

Received:
23 July 2015
Revised:
12 January 2016
Accepted:
15 January 2016

Heliyon (2016) e00066



Beneficial cyanobacteria and eubacteria synergistically enhance bioavailability of soil nutrients and yield of okra

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Abstract

Microorganisms in the rhizosphere mediate the cycling of nutrients, their enhanced mobilisation and facilitate their uptake, leading to increased root growth, biomass and yield of plants. We examined the promise of beneficial cyanobacteria and eubacteria as microbial inoculants, applied singly or in combination as consortia or biofilms, to improve growth and yields of okra. Interrelationships among the microbial activities and the micro/macro nutrient dynamics in soils and okra yield characteristics were assessed along with the changes in the soil microbiome. A significant effect of microbial inoculation on alkaline phosphatase activity was recorded both at the mid-crop and harvest stages. Microbial biomass carbon values were highest due to the *Anabaena* sp. - *Providencia* sp. (CR1 + PR3) application. The yield of okra ranged from 444.6–478.4 g⁻¹ plant and a positive correlation (0.69) recorded between yield and root weight. The application of *Azotobacter* led to the highest

root weight and yield. The concentration of Zn at mid-crop stage was 60–70% higher in the *Azotobacter* sp. and *Calothrix* sp. inoculated soils, as compared to uninoculated control. Iron concentration in soil was more than 2–3 folds higher than control at the mid-crop stage, especially due to the application of *Anabaena-Azotobacter* biofilm and *Azotobacter* sp. Both at the mid-crop and harvest stages, the PCR-DGGE profiles of eubacterial communities were similar among the uninoculated control, the *Anabaena* sp. - *Providencia* sp. (CW1 + PW5) and the *Anabaena-Azotobacter* biofilm treatments. Although the profiles of the *Azotobacter*, *Calothrix* and CR1 + PR3 treatments were identical at these stages of growth, the profile of CR1 + PR3 was clearly distinguishable. The performance of the inoculants, particularly *Calothrix* (T6) and consortium of *Anabaena* and *Providencia* (CR1 + PR3; T5), in terms of microbiological and nutrient data, along with generation of distinct PCR-DGGE profiles suggested their superiority and emphasized the utility of combining microbiological and molecular tools in the selection of effective microbial inoculants.

Keywords: Soil science, Agriculture, Ecology, Microbiology, Agronomy, Horticulture

1. Introduction

Reliance on chemical fertilizers, pesticides, and herbicides is extensive in modern agriculture (Santos et al., 2012; Pereg and McMillan 2015) and the resulting chemical residues are difficult to degrade, accumulative and harmful to plants, animals, and humans. These chemical inputs also slowly change the nutrient supplying capacity, making the soils even becoming infertile. Deficiencies of nutrients are becoming a major limiting factor in food production. In India, many agricultural soils in different geographical regions show deficiencies of most of the major and minor nutrients (Motsara 2012).

Soil microorganisms perform vital roles in the cycling of macro- and micronutrients and enhance plant health, soil fertility and productivity of the agro-ecosystems (Lian et al., 2010; Prasanna et al., 2012b). The biomass of microorganisms that store plant nutrients in soils acts, in fact, as slow release fertilizers, while their metabolic activities enhance the bioavailability of nutrients and their uptake by plants. Microorganisms help to release the fixed forms of nutrients and improve the carbon content of soil, by their growth, multiplication and metabolic activities (Malik et al., 2013; Gichangi et al., 2009; Malik et al., 2012; Ryazanova et al., 2009; Docampo et al., 2010; Manjunath et al., 2011). Application of microorganisms to soil or seed aids in their multiplication, facilitating nutrient cycling, and improving the productivity of agricultural crops (Singh et al., 2011). There is a definite need to integrate

different plant nutrient management practices for enhancing agricultural productivity and environmental sustainability (Adesemoye and Kloepper, 2009). In vegetable cultivation, the adverse environmental effects of chemical fertilizers and pesticides assume tremendous significance. Often, the vegetables are consumed fresh for their nutritive value, rather than for calorific requirements. Therefore, the application of biological inputs such as beneficial cyanobacteria, eubacteria and fungi may prove to be more environmentally safe as compared to chemical inputs.

Okra (*Abelmoschus esculentum* (L.) Moench.) belongs to the family Malvaceae and is commonly known as *bhindi* in India. It is an important foreign exchange crop as it contains a useful nutritional mix of carbohydrates, minerals, proteins and fibre; and constitutes upto 60% of the total exports of fresh vegetables, besides having industrial significance as a source of fibre in paper and food industry in India.

Okra is typically a warm season crop, which is grown in many tropical and subtropical parts of the world. Sandy loam or clay-loam soils are considered most suitable for its cultivation. Despite the large acreage of okra (upto 4.5×10^5 hectares in India with an annual production of 48.0×10^5 tons), yield losses as a result of insect pests, diseases and nematodes lead to low productivity of 10.6 t/ha. This crop is attacked by more than 70 insect pests, starting from the seedling to harvest stage (Kedar et al., 2013). Dawar et al. (2008) evaluated several biocontrol agents as seed dressings, especially to control nematode damage. They observed that *Trichoderma* spp. was as effective as chemical control/nematicide - fertinmakil. Most of the biocontrol agents comprise bacterial genera such as *Bacillus* and *Pseudomonas*, however, cyanobacteria are also globally gaining attention as inoculants for various crops (Mandal et al., 1998; Manjunath et al., 2010; Prasanna et al., 2009b; Prasanna et al., 2012a; Prasanna et al., 2015a, b; Zafar et al., 2012). Cyanobacteria are promising options, both as plant growth promoting and biocontrol agents in diverse crops including rice, wheat, cotton, legumes, and vegetables such as tomato (Manjunath et al., 2010; Prasanna et al., 2013a, b; Prasanna et al., 2014; Prasanna et al., 2015a, b; Swarnalakshmi et al., 2013). Although known primarily for their role as nitrogen supplements in rice paddies, several species of cyanobacteria can produce a spectrum of biocidal compounds and hydrolytic enzymes (Natarajan et al., 2013; Prasanna et al., 2008; Prasanna et al., 2010). The potential of these inoculants in okra has not been investigated.

Improving the bioavailability of macro and micronutrients in soil using microbial inoculants, thereby improving uptake and biofortification of crops or their produce can be cost-effective options to improve the nutritional quality and reduce malnutrition, particularly in the developing countries (Zhu et al., 2012).

