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Original article

Response of rhizospheric bacterial communities of *Cajanus cajan* to application of bioinoculants and chemical fertilizers: A comparative study

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ABSTRACT

Despite the long history of application of bioinoculants for improving crop productivity, very little information is available on their impact on native microbial communities in crops' rhizosphere. The present study compares the effects of a microbial consortium comprising of *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma harzianum*, with that of two commonly used chemical fertilizers, on microbial population in *Cajanus cajan*'s rhizosphere using cultivation-dependent (enumeration) and −independent (denaturing gradient gel electrophoresis, DGGE) methods at both DNA and RNA levels. The bioinoculants' application demonstrated stimulatory effects on rhizospheric microflora of phosphate solubilizing bacteria (2.5- fold), *Pseudomonas* spp. (1.9- fold), nitrogen fixing bacteria (1.2-fold) and fungi (1.6- fold), which was better than that of chemical fertilizers, rendering the consortium to be safe for release in agricultural fields. The qualitative profiling of microbial communities, based on DGGE, exhibited significant changes in treated rhizosphere samples as compared to the control, indicating the noticeable impact of bioinoculants. The study stresses upon the significance of beneficial role of the bioinoculants on crop productivity, which results due to their interaction with native rhizospheric microbial community.

1. Introduction

In sustainable agriculture, resources are utilized and managed to satisfy human needs, with special care being taken to improve environmental quality and conservation of natural resources for next generations. This concept involves the utilization of a number of eco-friendly techniques, viz. application of bioinoculants, use of organic farming, and mixed/inter cropping. Bioinoculants are culture concoctions of microbial isolates that are presently the most ecologically viable and economically feasible example of practical applications to fields for the benefit of modern day farmers.

The beneficial effects of bioinoculants for enhanced crop productivity and better plant health have been demonstrated for various crops [1–3]. However, prior to their application in field, it is absolutely critical to evaluate the effect of these artificially-introduced microorganisms on the indigenous microflora and other soil processes contributing to soil nutrient status. Such effects of bioinoculants on soil resident microbial community structure and function other than their target species, or on biogeochemical cycles, are known as non-target effects [4]. The present study aims to assess and compare the non-target effects of a microbial consortium, comprising of three selected bioinoculants, viz. *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma harzianum*, with that of chemical fertilizers at recommended dose, using both cultivation-dependent and −independent techniques. The strains selected were *Bacillus megaterium* MTCC 453, *Pseudomonas fluorescens* MTCC 9768 and *Trichoderma harzianum* MTCC 801. *B. megaterium* MTCC 453 is known for its biocontrol property against nematodes population by the production of neutral extracellular protease enzyme [5,6]. *P. fluorescens* MTCC 9768 acts as a plant growth promoting as well as biocontrol agent through phosphate solubilization, and production of siderophore, hydrogen cyanide, and indole acetic acid [7]. *T. harzianum* MTCC 801 is commonly used as antagonist in biocontrol of some important plant pathogenic soil-borne fungi [8].

A combination of microbial species belonging to the above-mentioned three genera has also been reported to promote plant growth and prevent disease occurrence in other legumes. Jain et al.

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[9,10] observed an increase in number of leaves, secondary roots and nodules, plant length, total biomass and yield in pea plants along with a decrease in plant mortality due to infection with *Sclerotinia sclerotiorum*, when treated with a microbial consortium of *Pseudomonas aeruginosa* PJHU15, *Trichoderma harzianum* TNHU27, and *Bacillus subtilis* BHHU100. Both the bacterial strains were isolated from rhizosphere of pea, while the fungal member of the consortium was isolated from an agricultural farm. Compatibility amongst the three, and their antagonistic activity against *S. sclerotiorum* led to their application as a consortium. *Cajanus cajan*, commonly known as pigeon pea or arhar, is known to be the major grain crop of semiarid tropics and is used as a substitute for meat because of its high protein content. The variety UPAS-120 is an extra early maturing variety. The plant enriches soil and prevents soil erosion. It is easy to grow, productive, and drought tolerant because of its deep tap root system. Both cultivation-dependent and -independent approaches were employed to assess the culturable fraction, and total microbial community, respectively with latter involving co-extraction of DNA and RNA followed by profiling using denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Soil characteristics, microbial strains and preparation of formulation

The soil used for the study was collected from a local site in New Delhi, India with the following properties: clayey loam, 0.42% organic matter content, 7.4 pH (in water), 0.06 dS m^{-1} electrical conductivity, and 11% water holding capacity. The nitrogen, phosphorus, potassium and iron content of the soil were 136.4, 98.5, 176.25 and 14.2 mg kg^{-1}, respectively.

