Applied Soil Ecology 76 (2014) 26–33

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Non-target effects of bioinoculants on rhizospheric microbial communities of *Cajanus cajan*

Rashi Gupta[a], Natarajan Mathimaran[b], Andres Wiemken[b], Thomas Boller[b], Virendra S. Bisaria[a], Shilpi Sharma[a,*]

[a] Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, New Delhi, India
[b] Zurich-Basel Plant Science Center, Institute of Botany, University of Basel, Basel, Switzerland

ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form
28 November 2013
Accepted 4 December 2013

Keywords:
Bioinoculants
Cajanus cajan
ARISA
qPCR
Rhizosphere

ABSTRACT

"Bioinoculants" have become a useful, environment-friendly tool in agriculture to increase crop yield. Previous work has shown that *Cajanus cajan*, India's most important pulse, can profit considerably from applications of the three bioinoculants, viz. *Bacillus megaterium* MTCC 453, *Pseudomonas fluorescens* LPK2 and *Trichoderma harzianum* MTCC 801. For careful "risk assessment", it is of interest to investigate the effect of application of such bioinoculants not only on the target crop, but also on the indigenous rhizospheric microbial community of that particular plant. To do so *C. cajan* treated with bioinoculants, individually as well as in combinations, was grown in pots under field conditions. Fingerprinting, using automated ribosomal spacer analysis showed distinct, highly diverse bacterial and fungal rhizospheric communities, which were differently influenced by the applied bioinoculants. Two important groups of soil microbes, actinomycetes and β-proteobacteria, were quantified using qPCR and shown to be little affected by the bioinoculants. Additionally, rhizosphere populations of groups to which the inoculants belonged were enumerated on selective media. An increase in abundance of phosphate solubilizing *Bacillus* sp. (73%), *Pseudomonas* sp. (42%), and fungi (53%) was observed with triple inoculation at maturity, as compared to control plants. Thus, there was no negative impact of the bioinoculants used in the study on specific groups of indigenous microbial community.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) and fungi, so-called "bioinoculants", have become an interesting possibility for sustainable, eco-friendly agriculture. To make use of such bioinoculants prudently, it is important to first assess their effects on the indigenous microbial community. The analysis of microbial counts on selective plates, a cultivation-dependent method has been used, since a long time to study soil microbial composition. However, 'the great plate count anomaly' (Staley and Konopka, 1985), which is due to the fact that majority of the soil microorganisms are effectively uncultivable (Brown et al., 2005), has led to the emergence of a number of cultivation-independent methods to analyze soil's complex microbial diversity.

Most importantly, various DNA fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993), terminal restriction fragment length polymorphism (T-RFLP) (Avaniss-Aghajani et al., 1994) and automated ribosomal spacer analysis (ARISA) (Fisher and Triplett, 1999), have been applied for unraveling environmental microbial diversity. In prokaryotes, the length of the intergenic transcribed spacer (IGS) region between the 16S-23S rRNA genes is inherently variable among species. ARISA exploits this length heterogeneity by utilizing PCR amplification across the IGS region to produce DNA fragment lengths characteristic of the taxa present in the sample (Brown et al., 2005). The method can be extended to fungi, where the species-specific size variation of the internal transcribed spacer region (ITS) is exploited (Ranjard et al., 2001).

In sustainable agriculture, the cultivation of legumes and their rotation with other crops is of great significance for increasing soil fertility. *Cajanus cajan* is an important legume crop with high N-fixation ability, with up to 79% N derived from atmospheric N_2 (Espana et al., 2006). Previous studies have reported the use of bioinoculants in different combinations to improve growth of *C. cajan* (Gupta et al., 2012; Niranjana et al., 2009). However, there is lack of knowledge of the "non-target" effects of these bioinoculants on native rhizospheric microbial community. Though in ecotoxicology, the term "non-target effects" refers only to undesired/negative implications of any application to treat a disease, in the manuscript we have included effects (both positive and negative), other than target effect on plant, as "non-target" as per the definition given by Winding et al. (2004), They define "non-target effects" as the effects

* Corresponding author. Tel.: +91 11 26596192; fax: +91 11 26582282.
E-mail address: shilpi@dbeb.iitd.ac.in (S. Sharma).

