Expl Agric. (2015), volume 51 (1), pp. 42–65 © *Cambridge University Press 2014*
doi:10.1017/S0014479714000143

PROSPECTING CYANOBACTERIA-FORTIFIED COMPOSTS AS PLANT GROWTH PROMOTING AND BIOCONTROL AGENTS IN COTTON

By RADHA PRASANNA†,‡, SANTOSH BABU†, NGANGOM BIDYARANI†, ARUN KUMAR†, SODIMALLA TRIVENI†, DILIP MONGA§, ARUP KUMAR MUKHERJEE¶, SANDHYA KRANTHI††, NANDINI GOKTE-NARKHEDKAR††, ANURUP ADAK†, KULDEEP YADAV†, LATA NAIN† *and* ANIL KUMAR SAXENA†

†*Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi 110012, India*, §*Central Institute for Cotton Research, Regional Station, Sirsa 125055, Haryana, India*, ¶*Central Rice Research Institute, Cuttack 753006, Odisha, India and* ††*Central Institute for Cotton Research, Nagpur 441108, Maharashtra, India*

(*Accepted 1 May 2014*; *First published online 17 June 2014*)

SUMMARY

The potential of cyanobacteria-based compost formulations was evaluated in cotton crop at two agro-ecological locations (Nagpur and Sirsa) as plant growth promoting (PGP) and biocontrol agents. Compost-based formulations fortified with *Calothrix* sp. or *Anabaena* sp. enhanced germination and fresh weight of plants, and microbiological activity by 10–15%, besides increased available nitrogen (by 20–50%) in soil at Nagpur. In the fungi-infected fields at Sirsa, *Anabaena–T. viride* biofilmed formulation performed the best, recording 11.1% lower plant mortality than commercial *Trichoderma* formulation. Scanning electron microscopy confirmed the colonisation of inoculated cyanobacteria/biofilms on roots. Significant correlation between mortality, increased activity of hydrolytic enzymes and fresh weight of plant roots were recorded. *Calothrix* sp. and *Anabaena* sp. proved promising as both PGP and biocontrol agents, while biofilmed formulations substantially reduced mortality of cotton plants in sick plots. This study illustrates the promise of cyanobacteria as viable inoculation option for integrated nutrient and pest management strategies of cotton.

INTRODUCTION

Microbes in soil catalyze essential transformations of major and minor elements and also help the crop plants combat abiotic and biotic stress (Brussaard *et al.*, 2007; Persello-Cartieaux *et al.*, 2003). Among various soil inhabitants, cyanobacteria are oxygenic photosynthetic prokaryotes and represent members of the first trophic level. Cyanobacteria have been widely employed as inoculants for enhancing soil fertility and improving soil structure besides their significant role as plant growth promoting (PGP) agents (Mandal *et al.*, 1999), especially in rice crop and more recently in wheat and vegetables (Karthikeyan *et al.*, 2007; Nain *et al.*, 2010; Prasanna *et al.* 2012,

‡Corresponding author: Email: radhapr@gmail.com

2013a, b, c; Venkataraman, 1981). But to our knowledge, there is scanty published literature on cotton crop, especially at field level.

The significance of cyanobacteria as producers of cyanotoxins and other novel bioactive molecules is globally recognised (Jaiswal *et al.*, 2008; Mundt *et al.*, 2001); however, their chemical potential is less explored in agriculture, especially as biocontrol agents. Few reports are available on the activity of hydrolytic enzymes such as chitosanases, xylanases and endoglucanases in *Anabaena* strains (Gupta *et al.*, 2010; Prasanna *et al.*, 2008) and their positive correlation with fungicidal activity. This has diversified the significant role of cyanobacteria beyond nitrogen fixation or phytohormones-mediated plant growth promotion (Nayak *et al.*, 2004; Prasanna *et al.*, 2009, 2012; Sergeeva *et al.*, 2002). The colonization of plants by cyanobacteria is known to trigger the release of a diverse array of biologically active metabolites in rhizosphere, which elicit induced systemic responses in plant, and enhance plant growth, even under conditions of abiotic or biotic stress (Prasanna *et al.*, 2013a, b, c). The use of cyanobacteria as a matrix for developing biofilmed biofertilizers is an economically attractive option (Prasanna *et al.*, 2011), providing multiple benefits, especially in terms of nutrient savings, as most cyanobacteria show independence for carbon and nitrogen and can support useful heterotrophic bacteria and fungi as consortia or biofilms. Carrier-based preparations using these biofilms have shown promise as PGP inoculants for wheat crop, tomato and legumes (Prasanna *et al.*, 2013b, c; Swarnalakshmi *et al.*, 2013), but need to be explored in other crops.

