity of glycerol (1 ml L⁻¹), trehalose, arabinose and FeEDTA; and nutrient glucose broth without any osmoprotectants. Liquid inoculant-2 also enhanced the P-uptake of cowpea plants significantly.

Keywords: High temperature and desiccation stress, Mussoorie rock phosphate, Osmoprotectants, Phosphate-solubilizing bacteria.

INTRODUCTION

Phosphorus (P) is the second key nutrient for plants and affects several characteristics of plant growth. Though P both in organic and inorganic forms are abundant, but due to its ability to form complexes with other soil constituents, it is not easily available for uptake by plants. However, organic farming is gaining popularity where bio-inoculants could play a key role in promoting the growth of plants. India has vast reserves of mussoorie rock phosphate (MRP), which is insoluble. Based on these circumstances, emphasis has been given to develop and use P-solubilizing biofertilizers, which have been found useful in

making P available to plants through solubilization of native P and converting low grade rock phosphate as fertilizer and increasing yield of different crops (Khan *et al.*, 2007; Khan *et al.*, 2010).

In India, presently, P-solubilizing bacterial inoculants are carrier based (Menaka and Alagwadi, 2007). In preparation of these carrier based inoculants, solid carriers like peat and lignite are used. In tropics, due to unavailability of peat, many alternate carriers including lignite (Kandaswamy and Prasad, 1971) vermiculite, compost, bentonite, Kaolinite (Graham-Weiss *et al.*, 1987), charcoal dust (Poi and Ghosh, 1985), perlite (Khavazi and Rejali, 2000) and, more recently, cassava wastes (Ogbo, 2010) have been

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explored. These carrier-based inoculants are inherent with certain constraints like lower shelf-life, poor survival under adverse environmental conditions, high degree of contamination, and inconsistent field performances. There have been many attempts to find alternatives for carrier based inoculants and, also, to enhance viability of microorganisms in the inoculants. Calcium alginate, when used as a carrier, was found superior to the conventional charcoal with soil (3:1) for *Bacillus polymyxa* and *Pseudomonas striata* (Viveganandan and Jauhri, 2000). However, serious constraints to the high efficiency of alginate entrapped soil inoculants are the limitation of activity caused by reduced oxygen transfer into the alginate beads and also application of these inoculants. Because of these constraints in the existing inoculants technology, there is a need to develop an alternate formulation that will support high microbial population.

In contrast, liquid inoculants are known to play an important role and have become popular. Earlier, we developed a liquid inoculant using osmo/cell-protectants for phosphate-solubilizing *B. megaterium* (Sridhar *et al.*, 2004), which supported a higher viable population up to a storage period of six months. In the present study, we made an attempt to determine the survival of phosphate-solubilizing *B. megaterium* in liquid inoculants containing various osmoprotectants under high temperature and desiccation stresses and their subsequent influence on P-uptake by cowpea plants.

MATERIALS AND METHODS

Bacterial Strain and Growth Media

The phosphate solubilizing *B. megaterium* was obtained from the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore. The culture was transferred onto nutrient agar slants, incubated at 30°C for 48 hours, properly packed and stored at 4°C for further studies. The slants were subsequently sub-cultured at

an interval of three weeks. Nutrient broth was used to grow the working culture. The culture was checked for its purity by Gram-staining and also tested for its ability to solubilize insoluble P by growing on Sperber's agar and subsequent formation of solubilization zone (halo) around the bacterial colony.

Preparation of Different Inoculant Formulations

Liquid inoculants 1 and 2 were prepared by weighing the ingredients as published earlier (Sridhar *et al.*, 2004). A lignite inoculant was prepared by employing finely powdered lignite whose pH was adjusted to 6.5-7 by using calcium carbonate (CaCO₃). *B. megaterium* inoculum was added at the rate of 10 ml 250 ml⁻¹ broth for liquid inoculants and 25 ml 50 g⁻¹ for lignite inoculant.

Determination of Viable Counts

The viable count was determined by using drop plate method (Somasegaran and Hoben, 1985). Serially diluted cultures were plated on nutrient agar plates and incubated in invert position for two days. The number of colonies was counted and the number of viable cells in one ml inoculum was calculated by using the following formula:

$$\text{Average number of CFU ml}^{-1} = \frac{(\text{Number of colonies}) \times (\text{Dilution factor})}{(\text{Volume of inoculum})}$$

Survival of *B. megaterium* in Different inoculants under High Temperature Stress

To study the survival of *B. megaterium* under the influence of high temperature, three different liquid inoculants *viz.*, nutrient glucose broth and liquid inoculants 1 and 2 were maintained in four replications for each treatment. In this experiment, a set of liquid inoculants was maintained at room

temperature ($26^{\circ}\pm 3^{\circ}\text{C}$) and another set was exposed to 48°C for 8 hours per day. These inoculants were maintained for four weeks. At weekly intervals, viable counts were determined using drop plate method.