0929-1393/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.apsoil.2013.12.001

of introduced biocontrol agents on organisms other than target organisms or on biogeochemical cycles. The present study assesses the effects of three bioinoculants (*Bacillus megaterium* MTCC 453, *Pseudomonas fluorescens* LPK2 and *Trichoderma harzianum* MTCC 801) individually, and in their non-conventional combinations, on specific rhizospheric populations using both cultivation-dependent method and cultivation-independent methods.

2. Materials and methods

2.1. Soil characteristics, microbial strains and preparation of formulation

Soil used in this study was collected from an agricultural field of Delhi, India with the following properties: clayey loam (40% clay, 35% sand and 25% silt), organic matter content of 0.42%, pH of 7.2 (in water), electrical conductivity of 0.04 dS m^{-1}, and water holding capacity of 14%. The bioinoculants used in this study were *B. megaterium* MTCC 453, *P. fluorescens* LPK2, and *T. harzianum* MTCC 801. All the three strains were compatible with each other (Gupta et al., 2013). The microbial strains were applied as seed coats, using appropriate formulations as described earlier (Gupta et al., 2012).

2.2. Plant growth experiment and sampling

Seeds of *C. cajan* variety UPAS 120, an early maturing variety, were procured from National Seed Corporation (NSC), Indian Agricultural Research Institute (IARI), New Delhi. Seeds were surface-sterilized with 70% ethanol for 30 s followed by sterilization with NaClO (0.01%) for 2 min. The seeds were then washed with 0.01 N HCl to remove NaClO (Abdul-Baki, 1974) rinsed 8 times with sterile water and then treated with the appropriate mixture of bioinoculants according to Sarma et al. (2009). The nomenclature used for the treatments were: *B. megaterium* (B), *P. fluorescens* (P), and *T. harzianum* (T) individually; *B. megaterium* + *P. fluorescens* (BP), *B. megaterium* + *T. harzianum* (BT), and *P. fluorescens* + *T. harzianum* (PT) in combination, and BPT as mixed consortium (*B. megaterium* + *P. fluorescens* + *T. harzianum*); control i.e. seeds without any inoculation (C), unplanted (and no inoculation) soil (US). Four replicates were set up for each treatment and for each sampling point, using a completely randomized design. The pots were kept in approximately 12 h of daylight and temperature ranging from 21 °C to 30 °C. Samples were taken at different developmental stages of crop namely, vegetative (1 month after sowing), pre-flowering (2 months after sowing), flowering (3 months after sowing), and maturity stage (4 months after sowing). Rhizosphere soil samples were collected carefully by uprooting the root system from four randomly selected *C. cajan* plants for each treatment. The roots were shaken vigorously to separate soil loosely attached to the roots. The soil, tightly adhered to the roots, was collected using a soft brush without damaging the root and root nodules and this was termed as "rhizosphere soil". Each sample was divided into two parts, one kept at 4 °C and other stored at −20 °C (after shock freezing in liquid nitrogen) for cultivation-dependent and cultivation-independent studies, respectively.

2.3. DNA extraction

DNA was extracted from rhizosphere samples using the FastDNA® SPIN Kit for Soil and FastPrep® Instrument (MP Biomedicals, Santa Ana, CA, USA), according to manufacturer's instructions, except that the final elution was done in 40 μl of elution buffer.