Our earlier work on rice, wheat and maize with microbial inoculants has shown immense promise (Rana et al., 2012a, b; Rana et al., 2015; Prasanna et al., 2015a) in terms of bioavailability of macro- and micronutrients, not only in soils but also their enrichment in the harvested grains. Our recent study also shows that these organisms can modulate the microbiome of plant and soil, leading to the improved growth of plants such as rice (Priya et al., 2015). The present investigation aimed to identify promising cyanobacterial- and eubacterial inoculants, which could influence the soil microbiome, lead to the enhanced bioavailability of macro- and micronutrients in soils, and contribute to the improved yield of okra.

2. Materials and methods

2.1. Experimental site

The study was carried out at the farm of ICAR-Indian Institute of Vegetable Research (IIVR), Varanasi, India (82.52 °E longitude; 25.10 °N latitude). The area receives an average rainfall of 1000 mm which is distributed over a period of more than 100 days, with peak period between July and August. Scattered showers occur during winter months. In general, the temperatures range from 5 °C to 42 °C. January is the coldest month, while the maximum temperature is observed during May and June.

2.2. Organisms and their maintenance

The microbial cultures of *Azotobacter* sp., *Anabaena* sp. – *Providencia* sp. (CW1 + PW5) consortium; *Anabaena* sp.– *Azotobacter* sp. biofilm (An-Az biofilm), *Anabaena* sp. – *Providencia* sp. (CR1 + PR3) consortium, and *Calothrix* sp. were collected from the germplasm of the Division of Microbiology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi. The characteristics of these strains are given in our earlier publications (Nain et al., 2010; Prasanna et al., 2008; Prasanna et al., 2013a; Rana et al., 2012a). The cyanobacterial biofilm *Anabaena*–*Azotobacter* sp. was developed and characterized (Prasanna et al., 2011; Prasanna et al., 2013c). These organisms were evaluated earlier in the rice–wheat cropping system, tomato, cotton and legumes (Nain et al., 2010; Prasanna et al., 2013a, b; Prasanna et al., 2014; Prasanna et al., 2015a, b; Rana et al., 2012a, b). For growth and multiplication, Jensen's medium for *Azotobacter* sp., and nutrient broth for *Providencia* sp. were used. The flasks were incubated at 30 ± 2 °C in a shaking incubator. The cyanobacterial cultures were grown in nitrogen-free BG11 medium (Stanier et al., 1971), under a temperature of 27 ± 1 °C (light: dark cycles 16: 8) with white light ($50\text{--}55 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in Haffkine flasks. The protocols for

developing cyanobacterium based formulations, using compost: vermiculite (1:1) as a carrier were given earlier (Prasanna et al., 2014; Prasanna et al., 2015a, b).

2.3. Experimental set up

The pot experiment was undertaken, using a completely randomized design, under net house conditions, at the research farm of ICAR- IIVR, Varanasi during the *kharif* (wet) season of 2014. Pots containing 4 kg sterilised soil (Silt loam soil with pH of 7.82, EC - 0.14–0.36 dsm^{-1} ; organic carbon 0.5%) were used after mixing with fertilizers (NPK:25:8:25 kg/ha). The treatments were – T1: Control (no inoculation), T2: *Azotobacter* sp., T3: *Anabaena* sp.– *Azotobacter* sp. biofilm, T4: *Anabaena* sp. – *Providencia* sp. (CW1 + PW5), T5: *Anabaena* sp. – *Providencia* sp. (CR1 + PR3) and T6: *Calothrix* sp. The seeds were coated with the respective formulations using 1% carboxy methyl cellulose (CMC) as a sticker, air dried for 30 min and sown in pots. Each treatment was triplicated.

2.4. Analysis of soil chemical properties

The available concentrations of nitrogen, phosphorus, iron, copper, manganese and zinc in the soil samples were estimated for different treatments by standard protocols (Olsen et al., 1954; Prasad et al., 2006; Subbiah and Asija, 1956).

2.5. Soil enzyme activities

Dehydrogenase activity was analysed using the method of Casida et al. (1964) and expressed as μg of triphenyl formazan (TPF) g^{-1} soil d^{-1} . Soil samples (1 g each) were suspended in modified universal buffer (pH 11) and mixed with 1 mL p-nitrophenyl phosphate (Tabatabai and Bremner, 1969) for measuring the alkaline phosphatase activity. Total polysaccharides and total glomalin (GRSPs-Glomalin related soil proteins) were measured by the methods given by Liu et al. (2005) and Wright and Upadhyaya, (1996) respectively. The organic carbon of the soil, expressed as a percentage of carbon was measured following the methodology of Hesse, (1971).

2.6. Soil microbial biomass carbon (MBC)

Microbial biomass carbon (C) was estimated by the method of Nunan et al. (1998), using K_2SO_4 extracts obtained through dichromate digestion and calculated after back titration with ferrous ammonium sulphate. The following equation was used:

Biomass C = $2.64 \times \text{CE}$, where CE = organic C from fumigated soil – organic C from the un-fumigated soil. The microbial biomass C was expressed as $\mu\text{g C g}^{-1}$ soil.

2.7. Percent disease index

Percent disease index (PDI) (for the okra yellow vein mosaic disease) was calculated on the scale of 0–5 (0 = immune and 5 => 75% disease) using the formula:

$$\text{Percent disease index} = \frac{\text{Total sum of numerical rating}}{\text{No. of plants observed} \times \text{maximum grade value}}$$

2.8. Total DNA extraction from soils

Soil samples stored at -80 °C were processed by removing visible root pieces and blotted dry with sterile paper towels. PowerSoil DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) was employed for the isolation of total soil DNA, based on the manufacturer's instructions. The quality of total DNA preparation was examined on a 2% agarose gel after staining with ethidium bromide; the extracted DNA was stored at -20 °C until PCR analysis.

2.9. PCR-DGGE analysis

The polymerase chain reaction for 16S rDNA was performed using specific primers 341F with GC-clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCC CGCCGCCCCGCCCCGCC TACGGGAGGCAGCAG-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3') (Muyzer et al., 1993). The reaction mixture contained 1 × Taq buffer, 2.5 mM MgCl₂, 0.3 mM each of the deoxy nucleotide triphosphate, 10 pmol of each primer, 1U *Taq* DNA polymerase, 50 ng of template DNA and MilliQ water to a final volume of 25 μL. These were incubated in a PEQ lab Primus 96 Thermal cycler. The PCR reaction profile included: an initial denaturation at 94 °C for 2 min., followed by 35 cycles at 94 °C for 30 sec., 59 °C for 1 min., 72 °C for 2 min., and a final cycle of 8 min. at 72 °C for chain elongation. The quality of PCR amplicons were examined on horizontal 1.2% (w/v) agarose gel (0.5 mg ethidium bromide mL⁻¹) in TAE buffer (20 mM Tris acetate, 0.5 mM EDTA, pH 8.0) using the DNA mass ladders (Fermentas). The amplicons were then stored at -20 °C until the DGGE analysis.