Survival of *B. megaterium* in Different Inoculants under Desiccation Stress

In order to investigate the effect of desiccation on the survival of *B. megaterium*, three different liquid inoculants viz., nutrient glucose broth, liquid inoculants 1 and 2 were employed. In this study, glass beads were coated with the above liquid inoculants separately and were subjected to drying at 45°C for four days. Later on, the coated beads were maintained in 4 replications for four weeks at room temperature ($26^{\circ}\pm 3^{\circ}\text{C}$). At weekly intervals, viable counts were determined using drop plate method by transferring equal number of coated beads into a fresh nutrient broth medium and incubated for 48 hours.

Effect of Mussoorie Rock Phosphate (MRP) and Different Formulations of *B. megaterium* on P-uptake by Cowpea

A pot culture experiment was conducted to study the effect of MRP and different formulations of *B. megaterium* on P-uptake by cowpea plants. Surface soil (alfisol) samples (0-12 cm) was collected from Regional Research Station (RRS), GKVK, University of Agricultural Sciences, Bangalore. The cowpea (*Vigna unguiculata* var. TVX-944) seeds were sterilized by rinsing with alcohol (95%) for one minute, sodium hypochlorite (0.9%) followed by washings with at least six changes of sterile water and, then, pre-germinated on water agar (0.9%) in petri plates. Five pre-germinated seeds were carefully sown in each pot (56 cm width \times 30 cm depth \times 22 cm height) having 3.8 kg of soil that was maintained at field capacity by regular watering. After germination, the number of seedlings was thinned to two plants per pot.

Recommended dosage of N (urea at 32.5 kg ha^{-1}) and K (muriate of potash at 21.38 kg ha^{-1}) were applied with each treatment. The treatments included two levels of P (with and without MRP), four inoculant formulations (nutrient glucose broth, liquid inoculant-1, liquid inoculant-2, and lignite based inoculant) and an un-inoculated control. MRP was added at the rate of 0.5 g pot^{-1} . Pots were arranged on green house bench with three replications of each treatment in randomized complete block design (RCBD) and were grown under natural sunlight and watered regularly. At maximum vegetative phase (48 days after planting), the plants were harvested and their phosphorus contents in shoot and root samples were estimated by the Vanadomolybdo-phosphoric acid method (Black, 1965).

Statistical Analysis

The data was subjected to statistical analysis and treatment means were compared using Duncan's multiple range test (DMRT) (Little and Hills, 1978).

RESULTS

In the present study, the survival of *B. megaterium* in different liquid inoculants was tested under high temperature and desiccation stress. The data presented in Table 1 indicates the population of *B. megaterium* in liquid inoculants stored at room temperature and at 48°C for 28 days (8 hours per day). No significant ($P \leq 0.05$) difference in the population measured at different incubation intervals in nutrient glucose broth was observed when stored at room temperature. But, the bacterial populations declined significantly ($\log_{10} 9.46\text{ CFU ml}^{-1}$) four weeks after a storage at 48°C . No significant difference was observed in the population of *B. megaterium* at different incubation intervals in the liquid inoculant-1 stored at room temperature, but, the population declined ($\log_{10} 10.21\text{ CFU ml}^{-1}$) at 48°C (Table 1). A similar trend was observed when *B.*

**Table 1.** Survival of *B. megaterium* in liquid inoculants under high temperature stress.

S. No.	Inoculants	<i>B. megaterium</i> (\log_{10} CFU ml ⁻¹)																				
		0				7				14				21				28				
		RT ^a		48°C		RT		48°C		RT		48°C		RT		48°C		RT		48°C		
1	Nutrient glucose broth	11.05 ^b	11.09 ^b	11.03 ^b	10.92 ^{ab}	11.00 ^b	10.66 ^c	10.93 ^c	10.20 ^c	10.93 ^{ab}	10.93 ^c	10.20 ^c	10.93 ^{ab}	10.93 ^{ab}	10.93 ^{ab}	10.93 ^{ab}	10.93 ^{ab}	10.93 ^{ab}	10.93 ^{ab}	10.93 ^{ab}	9.46 ^c	
2	Liquid inoculant -1	11.08 ^a	11.10 ^b	11.04 ^b	10.97 ^{ab}	11.00 ^b	10.86 ^b	10.99 ^b	10.61 ^b	10.97 ^{ab}	10.99 ^b	10.61 ^b	10.97 ^{ab}	10.97 ^{ab}	10.97 ^{ab}	10.97 ^{ab}	10.97 ^{ab}	10.97 ^{ab}	10.97 ^{ab}	10.21 ^b	10.21 ^b	
3	Liquid inoculant-2	11.08 ^a	11.14 ^a	11.10 ^a	11.07 ^a	11.08 ^a	10.96 ^a	11.05 ^a	10.78 ^a	11.08 ^a	10.96 ^a	11.05 ^a	10.78 ^a	11.03 ^a	11.03 ^a	11.03 ^a	11.03 ^a	11.03 ^a	11.03 ^a	11.03 ^a	10.62 ^a	10.62 ^a

