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Efficacy of rhizobacterial strains encapsulated in nontoxic biodegradable gel matrices to promote growth and yield of wheat plants

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ABSTRACT

One of the successful, safe and effective methods to introduce bioinoculants in soil is encapsulation of cells in biodegradable gel matrices which not only releases the microorganisms to the soil gradually but also helps to increase the survival rate by protecting them against many environmental stresses. Two bacterial strains viz. *Pseudomonas fluorescens* BAM-4 and *Burkholderia cepacia* BAM-12 were immobilized using sodium alginate and alginate + skim milk as carrier to check the phosphate solubilization *in vitro* and were found to have significantly higher activity than control. Their efficacy was also tested *in vivo* on wheat plants growth in semi arid environmental conditions in pots. The seed inoculation of rhizobacteria along with hardly soluble TCP and RP significantly ($P < 0.05$) enhanced the overall development of wheat plants in free and immobilized cell states in comparison to control. With a few scattered exceptions, plants amended with bacterial strains showed significantly ($P < 0.05$) better performance than the plants having chemical fertilizer, SSP and biofertilizer, biogold. When the two insoluble P sources were compared, TCP was found much better than RP treatments. Performance wise, *B. cepacia* BAM-12 was found to be the best.

1. Introduction

Plant growth promoting rhizobacteria (PGPR) represent a wide variety of free-living soil bacteria, which when grown in association with host plants, result in stimulation of growth of their hosts. Plant growth benefits, due to the addition of PGPR include increase in germination rates, shoot and root growth, weight, yield, leaf area, chlorophyll content, phosphorus, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought, and delayed leaf senescence. They may be used as biocontrol agents (Dobbelaere et al., 2003).

The potential use of PGPR especially the phosphorus solubilizing rhizobacteria as inoculants in soil for increasing P availability to plants has been extensively studied (Amara and Dahdoh, 1997; Canbolat et al., 2006). One of the successful, safe and effective methods to introduce bioinoculants in soil is encapsulation of cells in biodegradable gel matrices (Vassilev et al., 2001). Non-toxic natural polymers are generally recommended for use in soil. Alginate beads and alginate based preparations, carageenan, agar, etc. have been reported to preserve the beneficial properties of plant growth promoting rhizobacteria under storage (Bashan and Gonzalez, 1999). These formulations encapsulate the living cells, protect the microorganisms against many environmental stresses, and release them to the soil gradually but in large quantities when the polymers are degraded by soil microorganisms, usually at the time of seed germination and seedling emergence (Bashan, 1986). A major role of inoculant formulation is to provide more suitable microenvironment for the prolonged survival of bacteria in the soil. It also helps in segregating the bacterial cells from adverse environment, thereby, reducing cell loss (Rekha et al., 2007).

Van Elsas et al. (1992) reported that *Pseudomonas fluorescens* number decreased only moderately in the soil when the cells were encapsulated, whereas a large reduction in bacterial number was observed when free, non-encapsulated inoculum was used. The reason for this is when bacteria were encapsulated, they were washed down only slightly compared to free inoculated bacteria, hence remained in the root zone. Jain et al. (2010) evaluated free and encapsulated *Aspergillus awamori* for phosphate solubilization in fermentation and soil–plant system and found significantly higher growth in the presence of bioinoculant.

With this backdrop, an experiment was conducted to gain the insight into the possible influence of these strains on growth and yield of wheat plants in free and encapsulated forms.

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2. Materials and methods

2.1. Microorganisms and fermentation media

In order to test the efficacy of encapsulated cells with respect to free cells in terms of phosphate solubilization, 2 bacterial strains viz. *P. fluorescens* BAM-4 and *Burkholderia cepacia* BAM-12, previously isolated from rhizosphere of agricultural crops of Banasthali region, Rajasthan, India, were immobilized using sodium alginate and alginate + skim milk as carrier. The cultures were deposited in MTCC, Institute of Microbial Technology, Chandigarh, India and the accession numbers MTCC 7898 and 7100 were assigned. The strains were maintained on nutrient agar slants at 4 °C and subcultured periodically. The growth medium used was nutrient broth and the production medium used for repeated batch cultivation consisted of Pikovskaya (PVK) broth (Pikovskaya, 1948) with rock phosphate (RP) (P_2O_5 content—34% and mesh size—74 μm) or tricalcium phosphate (TCP), 0.2%.

2.2. Preparation of inoculum

Bacterial strains were inoculated in 100 ml of nutrient broth (Hi media-India) and incubated in shaker incubator (200 rpm) at 30 ± 2 °C for 24 h to obtain inoculum concentration of 10^9 cfu ml^{-1}.