The PCR amplicons were run on denaturing gradient gel using a Bio-Rad DCodeTM Universal Mutation Detection System (Bio-Rad, California). The gel solution of acrylamide/ N, N-methylene bisacrylamide (37.5:1, 40%) was filtered through 0.45 μm filter and stored at 4 °C. The buffer (50 × TAE Buffer, pH 8.0) was prepared using deionized water and filter-sterilized. The denaturing gradient gel (6% polyacrylamide gel prepared using the 40%-acrylamide/N, N-methylene bisacrylamide, 40% (v/v) formamide, 7 M urea and 1 × TAE) with 1 mm thickness was prepared with a linear gradient of the denaturant

concentration ranging from 30% to 70%. The amplicons with a uniform concentration of nucleic acids were loaded onto the gel and the gel was then run for 14 h at 60 °C and 150 V. The gel images after ethidium bromide staining were recorded and the gel pictures were imported to the GeneTools version 1.4.1.0 (Syngene, Cambridge) for band visualization, and cluster analysis. Analyses were repeated at least twice, using triplicate samples, to confirm the reproducibility of the data generated. The GeneTools software was used to calculate the Dice correlation coefficient, and for the construction of the dendrogram using the unweighted pair-group agglomeration method with arithmetic average (UPGMA).

2.10. Statistical analysis

The mean performances of different treatments for the specific parameter were analyzed using triplicate sets of data, by ANOVA using the Statistical Package for Social Sciences (SPSS, using WINDOWSTAT 8.0). The critical differences (CD at a probability of 0.05) among the treatments were calculated at 5% level of significance and given in Tables.

3. Results

3.1. Soil enzyme activities

Cyanobacterial inoculants had a significant effect on the soil enzyme activities (Table 1, Fig. 1 and Fig. 2). Dehydrogenase activity values ranged from 133.25–239.39 and 153.69–275.94 ($\mu\text{g g}^{-1}\text{d}^{-1}$) at mid-crop and at harvest stage respectively. All the microbial formulations, except T4 (CW1 + PW5) recorded significantly higher values for dehydrogenase activity, when compared to control, both at mid-crop and at harvest stage. Highest values were recorded in T6 (*Calothrix* sp.) followed by T5 (CR1 + PR3) and T3 (An-Az biofilm) at the mid-crop stage. At harvest stage, T6 (*Calothrix* sp.), T2 (*Azotobacter* sp.) and T5 (CR1 + PR3) recorded greater values. Alkaline phosphatase activity, glomalin and polysaccharide content were much higher in all the microbial treatments, as compared to control. The values of alkaline phosphatase activity ranged from 32.9–84.1 (mg g^{-1} soil) and 18.0–55.6 (mg g^{-1} soil) at mid-crop and at harvest stage respectively (Fig. 1a, b). At the mid-crop stage, highest values were recorded in T4 (CW1 + PW5), followed by T5 (CR1 + PR3). The treatments T6 (*Calothrix* sp.) and T3 (An-Az biofilm) recorded higher values at harvest stage. Polysaccharide values ranged from 3.47–9.68 and 2.93–9.40 (mg g^{-1} soil) at mid-crop and at harvest stage respectively, with T6 (*Calothrix* sp.) and T4 (CW1 + PW5) recording the highest values (Fig. 2a, b). Glomalin values ranged from 18.85–30.07 and 16.68–28.51 (mg g^{-1} soil) at mid-crop and at harvest stage respectively. T4 (CW1 + PW5), T6 (*Calothrix* sp.) and

Table 1. Comparative Analysis of variance (ANOVA) for soil parameters in okra crop, as influenced by microbial inoculants.

Source of Variance	df ^a	Parameters (Mean Square)							
		Alkaline Phosphatase Activity		Dehydrogenase Activity		Polysaccharide Content		Glomalin	
		Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage
Replicates	2	0.0002	0.0002	6.9206	4.7862	0.079350 ^a	0.0002	0.056929	0.22709
Treatment A	5	0.0874 ^b	0.0760 ^b	6111.7262 ^b	7829.4255 ^b	15.1677 ^b	15.6930 ^b	51.372997 ^b	58.80226 ^b
Error A	10	0.0002	0.0001	10.3074	39.32813	0.0142	0.0030	2.016303	0.95432
Total	17	0.0258	0.0225	1804.4438	2326.46945	4.4788	4.6174	16.302467	17.88286

^a Significant at 5%.

^b Significant at 1%.