Each value is an average of four replications.

Values with different superscripts between inoculant formulations within sample interval are statistically significant at $P = 0.05$ by the DMRT.

^a RT is the room temperature (26 \pm 3°C).

megaterium was grown in the liquid inoculant-2. The decline was maximum in the nutrient glucose broth (14.7 %) and the liquid inoculant-1 (8 %) compared to the liquid inoculant-2 (4.7 %).

However, higher viability of bacterial cells was observed in liquid inoculants-2 (\log_{10} 10.62 CFU ml⁻¹) (Table 1) at 48°C when stored for four weeks as compared to the other liquid inoculants.

The data pertaining to survival of *B. megaterium* in different liquid inoculants under desiccation stress monitored for 28 days is presented in Table 2. There was no significant difference in the population of *B. megaterium* grown in liquid inoculant-1 (\log_{10} 11.07 CFU ml⁻¹) and liquid inoculant-2 (\log_{10} 11.12 CFU ml⁻¹) at zero incubation period. On the other hand, liquid inoculant-2 protected significantly higher population as compared to nutrient glucose broth (\log_{10} 11.03 CFU ml⁻¹) at zero incubation period. At the end of 28 days incubation, liquid inoculant-2 had higher bacterial growth (\log_{10} 10.04 CFU ml⁻¹) compared to liquid inoculant-1 (\log_{10} 9.47 CFU ml⁻¹) and nutrient glucose broth (\log_{10} 8.98 CFU ml⁻¹).

A greenhouse experiment was conducted to investigate further the effect of MRP used either alone or along with *B. megaterium* on P-uptake by cowpea plants (Tables 3 and 4). The root-P content in the cowpea plants was higher (1.46 mg plant⁻¹) when inoculated with liquid inoculant-2 with MRP followed by liquid inoculant-1 with MRP (1.41 mg plant⁻¹), nutrient glucose broth with MRP (1.33 mg plant⁻¹) and lignite inoculant with MRP (1.21 mg plant⁻¹) (Table 3). Root-P content was significantly ($P \leq 0.05$) low in the case of un-inoculated control without MRP (0.50 mg plant⁻¹). A significantly ($P \leq 0.05$) higher shoot-P content was observed with liquid inoculant-2 when used with MRP (6.67 mg plant⁻¹) followed by liquid inoculant-1 with MRP (5.08 mg plant⁻¹) and lignite inoculant with MRP (3.79 mg plant⁻¹). Lower amount of shoot-P (1.1 mg plant⁻¹) was observed in case of the un-inoculated control without MRP (Table 4). No significant differences among different inoculant formulations without MRP

Table 2. Survival of *B. megaterium* in liquid inoculants under desiccation stress.

S.No.	Inoculant formulations	<i>B. megaterium</i> (log ₁₀ CFU ml ⁻¹)				
		Incubation (days)				
		0	7	14	21	28
1	Nutrient glucose broth	11.03 ^b	10.73 ^c	9.72 ^c	9.43 ^c	8.98 ^c
2	Liquid inoculant-1	11.07 ^{ab}	10.82 ^b	10.21 ^b	9.92 ^b	9.47 ^b
3	Liquid inoculant-2	11.12 ^a	10.92 ^a	10.43 ^a	10.23 ^a	10.04 ^a

Each value is an average of four replications.

Values with different superscripts between inoculant formulations within sample interval are statistically significant at P= 0.05 by the DMRT.

Table 3. Effect of inoculation of different formulations of *B. megaterium* with and without MRP on root P of cowpea plants.

