Biocatalysis and Agricultural Biotechnology 4 (2015) 456–464

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Original Research Paper

Synergistic action of PGP agents and *Rhizobium* spp. for improved plant growth, nutrient mobilization and yields in different leguminous crops

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form
10 September 2015
Accepted 10 September 2015
Available online 14 September 2015

Keywords:
Anabaena
Biofilm
Cyanobacteria
Leghaemoglobin
Legumes
Nodules
Rhizobium
Trichoderma
Yield

ABSTRACT

The effect of *Rhizobium* inoculation alone and in conjunction with plant growth promoting (PGP) microbial inoculants was evaluated in three leguminous crops – chickpea, pea and lentil. Significant enhancement in soil polysaccharide content and plant dry weight was recorded over control, in all those treatments receiving microbial inoculation. Nitrogen fixation (measured as ARA, acetylene reducing activity) in the treatment involving *Anabaena laxa* (RPAN8) showed a two folds increase over both *Rhizobium* inoculation and control in lentil, and in *Trichoderma–Rhizobium* treatment in pea crop. An increase of two-four folds in leghaemoglobin content and a doubling of yields was recorded in the combined microbial inoculation treatments, as compared to both *Rhizobium* inoculation or control, particularly in chickpea crop. A significant positive correlation was observed between nitrogen fixation with plant biomass and yields in all the three crops. Among the treatments, the use of cyanobacterium *Anabaena laxa* (RPAN8) and *Anabaena–Pseudomonas fluorescens* biofilmed inoculant, were most promisimg, besides biofilmed inoculants of *Trichoderma* or *Anabaena* with *Rhizobium* being among the top ranking treatments. These investigations illustrate the promise of cyanobacterial inoculants in various leguminous crops. Future focus needs to be towards understanding signalling mechanisms between *Rhizobium* and cyanobacteria and their positive feedback on plant growth and yield of legumes.
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1. Introduction

Plants and microorganisms are known to interact with one another and the rhizosphere is the key region influencing crop growth and yields. Rhizobacteria that benefit plant growth and development are known as Plant Growth Promoting Rhizobacteria (PGPR). The extensively evaluated PGPR belong to Gram-negative genera, mostly members of the fluorescent pseudomonads (Kloepper, 1993). Many reports also suggest that Gram-positive bacteria, such as *Bacillus* and their positive effect is also well documented (Beauchamp, 1993). Early studies on PGPR were mainly on root crops (Kloepper, 1993); however, later studies covered a wide range of hosts, including cereals, legumes and trees.

Legumes provide the essential proteins in vegetarian diet, particularly in India, therefore there is a need to improve their yields and sustain soil fertility using other microbial inoculants. They are grown approximately on 252 million hectares of land, leading to about 90 Tg (or 9×10^{13} g) of dinitrogen (N_2) being fixed per year, with major contributors to overall N_2 fixation through legume–*Rhizobium* symbiosis being the activities of *Rhizobium* spp. and *Bradyrhizobium* spp. (Smith, 1992). The growth of grain legumes such as field pea (*Pisum sativum* L.), followed by the subsequent decomposition of N rich residues helps to replenish N removed by harvesting. This leads to savings of fertilizer N and brings about enrichment of soil N, which is available to subsequent crops (Jensen and Hauggaard-Nielsen, 2003). Inoculation of plant growth-promoting bacteria led to improved nutrient uptake by pea grown in pots under semi-arid region of Uzbekistan (Egamberdiyeva and Höflich, 2004), besides enhancing N content of plant components.

A lot of information exists on the beneficial effect of *Rhizobium* and *Bradyrhizobium* on legumes in terms of biological nitrogen fixation (Werner, 1992); in cereal–legumes crop rotation systems, inoculation of rhizobia and bradyrhizobia in maize (*Zea mays* L.), significantly increased nodule volume, the dry weight of shoots, number of pods. The grain yield of the following green gram (*Vigna radiata* L.) or groundnut (*Arachis hypogea* L.). crops were also enhanced. Co-inoculation of PGPR and *Rhizobium*/*Bradyrhizobium*/*Mesorhizobium* species is known to increase root and shoot weight, plant vigour, nitrogen fixation and grain yield in various legumes

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http://dx.doi.org/10.1016/j.bcab.2015.09.004
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(Valverde et al., 2006; Yadegari et al., 2008; Verma et al., 2012) and indirectly showing antagonism to the surrounding plant pathogenic fungi in the rhizosphere.

PGPR are known to stimulate plant growth directly by producing phytohormones or eliciting plant defense mechanisms, which in turn, leads to increased nutrient uptake and growth (Lippmann et al., 1995) or induce systemic plant resistance towards pathogenic micro-organisms (Liu et al., 1995a, 1995b). By modulating and regulating the balance of deleterious vis a vis beneficial microbial activities in the rhizosphere, PGPR can stimulate plant growth (Lemanceau, 1992; Kloepper, 1993) and bring about biological control of root diseases. Therefore, synergistic consortia of microorganisms with various metabolic capacities (N_2-fixation, P-mobilization, production of phytohormones and bioactive molecules) can definitely perform better than single inoculations. However, type of inoculum, method of inoculation and agricultural practices can influence the effect of the inoculation. The effect of multiple inoculants with symbiotic N_2 fixing rhizobia, asymbiotic free- living N_2 fixing bacteria and phosphate solubilising bacteria or cyanobacteria has rarely been tested in legumes.

On the basis of the results drawn from the earlier investigations (Prasanna et al., 2013a, 2013b, 2014, 2015a, 2015b; Manjunath et al., 2011; Chaudhary et al., 2012), the aim of the present research was to investigate the effects of PGPR along with the specific *Rhizobium* for pea, lentil and chickpea and identify promising combinations for multi-location testing and use as inputs in integrated nutrient management strategies.

