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Survival, efficacy and rhizospheric effects of bacterial inoculants on *Cajanus cajan*

Richa Sharma[a], Jeny Singh Paliwal[a], Preeti Chopra[b], Deepshikha Dogra[a], Vijay Pooniya[c], Virendra Swarup Bisaria[a], Karivaradharajan Swarnalakshmi[b], Shilpi Sharma[a,*]

[a] Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India
[b] Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India
[c] Division of Agronomy, Indian Agricultural Research Institute, New Delhi 110012, India

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ABSTRACT

Bioinoculants serve as a promising, eco-friendly alternative to conventional chemical fertilizers and pesticides. While their direct positive effect on plant growth is well known, non-target effects of these agricultural amendments have so far not been extensively studied. The present study is an attempt to assess (a) the survival of *Bacillus megaterium*, *Pseudomonas fluorescens* and *Azotobacter chroococcum* in the rhizosphere of *Cajanus cajan* (pigeon pea), (b) the target effects of unconventional combinations of these bioinoculants on the crop, and (c) the non-target effects (on the resident soil microflora) of the bacterial supplements, when applied individually and in combination. Rifampicin-resistant strains were employed to follow the persistence of the bioinoculants in the rhizosphere. They could be detected until approximately two months after sowing. The effect of the bioinoculants in the field was assessed on various plant growth parameters. Triple inoculation competed well with chemical fertilizer with respect to plant growth parameters. Grain yield (kg ha^{-1}) was 1.5- and 1.7-fold higher with mixed consortium and chemical fertilizer, respectively, than that of the untreated control. A cultivation-dependent approach was employed to assess important microbial groups in the plant rhizosphere. In a comparison of the treatments with bulk soil, a clear effect on the rhizosphere was apparent. Apart from the inoculation effect, pronounced changes in the microbial diversity were observed during plant development. At the vegetative stage, the mixed consortium showed increases of 1.08-, 1.22- and 4.2-fold in the abundance of nitrogen fixers, *Pseudomonas* and *Actinomycetes*, respectively, as compared to the untreated control. Additionally, the bioinoculants were found to be compatible with other groups of plant growth promoting rhizobacteria.

1. Introduction

Sustainable agriculture involves the utilization of eco-friendly technologies, which include the application of bioinoculants for crop improvement. Bioinoculants are widely used in agriculture because of their beneficial interaction with plant roots, protection against soil borne pathogens, and improvement of grain yield. In the past decade, a large amount of data has been generated that support the efficacy of bioinoculants in enhancing plant growth and yield (Tilak et al., 2006; Niranjana et al., 2009; Kumar et al., 2010; Gupta et al., 2012). However, only limited information is available on the 'non-target' effects of bioinoculants on the resident microbial community (Naseby et al., 2000; Björklöf et al., 2003; Pereira et al., 2009; Gupta et al., 2014). Non-target effects can be defined as the effects caused by the introduction of bioinoculants on organisms other than the target organisms (Winding et al., 2004). When bioinoculants are released into the environment, they can induce transient disturbances in resident microbial community, which may be either positive or negative. Aseri et al. (2008) reported enhanced activities of the enzymes dehydrogenase, alkaline phosphatase and nitrogenase in the rhizosphere of *Punica granatum* upon inoculation with *Azospirillum brasilense*, *Azotobacter chroococcum*, *Glomus fasciculatum* and *G. mosseae*. Increase in microbial biomass and abundance of specific microbial groups have also been observed upon application of bioinoculants (Zhang et al., 2010; Trabelsi et al., 2011). These effects on the native microflora depend on various parameters, like type of soil, mode of application and other environmental conditions (Berg

* Corresponding author.
E-mail address: shilpi@dbeb.iitd.ac.in (S. Sharma).

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and Smalla, 2009). On the other hand, López-Valdez et al. (2011) could not see any positive effect of *Bacillus subtilis* at the harvest stage of *Helianthus annuus*. Costa et al. (2006) did not observe any significant rhizospheric effects on actinobacterial population in the rhizosphere of oilseed rape in the early growth season.

Besides the crucial aspect of non-target effects of bioinoculants, it is essential to know their persistence in the rhizosphere before application. This is a relatively under explored area, as tracking of bioinoculants at strain level is cumbersome due to a limited availability of tools. To monitor a bioinoculant, the specific strain must have a selective characteristic that does not interfere with its colonization of the rhizosphere and survival (Turco et al., 1986). Selection of strains with spontaneous antibiotic-resistances has provided a simple and effective method to monitor bioinoculants after their introduction into soil. Rifampicin resistance in bacteria is due to a rare mutation in the RNA polymerase gene. Due to its chromosomal nature, such a mutation is stable and non-transferable, in contrast to plasmid-borne markers (Sippel and Hartmann, 1968).