S. No.	Inoculant formulations	Root P content (mg plant ⁻¹)	
		- MRP	+ MRP
1	Control	0.50 ^d	0.63 ^{cd}
2	Nutrient glucose broth	0.86 ^{bc}	1.33 ^a
3	Liquid inoculant -1	0.95 ^b	1.41 ^a
4	Liquid inoculant -2	0.95 ^b	1.46 ^a
5	Lignite inoculant	0.87 ^{bc}	1.21 ^a

Each value is an average of three replicates, where each replicate constituted two plants per pot.

Interaction effects are compared both across the columns and rows.

Means with same superscripts are statistically on par at P= 0.05 by the DMRT.

Table 4. Effect of inoculation of different formulations of *B. megaterium* with and without MRP on shoot P of cowpea plants.

S. No.	Inoculant formulations	Shoot P content (mg plant ⁻¹)	
		- MRP	+ MRP
1	Control	1.10 ^f	1.39 ^{ef}
2	Nutrient glucose broth	2.09 ^{de}	3.68 ^c
3	Liquid inoculant -1	2.84 ^d	5.08 ^b
4	Liquid inoculant -2	2.74 ^d	6.67 ^a
5	Lignite inoculant	2.46 ^d	3.79 ^c

Each value is an average of three replicates, where each replicate constituted two plants per pot.

Interaction effects are compared both across the columns and rows.

Means with same superscripts are statistically on par at P= 0.05 by the DMRT.

were observed. Other plant growth parameters like plant height, number of leaves per plant, number of nodules per plant, nodule dry weight, shoot dry weight, and root dry weight were also high, when cowpea plants were inoculated with liquid inoculant-2 along with MRP (un-published data).

DISCUSSION

Results of survival of *B. megaterium* in different inoculant formulations under the

influence of high temperature stress revealed that there was no significant difference in the population at different incubation intervals in different liquid inoculants at room temperature (26±3°C). That was probably due to the congenial conditions of the media used for growing *B. megaterium*. There was adequate supply of nutrients and the incubation temperature was favorable. Whereas, at 48°C, it was observed that there was a decline in the population of *B. megaterium* in the liquid inoculants. The decline was higher in the nutrient glucose broth and liquid inoculant-1



as compared to that in liquid inoculant-2. This observation correlates with the fact that the growth rate of *Bacillus* sp. at higher temperature (45°C) was low (Gaind and Gaur, 1990; Kucerova *et al.*, 1999). A similar trend was noticed, when *B. megaterium* in the liquid inoculants were subjected to desiccation stress. After a storage period of 28 days, it was observed that the liquid inoculant-2 supported significantly higher number of viable bacterial cells as compared to that in liquid inoculant-1 and the nutrient glucose broth. In the present study, the *B. megaterium* in different liquid inoculant formulations was subjected to high temperature stress at 48°C only for 8 h per day and desiccation stress (45°C) for a final storage period of four weeks. The intention was to mimic the summer temperatures prevailing in most of the tropical countries where the temperature ranges between 45-50°C only for 8-10 h day⁻¹. Under these stress conditions, maintenance of high number of viable *B. megaterium* cells in liquid inoculant-2 could be attributed to the presence of PVP and high quantity of glycerol in the medium.

The PVP has a high water binding capacity and hence available moisture was high in the media. Moreover, PVP also has the ability to protect “proteins and enzymes” over a wide range of temperatures and the effect of glycerol is well known (Somasegaran and Hoben, 1985) as it protects the cells against adverse conditions. It has also a high water binding capacity and acts as an alternate carbon source (Lorda and Balatti, 1996). Probably, due to the presence of a combination of PVP and high quantity of glycerol (12 ml l⁻¹), the magnitude of the decline in the population of *B. megaterium* was less in liquid inoculant-2 compared to the nutrient glucose broth and liquid inoculant-1. In liquid inoculant-1, even though osmoprotectants like trehalose, arabinose, FeEDTA, glucose and PVP were present; the decline in viable count was more compared to the liquid inoculant-2. This was probably due to the fact that the sugars offered a moderate degree of protection to proteins, enzymes, and other bio-molecules of the bacterial cells at higher temperatures. The liquid inoculant-1 retained fairly high log

number of viable cells compared to the nutrient glucose broth due to the presence of PVP and, to some extent, due to the presence of smaller quantities of glycerol (1 ml l⁻¹), trehalose, arabinose and glucose. Judging by the results of this study, it was clear that the presence of osmoprotectants protected bacterial cells and maintained a high population, even though the organism has the tendency to form endospores under stressed environment.