2.4. Automated ribosomal spacer analysis (ARISA) fingerprinting

ARISA was used to study the genetic structure of microbial community in the rhizosphere samples. For ARISA of bacterial communities (B-ARISA), bacterial intergenic spacer located between the small- and large-subunit rRNA genes were amplified with the following primers: S-D-Bact-1522-b-S-20-FAM (5′-TGCGG CTGGATCCCCTCCTT-3′) and L-D-Bact-132-a-A-18 (5′-CCGGGT TTCCCCATTCGG-3′) (Ranjard et al., 2001). The fungal population was characterized by exploiting the length polymorphism of the internal transcribed spacer (F-ARISA), i.e. the ITS1-5.8S-ITS2 region, using the primers 2234C-FAM (5′-GTTTCCGTAGGTGAACCTGC-3′) and 3126T (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Ranjard et al., 2001). PCR conditions were as follows: 95 °C for 15 s, followed by 15 cycles of touchdown protocol from 60 °C to 55 °C for 45 s, 25 cycles at 55 °C, and extension at 72 °C for 45 s.

PCR products were run on 1% agarose gel and then analyzed using the ABI 3500 Genetic Analyzer. GeneScan™ 1200 LIZ® (Applied Biosystems, CA, USA) size standard was used to determine sizing up to 1200 bp.

Raw data generated by the ABI 3500 Genetic Analyzer were initially analyzed using GeneMapper® 4.1 (Applied Biosystems, CA, USA) to accurately determine fragment size. The data were then exported to Microsoft Excel® for further analysis. All values with fluorescence intensity less than 10 RFUs (relative fluorescence units) were excluded (Kovacs et al., 2010). Further, all fragments were assigned to bins of 3 bp for fragments up to 500 bp lengths, bins of 5 base pairs for fragments between 501 and 700 bp lengths, and bins of 7 bp for fragments between 701 and 1200 bp (Hewson and Fuhrman, 2004), and intensities were summed up for each bin. Each bin was considered an OTU (operational taxonomic unit). Next, relative intensities for each OTU in a given sample were calculated, and binned OTUs that contributed to less than 0.5% to the total intensity of the sample were excluded. Triplicates were compared among each other, and OTUs that appeared in only one of the triplicates were excluded, in order to minimize the effect of false peaks resulting from instrument noise, thus preventing overestimation of richness. Finally, richness was determined by counting the number of remaining OTUs in each sample (Kovacs et al., 2010). UPGMA cluster analysis was done using NTSYS software, version 2.1 (Applied Biostatistics Inc., New York) that required a 0-1 matrix prepared on the basis of presence or absence of the peak in the electrophoregrams, which in turn signifies the presence or absence of a specific microbial group in the rhizosphere samples.

2.5. Taxon-specific qPCR assays

Quantitative PCR (qPCR) was performed with primers specific for actinomycetes and β-proteobacteria (Fierer et al., 2005). Cycling conditions were: 15 min at 95 °C, followed by 40 cycles of 95 °C for 1 min, 30 s at 60 °C, and 72 °C for 1 min (Fierer et al., 2005). Each plate included triplicate reactions per DNA sample and the appropriate set of standards. Melting curve analysis of the PCR products confirmed that the fluorescence signal obtained was from specific PCR products and not from primer–dimers or other artifacts. Sequencing the PCR products followed by BLAST search confirmed the identity of target group, namely actinomycetes and β-proteobacteria, which corroborates to the result obtained from melt curve analysis. Serial dilutions of a known amount of the plasmid DNA containing the targeted genes were used to generate standard curves ($r^2 > 0.985$ for all assays; intercepts ranged between 24.8 and 27.5 and thresholds between 0.29 and 0.71 depending on the targeted genes).

2.6. Enumeration of specific rhizospheric microbial groups

One gram of rhizosphere soil was transferred to a 15 ml falcon tube containing 10 ml sterile distilled water and shaken vigorously. Serial dilutions were made and 0.05 ml aliquots (10^{-2} to 10^{-4}) were spread on plates containing Sperber (Molla and Chowdhury, 1984),