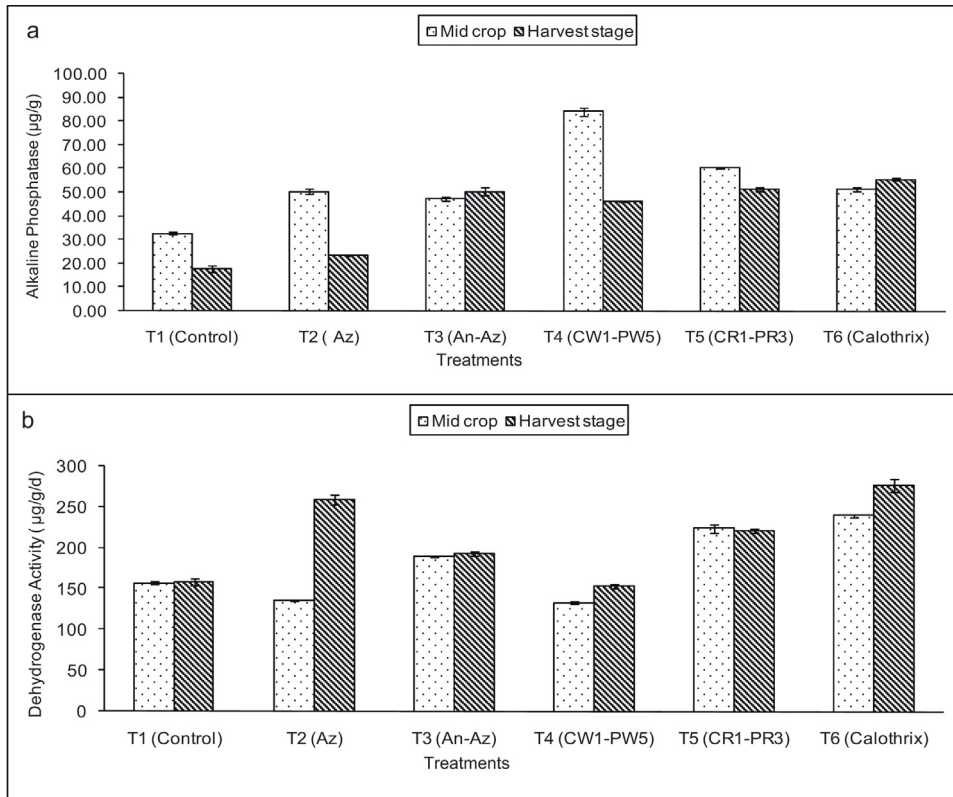


Fig. 1. Effect of microbial inoculants on soil enzyme activities in Okra rhizosphere. (a) Alkaline phosphatase activity; (b) Dehydrogenase activity.

T3 (An-Az biofilm) were top ranked at both stages of crop growth, in terms of glomalin content.

3.2. Bioavailability of soil macro- and micronutrients

Microbial treatments had a significant effect on the bioavailability of nutrients. Among the treatments, significant differences were observed in soil microbial biomass, % organic carbon, available N and available P, as well as micronutrients (Tables 2–5).

Available phosphorus values at mid-crop stage ranged from 34.35–65.15 (mg kg^{-1} soil). The treatment T3 (An-Az biofilm) recorded the highest values, which was on par with T1 (Control), T4 (CW1 + PW5) and T5 (CR1 + PR3). At harvest stage, available phosphorus values were much lower and ranged from 27.57–67.68 (mg kg^{-1} soil). T6 (*Calothrix* sp.) recorded a significantly higher value at harvest stage, which was almost two-folds higher as compared to mid crop stage. Carbon (%) values ranged from 1.7–2.13, with no significant differences observed among the treatments at both stages. Microbial biomass carbon values ranged from 462.2–1617.6 (mg kg^{-1} soil), with the highest values

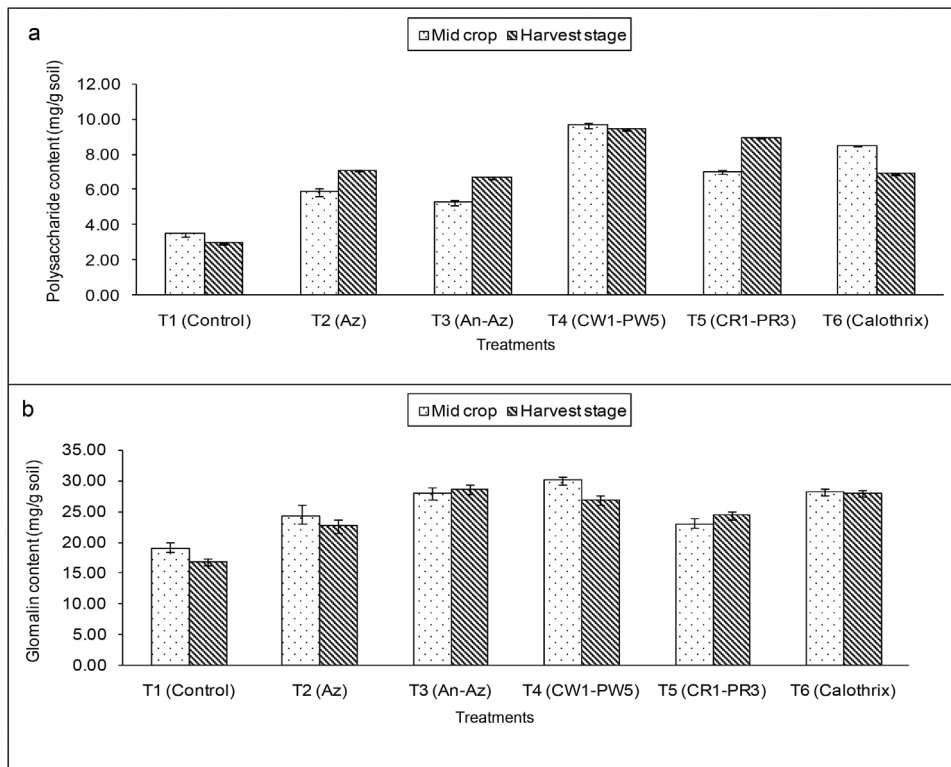


Fig. 2. Influence of microbial inoculation on soil attributes (a) Polysaccharides; (b) Glomalin.

recorded in T5 (CR1 + PR3), followed by T6 (*Calothrix* sp.) and T4 (CW1 + PW5). Microbial biomass carbon values at harvest stage, ranged from 407.1–2222.8 (mg kg^{-1} soil), with more than four-five-folds higher values in T2 (*Azotobacter* sp.), followed by T3 (An-Az biofilm) and T4 (CW1 + PW5), as compared to mid-crop values. Available nitrogen values ranged from 67.01–246.37 (kg ha^{-1} soil) at mid-crop stage. The treatment T6 (*Calothrix* sp.) recorded the highest values, which were significantly higher than the other treatments. At harvest stage, available nitrogen values ranged from 90.30–180.33 (kg ha^{-1} soil), with a sharp drop in control. The treatment T4 (CW1 + PW5) recorded the highest value, followed by T6 (*Calothrix* sp.) and T3 (An-Az biofilm). Although no general trend regarding the values at harvest stage could be surmised, T2 (*Azotobacter* sp.) and T6 (*Calothrix* sp.) showed an increase of 45–70 kg/ha at harvest stage, *vis a vis* mid-crop stage (Table 2 and Table 3).

At midcrop stage, all the micronutrients analysed (i.e., Fe, Cu, Zn, except Mn) in the microbial treatments showed an enhancement, compared to the respective controls (Table 4 and Table 5). The concentration of Zn was highest in T2 (*Azotobacter* sp.), followed by T6 (*Calothrix* sp.) and T4 (CW1 + PW5); more than 60–70% higher values, as compared to control. Iron concentration was more than 2–3 folds higher than control in

Table 2. Mean performance of microbial inoculants on the activity of soil macronutrient content in okra crop.

