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SHORT COMMUNICATION

Recovery of plant growth-promoting rhizobacteria from sodium alginate beads after 3 years following storage at 4 °C

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Abstract Two plant growth-promoting bacteria, *Bacillus subtilis* and *Pseudomonas corrugata*, immobilized in a sodium alginate based formulation were evaluated for their survival, viability and plant growth-promoting ability after 3 years of storage at 4 °C. Populations of both of the bacterial isolates recovered from the immobilized sodium alginate beads were in the order of 10^8 cfu g^{-1}. The plant-based bioassay indicated that the plant growth promotion ability of both of the bacterial isolates was equal to those of fresh broth-based formulations. The bacterial isolates retained the root colonization, and antifungal and enzyme activities in the alginate-based formulation during storage.

Keywords Plant growth-promoting rhizobacteria · Formulations · Viability · Recovery · Storage

Introduction

Plant growth-promoting rhizobacteria (PGPR) are characterized by a number of activities, which include improvement of plant establishment, increased availability of plant nutrients, enhancement of nutrient uptake, improvement of soil structure and protection against diseases [11, 15, 34]. The use of microbial technologies in agriculture is currently expanding quite rapidly with the identification of new bacterial strains, which are more effective in promoting plant growth. Novel technologies have also been developed for the optimization of biomass production, product formulation and delivery systems. Two major problems in expansion and commercialization of this microbe-based technology are: (1) identification of suitable microorganisms for a specific climate [22] and (2) the unavailability of the inoculants in ready-to-use forms [2]. A variety of formulations using different carriers have been devised [2, 33], but to date no universal carrier or formulation is available for release of microorganisms into the soil [29]. A good quality formulation should promote the survival of bacteria preserving the beneficial traits and when applied should protect the cells from various biotic and abiotic stresses [2, 10, 28]. Formulations based on polymers, especially alginate, have received considerable interest in recent times [4, 30, 31].

Efficient strains of *Bacillus subtilis* and *Pseudomonas corrugata* have been selected as microbial inoculants suitable for application in colder regions and developed in appropriate formulations [23, 30]. In the present study, the viability of these PGPRs in three formulations, viz alginate beads, coal and broth was determined after storage at 4 °C over a period of 3 years. The effect of storage on various plant growth promotion traits was determined from the cells recovered from alginate beads stored at 4 °C for 3 years and compared to fresh cultures from the culture collection. The efficiency of growth promotion of the 3-year-old beads containing bacteria was evaluated through a bioassay using wheat as the test plant.

Materials and methods

Bacterial isolates

Two bacterial isolates, viz *Bacillus subtilis* (NRRL B-30408) and *Pseudomonas corrugata* (NRRL B-30409), originally isolated from the temperate locations of the

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Indian Himalayan Region (IHR) were used in this study. The bacterial species have been reported for their efficiency to promote plant growth and disease control [18–23, 30].

Formulations

The bacterial isolates were raised in culture suspension and prepared in three formulations: (1) alginate beads, (2) thick coal slurry and (3) broth, following procedures as described in Trivedi et al. [30]. The formulations were stored in hermetically sealed flasks for 3 years at 4 °C in the refrigerator.

Enumeration of viable bacteria in various formulations under storage

The viability of bacteria in various formulations was enumerated by dissolving 1.0 g in case of alginate beads and coal formulations and by adding 1.0 ml of broth formulation in 9.0 ml of potassium phosphate buffer (0.25 M, pH 6.8) in a test tube for 16–24 h at 28 °C. Further enumeration was carried out by dilution plate technique using Tryptone yeast extract (TY) agar. The plates were incubated at 28 °C and the colony-forming units (cfu) were counted after 5 days. These viability tests were carried out with fresh as well as preparations stored at 4 °C at an interval of 6 months up to a period of 36 months of storage.

In vitro characterization of plant growth promotion ability of recovered bacterial cells from alginate beads

Cultures of *B. subtilis* and *P. corrugata* recovered from the plates used for enumeration of viable cells entrapped in alginate beads stored at 4 °C for 3 years were tested for various plant growth promotion abilities and compared with the fresh cultures of the respective strains stored in glycerol at −20 °C in a deep freezer. Quantitative estimation of phosphate solubilization was recorded in NBRIP broth [17] and assayed by molybdo-phosphoric acid blue method [1] after 7 days of incubation at 28 °C. Qualitative estimation of indole acetic acid (IAA) [24], chitinase [14], salicylic acid [16] and siderophore [25, 27] were performed following standard methods. Qualitative estimation of ammonia production was carried out by using the methods described by Dye [8]. *Fusarium oxysporum* was used as test phytopathogenic fungi to study the effect of diffusible and volatile antifungal compounds produced by the bacterial isolates following the procedure described in Chaurasia et al. [6].