The use of phosphate-solubilizing microorganisms (PSM) as biofertilizers in crop production has been encouraging (Khan *et al.*, 2010). The successful use of PSM along with indigenously available cheaper sources like low grade rock phosphate (RP) has been found economical. These PSM have the ability of releasing the native soil P as well as increasing the availability of P from the added RP (Alagawadi and Gaur, 1992; Sharma and Kamath, 1991). The solubilization of insoluble P by PSM is carried out by the production of organic acids such as formic, citric, acetic, propionic, malic, succinic, fumaric, glycolic and gluconic acids, which convert insoluble P into soluble orthophosphates, making it available to plants (Khan *et al.*, 2007,). Sometimes, these acids form unionized association with metal (chelation) and increase the concentration of soluble P. This solubilization of insoluble P as well as the added RP by PSM was greater in non-sterile soil, indicating that inoculation was more effective in the presence of normal soil microflora (Illmer and Schinner, 1992).

In this study, a remarkable influence of inoculation with *B. megaterium* in different formulations was observed on P-uptake by cowpea plants. The effect was more pronounced when *B. megaterium* was used with MRP. Significantly higher root- and shoot-P were observed following inoculation of liquid inoculant-2 with MRP over uninoculated control and the other inoculated treatments. The increased P uptake by inoculated cowpea plants grown in liquid inoculant-2 with MRP was mainly due to efficient solubilization of insoluble soil-P as well as the added MRP. This was mainly

attributed to increased metabolic activity of higher population of *B. megaterium* that might have established well in the rhizosphere. Due to increased metabolic activity, it was hypothesized that there was an increased production of organic acids by the tested bacterium that efficiently solubilized insoluble P leading to increased P uptake by the plants. Similar increase in P uptake when inoculated with *B. megaterium* along with MRP has been reported for cowpea (Nagaraju *et al.*, 1995) and chick pea (Zaidi *et al.*, 2003; Gull *et al.*, 2004).

In conclusion, liquid inoculants-2 favored higher survival of *B. megaterium* as compared to the other inoculants formulations used in the present study under high temperature and desiccation stress. The results clearly demonstrated that a combination of PVP and high quantity of glycerol used in the liquid inoculant-2 were very effective in protecting the bacterial cells in adverse environment. Inoculation of *B. megaterium* grown in the liquid inoculants-2 along with application of MRP resulted in enhanced P-uptake by the cowpea plants as compared to the other formulations. Future studies are needed to evaluate the feasibility and efficacy of the liquid inoculant-2 under field conditions.

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بقای باکتری‌های *Bacillus megaterium* در مایه تلقیح‌های مایع و توان آنها برای انحلال فسفات تحت درجه حرارت بالا و تنش خشکی

س. ولیننی و ج. پ. برهماپراکاش

چکیده

تلقیح میکروارگانیزم‌های حل‌کننده فسفات همراه با خاک فسفات به عنوان یکی از راه‌های افزایش دهنده فسفر خاک شناخته شده است. موفقیت این حل‌کننده‌ها عمدتاً به توانایی بقای سوش‌های تلقیح‌شده در شرایط غیرطبیعی بستگی دارد. در این رابطه، مایه تلقیح‌های مایع بخاطر ماندگاری بیشتر آنها، از اهمیت ویژه‌ای برخوردار می‌باشند. در تحقیق حاضر، مطالعه‌ای مقدماتی برای تعیین بقای *Bacillus megaterium* در محیط‌های مایع حاوی محافظ‌های اسمزی و تحت تاثیر درجه حرارت بالا و تنش خشکی اجرا شد و همچنین تاثیر متعاقب آنها بر روی جذب فسفر بوسیله لوبیا چشم‌بلبلی مورد بررسی قرار گرفت. در این بررسی سه محیط کشت مایع شامل: مایه کشت گلوکوزی بدون افزودن محافظ اسمزی، مایه تلقیح شماره ۱ شامل محافظ‌های اسمزی، پلی‌وینیل‌پیرولیدین (PVP)، مقدار کمتری از گلیسرول (یک میلی‌لیتر در لیتر)، تری‌هالوز، آربینوز و کلات آهن، و مایه تلقیح شماره ۲ شامل محافظ‌های اسمزی حاوی PVP، گلیسرول زیاد (۱۲ میلی‌لیتر در لیتر) و گلوکز که حاوی جمعیت بالایی از باکتری بوده و تا دمای ۴۸ درجه سانتیگراد بمدت ۴ هفته قدرت زنده ماندن داشته و تحت تنش خشکی بودند (log₁₀ 10.04 CFU ml⁻¹)، مقایسه گردید. نتایج نشان داد که مایه تلقیح شماره ۲ (مایع) میزان فسفر قابل جذب توسط لوبیا چشم‌بلبلی را به طور معنی‌داری افزایش داد.