Treatment	Available Phosphorus (mg/kg soil)		Carbon (%)		Microbial Biomass Carbon (mg/kg soil)		Available Nitrogen (kg/ha soil)	
	Mid-Crop stage	Harvest Stage	Mid-Crop stage	Harvest Stage	Mid-Crop stage	Harvest Stage	Mid-Crop stage	Harvest Stage
T1 Control	61.23	31.71	1.70	1.77	495.2	407.1	90.30	67.01
T2 <i>Azotobacter</i>	44.91	38.08	1.80	1.47	462.2	2222.8	101.13	145.24
T3 (An-Az biofilm)	65.15	34.35	2.13	2.13	550.2	2003.7	129.40	162.85
T4 (CW1 + PW5)	50.32	27.57	2.10	2.27	594.2	1617.6	180.33	175.50
T5 (CR1 + PR3)	60.77	38.37	2.06	2.33	1617.6	1144.4	133.20	142.27
T6 (<i>Calothrix</i>)	34.35	67.68	2.10	2.37	1479.5	1485.5	175.40	246.37
CD 5%	14.04	7.39	0.50	0.39	291.5	270.2	32.9343	28.6708
CV %	14.62	10.25	13.80	10.42	18.5	10.0	13.413	10.067

T3 *Anabaena* sp.– *Azotobacter* sp. biofilm (An-Az biofilm), T4 *Anabaena* sp. – *Providencia* sp. (CW1 + PW5) consortium, T5 *Anabaena* sp. – *Providencia* sp. (CR1 + PR3) consortium.

Table 3. Comparative Analysis of variance (ANOVA) for soil macronutrients in okra crop, as influenced by cyanobacterial inoculants.

Source of Variance	df ^a	Parameters (Mean Square)							
		Microbial Biomass Carbon (mg/kg)		Available Phosphorus (mg/kg soil)		Carbon (%)		Available Nitrogen (kg/hectare soil)	
		Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest stage	Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage
Replicates	2	16334.90	3363.97	68.0971	64.3659	0.04167	0.0706	28.4955	144.6686
Treatment A	5	849262.60 ^b	1265169.00 ^b	41760152 ^b	616.1041 ^b	0.10233	0.3929 ^b	4120.1208 ^b	10089.4325 ^b
Error A	10	25683.44	22058.60	59.5859	16.5071	0.07500	0.0459	327.7162	248.3586
Total	17	266812.80	385480.10	165.8859	198.4896	0.0791	0.1509	1407.9330	3130.5931

^a Significant at 5%.^b Significant at 1%.

Table 4. Mean performance of microbial inoculants on the activity of soil micronutrient concentration in okra crop.

Treatment	Zinc (mg/kg)		Iron (mg/kg)		Manganese (mg/kg)		Copper (mg/kg)	
	Mid-Crop stage	Harvest Stage	Mid-Crop stage	Harvest Stage	Mid-Crop stage	Harvest Stage	Mid-Crop stage	Harvest Stage
T1 Control	0.891	0.778	0.505	0.258	0.381	0.275	0.300	0.478
T2 <i>Azotobacter</i>	1.403	1.215	1.174	0.659	0.388	0.687	1.174	1.327
T3 (<i>An-Az biofilm</i>)	1.240	1.010	2.445	1.872	0.405	0.202	1.147	0.625
T4 (CW1 + PW5)	1.262	0.734	0.892	0.635	0.685	0.726	1.024	0.350
T5 (CR1 + PR3)	1.151	0.545	0.822	0.992	0.347	0.280	1.071	0.417
T6 (<i>Calothrix</i>)	1.302	0.651	0.873	1.231	0.268	0.264	0.477	1.220
CD 5%	0.231	0.165	0.495	0.706	0.128	0.067	0.141	0.091
CV %	10.54	11.03	24.34	13.97	17.04	9.12	8.984	6.77

T3 *Anabaena* sp.– *Azotobacter* sp. biofilm (An-Az biofilm), T4 *Anabaena* sp. – *Providencia* sp. (CW1 + PW5) consortium, T5 *Anabaena* sp. – *Providencia* sp. (CR1 + PR3) consortium.

Table 5. Comparative Analysis of variance (ANOVA) for soil micronutrient in okra crop, as influenced by microbial inoculants.

Source of Variance	df ^a	Parameters (Mean Square)							
		Zinc Concentration (mg/kg)		Iron Concentration (mg/kg)		Manganese Concentration (mg/kg)		Copper Concentration (mg/kg)	
		Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage	Mid-crop stage	Harvest Stage
Replicates	2	0.045254	0.0264	0.01207	0.0076	0.00267	0.0045	0.0133	0.0038
Treatment A	5	0.0927 ^b	0.1832 ^b	1.4031 ^b	0.6888 ^b	0.0607 ^b	0.1657 ^b	0.4273 ^b	0.5477 ^b
Error A	10	0.0162	0.0082	0.0741	0.0201	0.0049	0.0014	0.0060	0.0024
Total	17	0.0421	0.0618	0.4577	0.2153	0.0211	0.0500	0.1308	0.1630

^a Significant at 5%.^b Significant at 1%.

T3 (An-Az biofilm) and T2 (*Azotobacter* sp.) and significantly higher in the other inoculated treatments. Manganese concentration was highest in T4 (CW1 + PW5), T3 (An-Az biofilm) and T2 (*Azotobacter* sp.), but very low in T6 (*Calothrix* sp.). Copper concentration was more in T2 (*Azotobacter* sp.), followed by T3 (An-Az biofilm) and T5 (CR1 + PR3).

At the harvest stage, the concentration of Zn was highest in T2 (*Azotobacter* sp.), and T3 (An-Az biofilm), T4 (CW1 + PW5) and T1 (control) recorded at par values. Iron concentration was almost 7–8 folds higher than control (T1), in T3 (An-Az biofilm) followed by T6 (*Calothrix* sp.) and T5 (CR1 + PR3). Manganese concentration was 40–50% higher in T4 (CW1 + PW5), T2 (*Azotobacter* sp.) T5 (CR1 + PR3) and T1 (control) recorded at par values. Copper concentration was 2.8 folds higher than control (T1) in T2 (*Azotobacter* sp.), followed by T6, T3 and T5.