Bioassay for evaluation of growth promotion by bacterial inoculants in alginate beads formulation

The bioassay was conducted using wheat (*Triticum aestivum*) as a test species. Three-year-old alginate beads (stored at 4 °C) and fresh broth-based formulations of both the bacterial isolates were used for inoculation. Formulation application and experimental layout were followed as described previously [30]. Measurements on growth parameters, namely root and shoot length and biomass, were recorded after 42 days of plant growth. In addition to growth measurements, other parameters such as acid phosphatase [13], peroxidase [7], phosphorus content [1] and rhizosphere colonization [30] were also determined.

Results and discussion

Evaluation of the survival of the organism(s) in the carrier-based preparation under storage over a period of time is essential for the determination of the suitability of the "carrier". In the present study, the rate of bacterial survival in the inoculants over time was taken as a parameter for determining the capacity of the formulations to support the

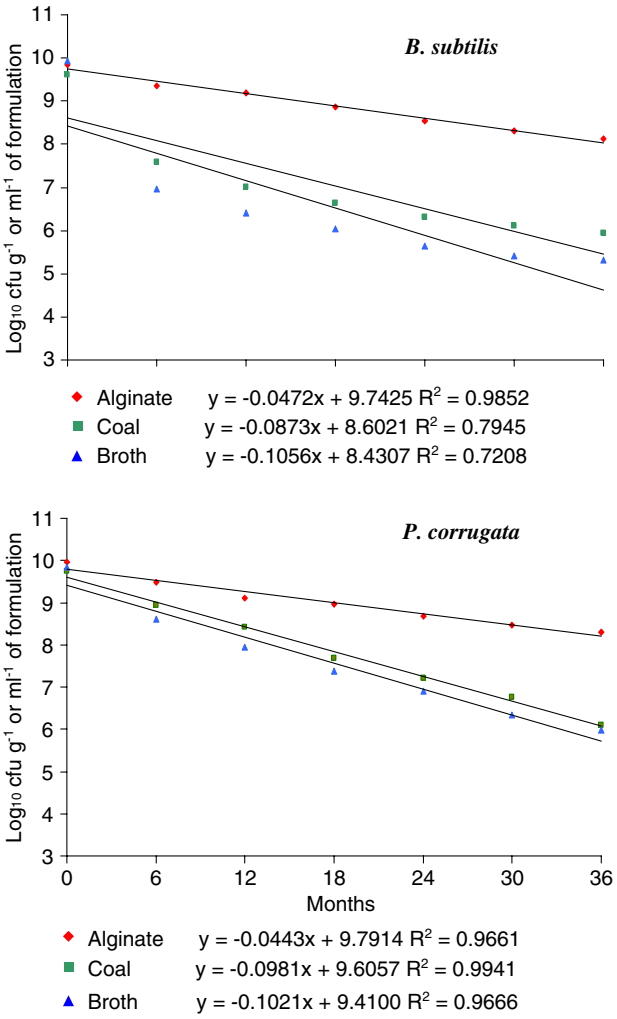

Fig. 1 Recovery of bacterial cells from different formulations during storage at 4 ± 2 °C

survival of *B. subtilis* and *P. corrugata*. The survival rate of both the bacteria was highest in alginate beads for both the isolates when compared with coal or the broth-based formulation (Fig. 1). The population level of the alginate beads was well above 10^6 cfu g^{-1} bead after 3 years, at par with the Canadian standard for freshly prepared *Rhizobium* inoculant [5]. PGPR(s) can survive in alginate beads for longer periods of time [3]. Viveganandan and Jauhri [32] reported the superiority of alginate-based formulations over charcoal-based ones in maintaining the population of two phosphate solubilizing bacteria during storage at different temperatures and moisture content.

In the process of inoculant formulation along with longer shelf life, preservation of strain characteristics is also desirable [10, 28]. In qualitative estimations, the plant growth promotion and biocontrol-related characteristics in the bacterial cells recovered from the alginate beads were assessed at par with those of fresh cultures obtained from the culture collection (Table 1). The capability of *Pseudomonas fluorescens* F113 Lac ZY entrapped in alginate beads to produce the antifungal metabolites 2,4-diacetylphloroglucinol (Phl) was not significantly affected after 12 months of storage [26].

A quality formulation should deliver a high number of cells for the effective colonization of the rhizosphere of target plants in order to facilitate the intended plant growth [12]. In the wheat-based bioassay, the bacterial inoculations with alginate beads formulations positively affected (statistically significant) the plant growth parameters (Table 2). Higher values for growth parameters were recorded in alginate beads treatments than in the case of broth-based formulations. The root colonization capacity of *B. subtilis* and *P. corrugata* entrapped in the alginate beads was also found to be superior to that of the fresh broth formulation. After 42 days, cfu g^{-1} dry weight of root was 5.60 and 5.10 for alginate beads as compared to 3.24 and 3.31 for fresh broth formulations of *B. subtilis* and *P. corrugata*, respectively. Inoculation of wheat plants with cells recovered from 14 years old dry beads of *Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313 resulted in an equal effect in terms of colonization and increment in plant growth as compared to those of the contemporary strain of a culture collection or to their old records [3]. Alginate beads formulations of *Pseudomonas fluorescens* F113 Lac ZY stored at 4 ± 2 and 28 ± 2 °C for 1 year showed effective root colonization and protection of sugar beet against two phytopathogenic fungi [26].