India is the largest producer and consumer of *Cajanus cajan*, accounting for about 70% of the global production (Odeny, 2007). After chickpea (*Cicer arietinum*) and field pea (*Pisum sativum*), pigeon pea is the third most important legume crop in India (Sharma et al., 2012). It also has high nitrogen fixation ability (España et al., 2006). As shown by Saxena and Nadarajan (2010), in India, the average productivity of pigeon pea of around 700 kg ha^{-1} has not changed in the last five decades. Given the immense overall technological improvement of agricultural productivity, this stagnation is a serious concern. The present study aims to attain a deeper understanding of the interactions between non-conventional combinations of bioinoculants with pigeon pea, and with its resident rhizospheric microflora with respect to enhancement of grain yield. The bioinoculants selected for the study were *Azotobacter chroococcum* A-41, *Bacillus megaterium* MTCC 453 and *Pseudomonas fluorescens* MTCC 9768. *A. chroococcum* is a free-living diazotroph, *B. megaterium* is a potential agent for the biocontrol of plant diseases, and *P. fluorescens* has multiple plant growth promoting properties like production of siderophores, hydrogen cyanide and indole acetic acid (Gupta et al., 2016). We hypothesized that the positive impact of bioinoculants on plant growth and yield is a cumulative effect of their direct contributions, as well as their effects (both positive as well as negative) on the resident microbial community of the plant. This could explain that the bioinoculants can have a lasting effect on the plant, despite their relatively short-term persistence in the soil. This would in turn have an impact on plant growth. Our objectives were thus (i) to monitor the persistence of plant growth-promoting *A. chroococcum*, *B. megaterium* and *P. fluorescens* in the rhizosphere of *C. cajan*, (ii) to quantify the target effect of the bioinoculants on plant growth parameters in the field, and (iii) to assess the non-target effects of the bioinoculants on the culturable rhizospheric microbiome.

2. Materials and methods

2.1. Plant system, microbial strains and compatibility assay

Seeds of pigeon pea (*C. cajan*) cultivar UPAS-120 were obtained from the National Seed Corporation, Pusa, New Delhi, India. Bioinoculants used were *B. megaterium* MTCC 453, *P. fluorescens* MTCC 9768 and *A. chroococcum* A-41. *B. megaterium* MTCC 453 and *P. fluorescens* MTCC 9768 were procured from the Institute of Microbial Technology, Chandigarh. *A. chroococcum* A-41 and *Bradyrhizobium* sp. (rhizobium recommended for the crop) were obtained from the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi, India. The three strains of bioinoculants, and rhizobium, were assessed for compatibility using standard cross streak assay method (Anandaraj and Leema Rose Delapierre, 2010).

2.2. Isolation of spontaneous rifampicin resistant bacterial strains

Spontaneous rifampicin resistant mutants of the three bioinoculant strains were isolated by plating overnight-cultures on the respective agar medium containing 100 μg mL^{-1} rifampicin (Nautiyal, 1996). Cultures of rifampicin resistant mutants with plant growth promoting properties comparable to those of the respective wild types (data not shown), were serially diluted and plated on agar plates containing 0, 5, 25, 50, and 100 μg rifampicin mL^{-1}. Colonies were observed on the plates after 24–36 h. The mutants were stored as glycerol stocks at −80 °C in antibiotic-supplemented (100 μg mL^{-1}) Luria broth.

2.3. Preparation of formulation, seed surface sterilization and bacterization

Culture broths containing 1.0×10^{10} cfu (colony forming unit) mL^{-1} of each bacterial strain were used for preparation of formulations. For approximately 100 g formulation, 80 g autoclave-sterilized talcum powder (inorganic carrier) was mixed with 18 mL of bacterial culture broth, 1 mL of 50% autoclaved glycerol and 1.0 mL of 0.1% filter sterilized carboxy methyl cellulose. The formulation was dried in an incubator at 27 °C to reduce the moisture content, packed in sterile polythene bags and sealed. For the combination of two or three bioinoculants (as specified in Section 2.4), individual cultures were mixed in equal quantities, resulting in formulations containing ~2.0×10^9 cfu g^{-1} of each bioinoculant. Seeds of *C. cajan* were surface-sterilized using 70% ethanol for 30 s, followed by soaking in 0.01% NaClO for 2 min. The seeds were then washed with 0.01 N HCl to remove excess NaClO (Abdul-Baki, 1974), and finally rinsed eight times with sterile distilled water. The sterilized seeds were then soaked in autoclaved water and kept overnight. Seeds of similar size and shape (visual observation) were selected for bacterization. Standard dilution and plate counting techniques were used to determine the cfu of the formulations prior to sowing of the seeds. The average cfu per seed was calculated for all treatments and were found to be in the range of 4–8 × 10^8.

2.4. Pot experiment for tracking bioinoculants in the rhizosphere of Cajanus cajan

The soil used in this study was collected from an agricultural field in Delhi, India. It had the following properties: clay loam (40% clay, 35% sand and 25% silt), 0.42% organic matter content, 7.2 pH (1:2.5 soil and water ratio), electrical conductivity of 0.04 dS m^{-1}, and moisture content of 14%. Nine different formulations were prepared: (1) *B. megaterium* (B), (2) *B. megaterium* rifampicin resistant mutant (Br), (3) *P. fluorescens* (P), (4) *P. fluorescens* rifampicin resistant mutant (Pr), (5) *A. chroococcum* (A), (6) *A. chroococcum* rifampicin resistant mutant (Ar), and three combinations of rifampicin resistant mutants: (7) *A. chroococcum* and *B. megaterium* (Ar + Br), (8) *B. megaterium* and *P. fluorescens* (Br + Pr), (9) *A. chroococcum* and *P. fluorescens* (Ar + Pr). Pots of 40.6 cm diameter were filled with soil described above, and three seeds per pot were sown at a depth of approximately 4–5 cm. The set-up was completely randomized (CRD). Seeds without inoculation (C) and bulk soil (S) served as control.

Five samplings points were chosen: pre-vegetative [9 and 16 days after sowing (DAS)], vegetative (25 DAS) and pre-flowering (45 and 59 DAS). At the time of each sampling, rhizospheric soil (soil tightly adhering to the roots) was collected. These samples