3.3. Plant parameters at harvest stage

Microbial treatments had a significant effect on root weight, yield and disease indices (Table 6). Root weight values ranged from 10.59–14.97 g⁻¹ plant. The treatment T3 (An-Az biofilm) recorded highest values, followed by T2 (*Azotobacter* sp.) and T6 (*Calothrix* sp.), however treatment T4 (CW1 + PW5) showed lower values than control (Fig. 3a). Yield values ranged from 444.6–478.4 g⁻¹ plant; highest values were recorded in T2 (*Azotobacter* sp.), followed by T5 (CR1 + PR3) and T3 (An-Az biofilm) (Fig. 3b; Table 6). Indices of okra (*bhendi*) yellow vein mosaic disease ranged from 30.0–46.7%. No significant differences were observed with respect to control; however, the treatments T3 (An-Az biofilm), T5 (CR1 + PR3) and T2 (*Azotobacter* sp.) recorded 7–13% lower percent disease indices, as compared to control (Fig. 3c).

Table 6. Analysis of variance (ANOVA) for plant parameters in okra crop, as influenced by microbial inoculants.

Source of Variance	df ^a	Parameters (Mean Square)		
		Root weight (Harvest stage)	Yield (Harvest stage)	Disease % (Harvest stage)
Replicates	2	0.43844	174.47053	216.66667
Treatment A	5	8.56666 ^b	639.74799 ^b	116.66667
Error A	10	0.99575	84.13200	63.33333
Total	17	3.15692	258.17644	97.05882

^a Significant at 5%.

^b Significant at 1%.

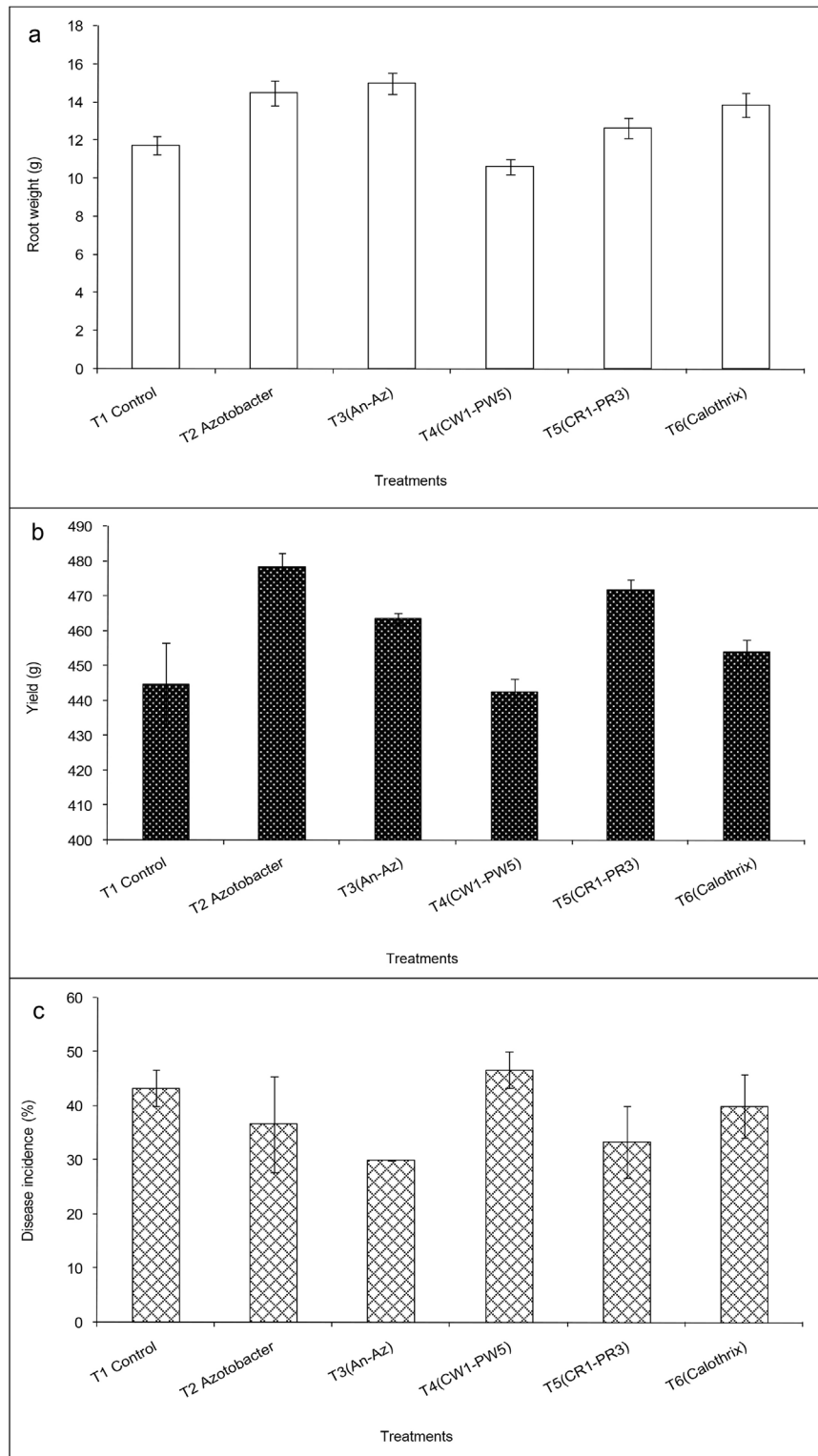


Fig. 3. Influence of microbial inoculation on (a) Root weight; (b) Yield; (c) Disease indices. Details of treatments given in Tables and Materials and Methods.