Although the alginate beads entrapping *P. corrugata* showed high viability and maintained the PGPR ability of the cells, liquefaction at the bottom of the flasks was observed after 3 years of storage. This might be due to the production of certain acids by *P. corrugata*, which caused the deformation and subsequent degradation of the beads. No such loss of integrity was observed in the case of beads entrapping *B. subtilis*. This observation highlights the superiority of *Bacillus* species in the development of carrier-based formulations of PGPR(s) as compared to *Pseudomonas* species. The superior storage of immobilized *B. subtilis* beads is in relation to alginate-based formulation. Various traits and properties of the genus *Bacillus*, which makes them suitable for developing commercial formulations has been described [9].

Table 1 Comparative assessment of plant growth promotion and biocontrol related parameters of *B. subtilis* and *P. corrugata* carried out on fresh cultures and cells recovered from alginate beads (after 3 years of storage at 4 °C)

Characteristics	*B. subtilis*[a]	*B. subtilis*[b]	*P. corrugata*[a]	*P. corrugata*[b]
P solubilization (μg ml^{-1})	54	52	101	103
Production of IAA[c]	–	–	+ (17.6)	+ (17.2)
Biocontrol properties (production of)				
Diffusible antifungal metabolites[d]	+ (68.5)	+ (66.0)	–	–
Volatile antifungal metabolites[d]	+ (36.3)	+ (34.8)	+ (32.6)	+ (34.7)
Ammonia	+	+	+	+
Chitinase[e]	+ (22.3)	+ (24.2)	+ (29.6)	+ (30.8)
Salicylic acid[f]	+ (5.0)	+ (4.8)	+ (16.9)	+ (16.4)
Siderophore[e]	+ (2.1)	+ (2.0)	+ (9.8)	+ (9.7)

[a] Fresh culture raised from culture collection stored in 10% glycerol at −20 °C

[b] Cells recovered from aliginate beads

[c] IAA production in μg ml^{-1}

[d] Percent reduction in the growth of *Fusarium oxysporum* as compared to control

[e] Chitinase activity in µmol min^{-1} mg^{-1} of protein

[f] Salicylic acid production in μg ml^{-1}

[g] Siderophore production in µmol benzoic acid ml^{-1}

Table 2 The effect on variables of wheat plant growth after inoculating with recovered (alginate beads, after 3 years of storage at 4 °C) and collection-based *B. subtilis* and *P. corrugata*

Treatments	Length (cm)		Dry weight (mg)		Enzyme activity (unit g^{-1} fresh weight)		Phosphorus content (%)	
	Root	Shoot	Root	Shoot	Acid phosphatase	Peroxidase	Root	Shoot
Control	09.3 ± 0.5a	40.6 ± 1.1a	10.3 ± 1.2a	60.0 ± 2.2a	410 ± 4.2a	132 ± 6.5a	0.019a	0.048a
B. subtilis[a]	12.3 ± 0.3b	46.8 ± 1.0b	18.4 ± 3.1b	77.0 ± 2.1b	569 ± 2.8b	185 ± 3.1b	0.035b	0.071b
B. subtilis[b]	13.5 ± 0.3bc	52.1 ± 1.8c	22.3 ± 2.2c	82.3 ± 3.1b	573 ± 2.8b	190 ± 2.0c	0.035b	0.067b
P. corrugata[a]	12.2 ± 0.3b	48.7 ± 1.9bc	19.3 ± 2.1b	76.7 ± 4.2b	791 ± 3.3c	210 ± 3.4d	0.035b	0.071b
P. corrugata[b]	13.8 ± 0.4c	51.5 ± 2.9c	20.7 ± 1.3b	81.9 ± 3.1b	770 ± 5.4d	215 ± 5.5e	0.037b	0.073b

For each factor, the values in the same column followed by the same letter are not significantly different according to the Fisher test ($P \leq 0.05$)

[a] Fresh broth formulation

[b] Alginate beads

B. subtilis and *P. corrugata*, originally isolated from the soil samples of temperate regions, have been thoroughly investigated for their various plant growth-related properties [23] and have been prepared in carrier-based formulations [30]. In the present investigation, the bacterial species have been evaluated for their shelf life under storage and their ability to retain the desirable traits related to plant growth promotion. From this study, it can be concluded that alginate can be used for the production of high-quality bacterial inoculants, which are required to be produced in large scale for the field application and commercialization of this microbe-based technology.

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