As a fibre crop, cotton (*Gossypium* sp.) is economically very important and India represents the 3rd largest country in terms of its production. Cotton crop is affected by several diseases, particularly root rot disease, caused by a complex of pathogenic soil inhabiting fungi comprising mainly *R. solani*, *Pythium* spp. and *Fusarium* spp., which also leads to severe losses in a wide variety of crop plants. The increased pressure from public and environmental scientists on the environmental concerns of transgenic cotton (having gene from Bt *or Bacillus thuringiensis*) and ill effects of chemical pesticides has stimulated the genesis of biocontrol agents (Nakkeeran *et al.*, 2005). *Trichoderma*, fluorescent *Pseudomonas* and endophytes have been developed and used in field experiments with a number of crops as biocontrol agents (Singh *et al.*, 1999). The extracts of cyanobacteria are known to reduce the incidence of *Botrytis cinerea* on strawberries and *Erysiphe polygoni* causing powdery mildew on turnips and damping off in tomato seedlings, besides reducing the growth of saprophytes – *Chaetomium globosum*, *Cunninghamella blakesleeana* and *Aspergillus oryzae* and plant pathogens such as *Rhizoctonia solani* and *Sclerotiana sclerotium* (Kulik, 1995). The tripartite interaction of tomato–*Fusarium*–*Anabaena* sp. as a bio-protection strategy against *Fusarium* wilt was illustrated in terms of higher nutrient content, build up of defence against phyopathogenic fungi and improved yield in tomato plants treated with *Anabaena* sp.-amended formulations (Prasanna *et al.*, 2013b). It is well recognized that biofilms possess the capacity to maintain metabolic activity under adverse environmental conditions and exhibit increased survival in a competitive environment. The presence of mucilaginous matrix of cyanobacteria when used individually or as a member of consortia or biofilmed preparations can provide an effective strategy for growth in crops besides rice, as

recorded earlier with legumes (Prasanna *et al*., 2013c). Notwithstanding a steady increase in the number of publications on the role of cyanobacteria in disease reduction and protection against fungal diseases (Dukare *et al*., 2011; Manjunath *et al*., 2010; Zulpa *et al*., 2003), no published reports on cyanobacterial formulations for a dryland (or rainfed) crop such as cotton are available.

The aim of the present study was, therefore, to evaluate selected cyanobacterial strains, consortia and biofilms-amended compost formulations as PGP and disease suppressive agents in cotton crop under field conditions.

MATERIALS AND METHODS

Organisms used in this study, their growth and maintenance

The cyanobacterial strains – *Calothrix elenkinii*, *Anabaena laxa* RPAN8, *Anabaena variabilis* (RPAN59), *Anabaena* sp. (CW1), members of the cyanobacterial consortium – BF1 *Anabaena torulosa*; BF2 *Nostoc carneum*; BF3 *Nostoc piscinale* and BF4 *Anabaena doliolum* (Nain *et al*., 2010; Prasanna *et al*., 2008; 2013a) were obtained from the germplasm of the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi, India. The details of these strains and their ecological habitats are given in earlier investigations (Prasanna *et al*., 2008; 2013a). *Calothrix elenkinii*, *Anabaena laxa* RPAN8 and *Anabaena variabilis* (RPAN59) have been evaluated for their fungicidal properties (Natarajan *et al*., 2012; Prasanna *et al*., 2008; 2013a) and as an inoculant for rice, wheat, tomato and legumes in earlier investigations (Nain *et al*., 2010; Prasanna *et al*., 2012, 2013a, b, c). The bacterial strains viz. *Azotobacter* sp. (CBD15), *Pseudomonas fluorescens* (PF1), *Bacillus subtilis* (RP24) and *Providencia* sp. (PW5) were obtained from the germplasm of the Division of Microbiology, IARI, New Delhi. The fungal strain, *Trichoderma viride* (ITCC 2211) was obtained from the Indian Type Culture Collection (ITCC), Division of Pathology, IARI, New Delhi. Cyanobacterial biofilms were prepared using *Anabaena torulosa* (BF1), which is a PGP bacterium, characterized previously for its biochemical attributes and agronomic efficacy, including fungicidal activity (Prasanna *et al*., 2011, 2013a; Swarnalakshmi *et al*., 2013). In the present investigation, the biofilms of *Anabaena–T. viride*, *Anabaena–B. subtilis*, *Anabaena–Azotobacter* and *Anabaena–Pseudomonas* were used. The growth media used were Jensen's medium for *Azotobacter* sp., King's B medium for *Pseudomonas* sp., Nutrient broth for *Bacillus* sp. and *Providencia* sp. and Potato Dextrose Agar for *Trichoderma viride*. The flasks were incubated at 30 ± 2 °C in a shaking incubator, except for *T. viride*, which was maintained as a static culture at 30 °C. The cyanobacterial cultures were axenized by standard procedure employing a set of antibiotics, after the optimization of dose of streptomycin, chloramphenicol and penicillin for each isolate (Kaushik 1987), grown and maintained in nitrogen-free BG11 medium (Stanier *et al*., 1971) under a temperature of 27 ± 1 °C and 16:8 (light:dark cycles) white light (50–55 μmol photons m^{-2} s^{-1}) as static cultures in Haffkine flasks.