3.4. PCR-DGGE analyses

The eubacterial communities of rhizosphere soil samples from different treatments [two stages: Mid-crop (M) and Harvest (H) stages with Control (T1), *Azotobacter* (T2), *Anabaena-Azotobacter* biofilm (T3), *Providencia* (CW1 + PW5) (T4), *Providencia* (CR1 + PR3) (T5) and *Calothrix* (T6)] were analysed by the PCR-DGGE fingerprints (Fig. 4a). The profiles of eubacterial communities of Control and those treated with CW1 + PW5 (T4) and An-Az biofilm (T3) both at the mid-crop and harvest stages were almost similar. Likewise, the profiles of those treated with Az (T2), *Calothrix* (T6) and

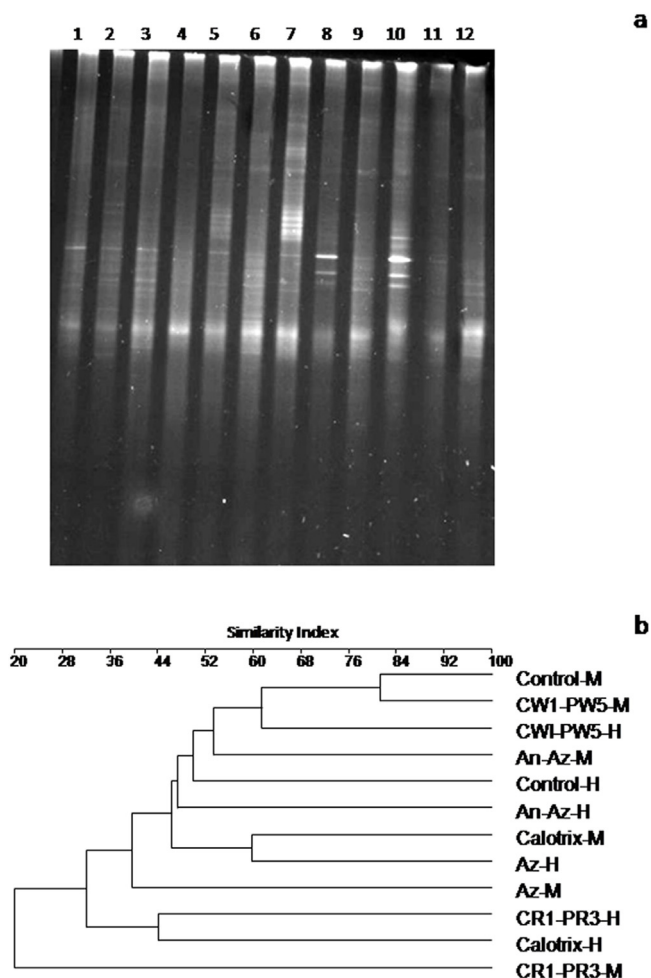


Fig. 4. (a) PCR-DGGE profiles of eubacterial communities from the rhizosphere soil treated with different bioinoculants: Lanes: 1- Control-Mid Crop Stage (M); 2-*Azotobacter*-M; 3-*Anabaena-Azotobacter* (An-Az biofilm)-M; 4- *Providencia* (CW1 + PW5)-M; 5-*Providencia* (CR1 + PR3)-M; 6-*Calothrix*-M; 7- Control-Harvest Stage (H); 8-*Azotobacter*-H; 9-*Anabaena-Azotobacter* (An-Az biofilm)-H; 10- *Providencia* (CW1 + PW5)-H; 11-*Providencia* (CR1 + PR3)-H; 12-*Calothrix*-H; (b) Dendrogram from the multivariate cluster: Dice's cluster analysis of the eubacterial DGGE community patterns of rhizosphere soil treated with different inoculants.

CR1 + PR3 (T5) both at the mid-crop and harvest stages were similar. However, the profile of CR1 + PR3 was distinct (Fig. 4b).

4. Discussion

In this study, microbial inoculation brought about significant differences in the activities of alkaline phosphatase and dehydrogenase, and the concentrations of glomalin and polysaccharides in the soil. Mader et al. (2011) observed up to 74% increase in root weight due to the inoculation of beneficial microorganisms. The treatment T5 (CR1 + PR3) recorded highest microbial biomass carbon and ranked second for alkaline phosphatase, dehydrogenase, and ranked third for polysaccharide, carbon (%) and available nitrogen at mid-crop stage. This treatment also exhibited the highest values for available phosphorus, second for yield, and the third for iron and copper concentration at harvest stage. A lowering of disease incidence by 10% over control was observed in this treatment. The potential of this treatment may be due to the synergistic action of bacteria and cyanobacteria (CR1 + PR3) in this treatment.

Prasanna et al. (2009a, b; 2012a; 2013b; 2014; 2015b) reported for the first time, the existence of cyanobacteria in the rhizosphere of crops and their positive influence on plant growth and availability of nutrients. Earlier, Prajapati et al. (2013) reported that the seed and soil application of microbial inoculants improved the growth of okra plants. In the present investigation, *Azotobacter* (T2) recorded highest root weight, followed by the An-Az biofilm (T3). The highest yield was recorded due to the application of *Azotobacter* (T2). A positive correlation (0.69) between the yield and root weight was also recorded in the present study. A significant enhancement in microbial biomass C and crop yield were observed in the treatments *Azotobacter* (T2) and An-Az biofilm (T3), as compared to control. The available nitrogen ranged from 101.13–246.37 kg ha⁻¹ soil in the microbial treatments, whereas in the control only values of 90.30 and 67.0 kg ha⁻¹ at mid-crop and harvest stages respectively were observed. This increase in nitrogen content may be ascribed to nitrogen fixation ability of the inoculated microorganisms. The multifaceted beneficial effects of *Azotobacter* sp. are widely reported across the globe. They are known to fix nitrogen and produce plant hormones (IAA) and vitamins (Abd El-Fattah et al., 2013; Revillas et al., 2000; Gholami et al., 2009). The role of cyanobacteria and their biofilm inoculants in disease suppressiveness or as biocontrol agents, besides nitrogen fixation, plant growth promotion and improving the availability of macro and micronutrients is also well established (Dukare et al., 2011; Venkataraman, 1972; Mandal et al., 1998; Prasanna et al., 2013a; Prasanna et al., 2015a, b). The present study demonstrated their multiple benefits, in the growth and development of okra.

Alkaline phosphatase is a key enzyme of phosphorus cycle, which is intimately related to the content of soil organic matter. Most of the earlier reports link this enzyme activity with microbial biomass C; hence, is considered one of the best indicators of relative numbers of microbial communities in soils (Richardson et al., 2009; Nain et al., 2010). In the present study, the treatments T6 (*Calothrix* sp.), T3 (An-Az biofilm) and T5 (CR1 + PR3) exhibited high values for this enzyme, reflective of their positive effects on P mobilization in the soil. A similar enhancement has been reported by Mader et al. (2011) and Pramanik et al. (2007). Total microbial activity in soil is better elucidated by the estimation of dehydrogenase activity, which is associated with respiratory processes (Bolton et al., 1985), therefore considered as an index of microbial activity (Nannipieri et al., 1990). In our study, an increase of 51.86% in dehydrogenase activity over control was observed.