2.3. Immobilization of bacterial strains in sodium alginate

Entrapment of bacteria within beads was carried out under sterile conditions in a laminar airflow hood (LAF). Sodium alginate solution (2%, w/v; S.D-fine, Biosar, India) and calcium chloride (0.1 M) were autoclaved separately. Fifty mililitres of bacterial suspension was aseptically mixed with sodium alginate solution and stirred gently for 1 h in shaking incubator. The mixture was vigorously stirred to allow a homogenous dissolution of alginate. Then the mixture was extruded through sterile syringe (syringe size; 10 ml, needle; 0.65 mm × 22 mm) into gently stirred, sterilized 0.1 M $CaCl_2$ at room temperature. The resulted alginate beads had a mean diameter of 2 mm which entrapping the bacterial cells. The beads were kept in $CaCl_2$ solution at room temperature for 1–3 h to obtain regular solid beads. The $CaCl_2$ solution was drained and the beads were washed twice with the sterile tap water. After washing step, beads were incubated in fresh growth medium (nutrient broth) for an additional 24 h in shaker incubator at 30 ± 2 °C to allow the bacteria to multiply inside the beads. Then the beads were washed twice with distilled water, collected and allowed to dry overnight in a LAF. These beads were further used for *in vitro* and *in vivo* experiments.

2.4. Immobilization of bacteria in sodium alginate + skim milk

Alginate formulation containing skim milk as a food supplement was prepared as previously mentioned steps of production beads formulations using 10% (w/v) sterilized skim milk to the bacterial broth.

2.5. Cultivation conditions

Fermentation experiment was carried out in 250 ml Erlenmeyer flasks containing 100 ml of production medium, PVK broth pH 7.0 before autoclaving. Triplicate flasks were inoculated with 1 g beads containing 10^8 cfu $flask^{-1}$. Autoclaved and inoculated with free cells media was used as control. The flasks were incubated at 30 ± 2 °C in an orbital shaker incubator at 130 rpm. Sampling was done after every 24 h, centrifuged and the phosphorus present in supernatant was estimated by Olsen's method (Olsen et al., 1954). Decline in pH value was also recorded.

2.6. Re-use of immobilized bacterial strains

Reusability of the immobilized cultures was tested by replacing the production medium following the same procedure with a fresh sterile one every 6th day. Phosphorus estimation and pH recording were performed as described by Olsen et al. (1954).

2.7. Pot experiment

A pot experiment was conducted with same bacterial strains (BAM-4 and BAM-12) in order to test the efficacy of encapsulated cells in P solubilization compared with the other free cells. Immobilization was carried out using sodium alginate + skim milk as carrier selected on the basis of its better performance in *in vitro* experiment.

2.7.1. Preparation of inoculum and immobilization of bacterial strains in sodium alginate + skim milk

Preparation of inoculum and entrapment of bacteria within carrier were carried out under sterile conditions as described for *in vitro* experiment. The population of bacterial strains was recorded as 1×10^8 cfu g^{-1} beads by dilution plate technique before sowing. In case of free cell treatment no entrapment was done.

2.7.2. Seed sterilization

Wheat seeds were obtained from KVK, Banasthali University and surface sterilized by immersing them in 0.1% of $HgCl_2$ prepared in sterile distilled water for 3 min. These seeds were rinsed with sterile distilled water for several times and then blotted on a sterile filter paper, dried and kept for use. All the steps were carried out taking all possible aseptic measures in LAF.

2.7.3. Seed sowing

Bacterial strains encapsulated in beads were mixed with seeds as 5 beads $seed^{-1}$ before sowing. Wheat seeds were sown at 2 cm depth (8 seeds pot^{-1}) in 1 kg plastic pots (length, 12.5 cm, upper diameter, 12.0 cm, lower diameter 8.5 cm) filled with unsterilized soil (clay, 0–25 mm; pH, 7.5; electric conductivity, 0.28 dS m^{-1}; organic matter, 0.78%; available P, 5.08 kg ha^{-1}; K, 280 kg ha^{-1}). Soil was thoroughly mixed and passed through a 2 mm sieve and then filled in plastic pots for experiment.

Seedlings were thinned down to 6 in number in each pot. The experiment was set up in a wire house from 15th December 2007 to 4th April 2008 and repeated from 17th December 2008 to 6th April 2009. Plants were irrigated with tap water daily to maintain moisture at field capacity. Three samples from each pot were taken at the time of harvesting to record the shoot length, root length, fresh shoot weight, fresh root weight, dry shoot weight, dry root weight, microbial count, spike length, weight of spike, and number of seeds $spike^{-1}$, 100 grains weight. The shoot samples were oven dried and stored for total P and N analyses.

2.7.4. Experimental design

The experiment was set in a randomized block design (RBD) and four replicates were taken for each set of experiment and it was done for 2 consecutive years. Tri-calcium phosphate (TCP) and rock phosphate (RP) were used as the source of insoluble inorganic phosphate, single super phosphate (SSP) as a chemical fertilizer and biogold as commercial biofertilizer as all these being used in agricultural practices frequently now-a-days. For combined inoculation liquid cultures of each organism were mixed in equal proportion and entrapped in the carrier. There were 23 treatments in the experiment which are as follows: (T1) soil