The cultures, *Bacillus megaterium* MTCC 453, *Pseudomonas fluorescens* MTCC 9768 and *Trichoderma harzianum* MTCC 801, were procured from the Institute of Microbial Technology, Chandigarh, India. Prior to their use as bioinoculants, the three microbial strains were checked for their compatibility with each other using cross-streak assay [11,12]. The mixture of three microbial strains was applied as seed coat, using appropriate formulation as described earlier [13]. The formulation contained approximately 2×10^7 cfu g^{-1} of each bioinoculant.

2.2. Seed sterilization, bacterization and sowing

Seeds of *Cajanus cajan*, commonly known as pigeon pea, variety UPAS 120 were procured from National Seed Corporation, Indian Agricultural Research Institute, New Delhi, India. *C. cajan* seeds were surface-sterilized with 0.01% sodium hypochlorite [13]. The sterilized seeds were then bacterized by mixing 50 g of seeds with 0.5 g of the formulations prepared for microbial treatment. The nomenclature used for the treatments was: BPT as microbial consortium (*B. megaterium* MTCC 453 + *P. fluorescens* MTCC 9768 + *T. harzianum* MTCC 801); C as control i.e., seeds without any inoculation; US as unplanted (and un-inoculated) soil, and NP, i.e. chemical fertilizers at recommended dose of N @ 8 kg/acre, (as Urea with >48% N content) and P @ 16 kg/acre (as super phosphate with >40% P) (http://agriharyana.nic.in/variouscrops.htm).

Before sowing, cfu per seed were counted in the formulations using serial dilution and plate count method and it was found to be ~1×10^6 $seed^{-1}$ for each of the three bioinoculants. Sixty four pots (4 treatments × 4 sampling points × 4 replicates) of approximately 40 cm diameter were filled with approx. 10 kg soil (with properties as described above) per pot and mixed well with host specific *Rhizobium* strain as recommended by the manufacturer (procured from IARI, New Delhi). Control and treated seeds were sown in pots at a depth of about 4–5 cm. Four replicates were set up for each treatment and sampling point. The design used for the experiment was completely randomized block design. The pots were kept under sunlight (approximately 16/8 photoperiod) in I.I.T. Delhi nursery, which experienced a maximum temperature ranging from 34 °C (minimum 26 °C) to 23 °C (minimum 8 °C). The pots were irrigated at regular intervals to maintain constant moisture level in the soil (approximately 14%).

2.3. Sampling

Samples were taken at four stages of plant's growth namely, vegetative stage (1 month after seed sowing), pre-flowering (2 months after seed sowing), flowering (3 months after seed sowing) and maturity stage (4 months after seed sowing). At each time point, samples of four randomly selected *C. cajan* plants, including roots and above ground parts, were collected for each treatment and control. The roots of the uprooted plants were shaken and the soil, bound to the roots, was collected without damaging the root and root nodules, and this soil was termed as "rhizosphere soil". Each rhizosphere sample was divided into two parts; one was kept at 4 °C and the other stored at −20 °C (after shock-freezing in liquid nitrogen) for cultivation-dependent and cultivation-independent studies, respectively.

2.4. Soil parameters

The micro- (Fe) and macro-nutrients (N, P, K) of the control as well as treated soil samples were analyzed using different analytical methods. The total available N was quantitatively determined using the analytical method of dynamic flash combustion system coupled with a gas chromatographic separation system and thermal conductivity detection system, while Olsen method [14] was used for determining the total P. The K cations were quantitated by inductively coupled plasma atomic emission spectrometry [15]. For measuring Fe content, the DTPA (diethylenetriaminepentaacetic acid) micronutrient extraction method was used [16].

2.5. Enumeration of specific rhizospheric microbial groups

Groups of plant growth promoting rhizospheric microorganisms, i.e., phosphate solubilizing bacteria, *Pseudomonas* spp., nitrogen fixing bacteria, and total fungal population were assessed on specific media, namely Sperber [17], King's B [18], Burk's [19] and Rose Bengal Chloramphenicol media [20], respectively.

One gram of the rhizosphere soil samples was transferred to a 15 ml falcon tube containing 10 ml sterile distilled water and shaken vigorously. Serial dilutions were prepared and then 0.05 ml aliquots from each tube were spread on all four types of media. Each rhizosphere sample was assessed in triplicates. The inoculated plates were incubated at 30 °C for 48 h in case of Sperber, King's B and Burk's media, and for 72 h in case of Rose Bengal Chloramphenicol medium. The colonies appearing on the media were counted and expressed as cfu/g dry soil.

2.6. Total nucleic acid extraction

Total nucleic acid extraction from rhizosphere soil samples stored at −20 °C was performed as described earlier [21]. All precautions were taken to prevent the degradation of RNA by RNases. Glassware were baked overnight at 200 °C and rinsed with diethylene pyrocarbonate (DEPC) - treated water.