The rhizosphere is a complex environment which plays an important role in the ecological fitness of the plant hosts, by providing a niche where microorganisms can play diverse roles in growth promotion, colonization, geochemical cycling of nutrients and their mobilization for plant uptake. Among the major properties of PGPRs relevant to nutrient acquisition or transformations, the production of siderophores, which sequester iron from the soil and make it available to the plants (Schwyn and Neilands, 1987) are extremely important. The bacterial strains used in this study are known to be siderophore producers (Rana et al., 2012a), which can lead to not only higher iron concentration in the root zone of the plants, but also facilitate increased root activity and higher uptake of Fe in the plants, and its translocation to the various parts of the plant. Zinc (Zn) is one of the most common micronutrients that have been found deficient predominantly in cereal-based diets (Cakmak et al., 2010) which is ranked as the fifth most important risk factor and health indicator in the developing world (WHO, 2011). In the present study, T2 (*Azotobacter*) and T3 (*Anabaena-Azotobacter* biofilm) recorded highest values for the availability of Zn, Fe and Copper in the soil, at both at the mid-crop and harvest stages, respectively. Tariq et al. (2007) showed that a commercial mixed PGPR consortium (containing *Pseudomonas* sp. and other strains of PGPR) was effective as a Zn solubilizer, increasing Zn concentration up to 157%. Even the application of *Pseudomonas* and *Acinetobacter* strains resulted in the enhanced uptake of Fe, Zn, Mg, Ca, K, and P by crop plants (Khan, 2005). Being the third most limiting nutrient for plant growth, iron, primarily due to the low solubility of the oxidized ferric form in aerobic environments (Samaranayake et al., 2012) is a major problem. However, several cyanobacteria, including unicellular marine forms possess the ability to sequester Fe or Zn using metallothioneins (Barnett et al., 2012). Prasanna et al. (2015a) evaluated a set of maize hybrids and found that cyanobacterial inoculants can bring about an enhancement in the

mobilization of Zn to flag leaf in maize hybrids, without any negative effects on plant vigour and yields.

Rana et al. (2012a, b, 2015), observed that in wheat, besides the enhancement in the uptake of N, P and micronutrients by bacterial inoculants and cyanobacteria, a positive involvement of such traits was observed in the increase of yield, micronutrient concentration and their uptake in wheat grains. The combined inoculation of *Anabaena oscillarioides* CR3, *Brevundimonas diminuta* PR7 and *Ochrobactrum anthropi* PR10 was found to significantly increase NPK content and improve rice yield by almost 21.2%, as compared to the application of recommended dose of NPK fertilizers alone. Analysis of variance (ANOVA) illustrated the significant effect of the microbial treatments, in terms of dehydrogenase activity in soil and Fe / Zn concentration in soil illustrating the positive correlation of microbial activity with the enhanced bioavailability of micronutrients. de Santiago et al. (2011) observed that inoculating a siderophore producing strain *Trichoderma asperellum* led to significant enhancement in the Fe concentration in the aerial parts of wheat. Sharma et al. (2013) observed two-fold higher translocation efficiency of iron from roots to grains in the treatments involving the inoculation of PGP bacterial strains - *P. putida*, *P. fluorescens* and *Azospirillum lipoferum*.

The present study is a beginning towards the deployment of microorganisms to enhance the bioavailability of macro/micronutrients in soils, which can be then be taken up more effectively by plants. The aspects related to microbe mediated biofortification is discussed only in scattered reports, mainly in cereals (Mader et al. 2011; Prasanna et al., 2012a, b; Prasanna et al., 2015a, b; Rana et al., 2012a, b; Rana et al., 2015) and the results of the present investigation, further emphasize the promise of microbial consortia in vegetable crops.

Yellow vein mosaic disease (YVMD) is a key limitation to the production of okra; this disease is caused by whitefly-transmitted begomoviruses (Venkataravanappa et al., 2015). The application of rhizobacterial formulations to seed, soil and foliage significantly reduced the occurrence of *bhendi* yellow vein mosaic virus (BYVMV) and enhancement of growth in the glasshouse experiment with okra (Jagadeesh et al., 2011). No significant differences were observed in the incidence of BYVMV in the present study, but lower disease incidence (7–13%) was recorded in the inoculated treatments. This illustrates that these formulations may show anti-viral properties, which can be an interesting lead for further investigation. Though cyanobacteria are well known for their antifungal properties (Manjunath et al., 2010; Natarajan et al., 2013; Prasanna et al., 2008; Prasanna et al., 2010; Prasanna et al., 2013b), there is a strong need to understand the mechanisms of interactions of cyanobacterial inoculation on viral diseases.

Interactions between plant-roots, surrounding soil and the microbial populations within the soil strongly influence the growth and productivity of plants (Pereg and McMillan 2015). The soil microbiome was observed to be altered by the application of microbial formulations. In the present study, the PCR-DGGE followed by the cluster analysis of eubacterial profiles suggested that the *Calothrix* (T6) and a consortium of *Anabaena* and *Providencia* (CR1 + PR3; T5) based inoculants influenced the rhizosphere eubacterial communities till the harvest stage. This was supportive of these two treatments being the top performers in terms of both the microbiological activity and bioavailability of nutrients.

5. Conclusions

The inoculation studies with beneficial cyanobacteria and eubacteria led to significant enhancement in the bioavailability of macro and micronutrients in the rhizosphere region of okra. Besides the differences related to biocontrol, there were increased root weight, yield and soil enzyme activities. The soil microbiome analyses showed the significant role played by cyanobacterial and/or eubacterial inoculation on the native microbial communities, and their interactions towards improved nutrient availability, growth and crop yields of okra.

Declarations

Author contribution statement

Mallappa Manjunath: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Amrita Kanchan, Kunal Ranjan, Siddarthan Venkatachalam, Firoz Hossain, and Yashbir Singh Shivay: Analyzed and interpreted the data.

Radha Prasanna: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analytical tools or data; Wrote the paper.

Balasubramanian Ramakrishnan: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lata Nain: Conceived and designed the experiments.

Awadhesh Bahadur Rai, Bijendra Singh: Contributed reagents, materials, analytical tools or data.

Funding statement

This work was supported by the ICAR-AMAAS and DST-SERB projects.

Conflict of interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors are grateful to the authorities of the ICAR-IIVR, Varanasi and Division of Microbiology, ICAR-IARI, New Delhi for providing the facilities towards undertaking this study. The authors gratefully acknowledge the Division of Agronomy, ICAR-IARI, New Delhi for providing necessary facilities for the analyses of soil samples.

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