2. Materials and methods

2.1. Organisms used in this study and their maintenance

The microbial strains viz. *Bacillus* sp., *Azotobacter chroococcum* and *Rhizobium* spp., specific for each leguminous crop (*Rhizobium* sp. P10 for lentil, *Rhizobium* sp. 2–28 for pea and *Mesorhizobium ciceri* IC4059/SP9 for chickpea), and the set of two cyanobacterial strains – *Anabaena torulosa* and *Anabaena laxa* (RPAN8) were obtained from the germplasm of the Division of Microbiology, ICAR-IARI, New Delhi (Prasanna et al., 2008, 2013a), Indian Agricultural Research Institute, New Delhi. *Trichoderma viride* (ITCC 2211) was obtained from the Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi, India. The growth media used were Nutrient Broth for *Bacillus* sp. and Jensen's media for *Azotobacter*. The flasks were kept at 30 ± 2 °C in a shaking incubator at 120 rpm for 48 h. The cyanobacterial cultures were axenized by the standard procedure. These cultures were grown and maintained in N_2 free BG11 medium employing a set of antibiotics (Kaushik, 1987), under conditions of temperature – 27 ± 2 °C, light: dark cycles (16:8) and white light (50–55 μmol m^{-2} s^{-1}) in Haffkine flasks. The biofilms were prepared using co-inoculation technique taking *Anabaena torulosa* (BF1) and *Trichoderma* sp. as a matrix with the bacterial strains according to procedure as described in Prasanna et al. (2013b, 2015a). *Anabaena torulosa* was first grown for 7 days under the optimal conditions (27 ± 2 °C, light: dark cycles (16:8) and white light (50–55 μmol m^{-2} s^{-1}) in Haffkine flasks and inoculated with 48 h old bacterial/fungal inocula. After visible growth of biofilms in the flasks, the counts of bacteria/fungus were measured and after it reached 10^6–10^8/10^3 respectively, the biofilms were harvested manuallly by sieving, using layers of muslin cloth. The CFU/ml ranging from 10^7 to 10^8 was maintained for the bacterial partners in the cyanobacterial biofilms. Chlorophyll content was measured as in index of growth (Mackinney, 1941).

2.2. Preparation of formulations

Paddy straw compost prepared and used as carrier after mixing with vermiculite (1:1) (Chaudhary et al., 2012). For cyanobacterial cultures and their combination with other bacterial strains/biofilms, the chlorophyll content of 21 d old cultures (Mackinney, 1941) was measured and maintained as 100 μg g^{-1}carrier. These formulations were used to coat the overnight soaked seeds at the rate of 300 g for seeds equivalent to one acre, using 1% carboxy methyl cellulose (CMC).The water holding capacity of the carrier was measured and maintained as 40% by adding cultures and suitable amount of water.

2.3. Experimental set up

The pot experiment was conducted at the net house of the Division of Microbiology, Indian Agricultural Research Institute, New Delhi. This is situated at a latitude of 28°40′N and longitude of 77°12′E, having an altitude of 228.6 m above the mean sea level (Arabian sea). Delhi has a mean annual rainfall of 650 mm and more than 80% generally occurs during the south-west monsoon season (July–September). Three crops – pea (GP473), chickpea (Pusa 1103) and lentil (L4076) were used in this study. There were seven treatments per crop, viz., T1 (Control), T2 (*Rhizobium* sp.), T3 (*Trichoderma–Rhizobium*), T4 (*Trichoderma–Bacillus*), T5 (*Anabaena–Rhizobium*), T6 (*Anabaena–Pseudomonas*) T7 (RPAN8). *Trichoderma–Azotobacter* was used as an additional treatment for chickpea. Three replicates were taken for each treatment. 10 kg of soil was taken per pot with seed rate of six seeds/pot. Twenty gram formulation/pot was added after sowing of seeds.

2.4. Biometric observations

Samples were taken at mid-crop stage (after 30 days of sowing). Plant parameters such as total (root and shoot) weight were taken both as a fresh and dry weight. Dry weight of plants was measured by keeping the plants in hot air oven at 60 °C till the constant weight is obtained.

2.5. Soil parameters

Microbial biomass carbon was measured according to the protocol given by Nunan et al. (1998), taking approx. 17.5 g soil samples. Two sets of triplicate samples were taken and one was kept for fumigation with chloroform. All the samples were incubated in dark for 24 h. After 24 h, chloroform completely evaporated at 40–50 °C. 70 ml of 0.5 M K_2SO_4 was added to all the samples and kept for 30 min on shaker. The extracts were filtered through Whatman 42 filter paper and the K_2SO_4 extracts was digested with dichromatic reagent. MBC was calculated through back titration with ferrous ammonium sulphate and calculated using the equation: $-2.64 \times C_E$, where C_E=organic C from fumigated soil – organic C from nonfumigated soil. Microbial biomass C was expressed as μg C g^{-1} soil.

Available N was estimated using alkaline permanganate method (Subbiah and Asija, 1956). The procedure involves the distilling of 10 g of soil with alkaline potassium permanganate solution and determination of ammonium liberated by back titration with 0.2 N H_2SO_4. This serves as an index of the available nitrogen status.

The polysaccharides in soil (0.5 g) were estimated after suspending in 4 ml (12 N H_2SO_4), following the method of Lin (2005). After dilution, to 0.5 N by adding distilled water and autoclaving, the samples were filtered. 1 ml of the filtrate was withdrawn and was mixed with 1 ml of phenol solution (5%) and 5 ml of H_2SO_4 was added finally, The OD was recorded at 490 nm. The polysaccharide

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