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

Preparation of biofilms/cyanobacteria/consortia-amended formulations and pre-inoculation testing of formulations

All the biofilms were extracted from flasks and thoroughly washed with distilled water and centrifuged to remove individual cells, and such uniform suspensions were mixed with carrier (vermiculite:compost, 1:1) and used as described earlier with Colony Forming Units (CFU) maintained at 10^7–10^{10} g^{-1} of bacterial/fungal partners and chlorophyll *a* content of 100 μg chlorophyll g^{-1} carrier (Prasanna *et al.*, 2013b; Swarnalakshmi *et al.*, 2013). The germination of cotton seeds has been checked using triplicate samples of 10 seeds each before sowing using 0.8% water agar plates in a growth chamber under controlled conditions. The germination and fungicidal activity of formulation-coated seeds of all the treatments was evaluated under laboratory conditions and compared with the field data. Seed vigour index was calculated using the following formula: (root length + shoot length) × germination percentage. The populations of different microorganisms used in the formulations were evaluated in a growth chamber under controlled conditions before their application in field. Such formulations were amended with 1% carboxymethyl cellulose (CMC; Himedia, India) as a sticker prior to application on seeds. The coated seeds were air-dried in shade before sowing.

Experimental setup with cotton crop at Nagpur

The effect of selected cyanobacteria, *Anabaena*-based biofilms, cyanobacterium-bacterium consortia and bacterial strains were investigated with cotton crop as PGP agents in the fields of Central Institute for Cotton Research (CICR), Nagpur, Maharashtra, India (21°N; 79°E; altitude of 301 m above mean sea level; mean annual rainfall 1095 mm). The physico-chemical characteristics of soil and other parameters at the initial stage of crop at Nagpur were as follows: soil type – Vertic Haplustept; available N – 90–105 kg ha^{-1}, available P – 7.0–7.9 kg ha^{-1} and available K – 220–280 kg ha^{-1}; electrical conductivity (EC) – 1.2 to 1.4 dS m^{-1}, organic carbon – 0.4%, pH – 7.8–8.2. The mean temperature of the area during the crop growth ranged from 30–33 °C in June to 26–28 °C in December.

The cotton variety PKV081 (*Gossypium hirsutum*) was selected, which represents an improved variety, suitable for organic cultivation in Maharashtra. The experimental design was arranged as a randomized block design (RBD), which includes 11 treatments, including controls – recommended chemical fertilizer (RDF) and 50% N + full dose of P and K fertilizers. All treatments included three replicates in a plot size of 35 m^2. T1: IARI *Azotobacter* inoculant was used as a check because of its widespread use in India for cotton crop as a biofertilizer. The recommended dose of fertilizers (120:40:40 NPK kg ha^{-1}, prilled urea, single super phosphate and muriate of potash) was used as a control, with all microbial inoculation treatments receiving 50% N along with full dose of P and K fertilizers. All the treatments were taken in triplicate.

The details of treatments are as follows: T1: IARI *Azotobacter* inoculant; T2: *Anabaena* sp.-based formulation; T3: *Providencia* sp.-based formulation; T4: *Anabaena*

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

sp.–*Providencia* sp.-based formulation; T5: *Anabaena* sp. *Azotobacter*-based formulation; T6: *Anabaena* sp.–*Pseudomonas* sp. Biofilm-based formulation; T7: *Anabaena–Nostoc* spp. Consortia-based formulation; T8: RPAN59 *Anabaena* sp.-based formulation; T9: *Calothrix* sp.-based formulation; T10: 50% N + full dose of P and K fertilizers; T11: given 100% dose of N + PK – RDF. In the treatment involving biofilms, inclusive of nitrogen fixers *Azotobacter* or *Anabaena* sp., only 50% of recommended nitrogen application was given along with full dose of P and K fertilizers.

Experimental setup with cotton crop at Sirsa

The experiment was set up in *Rhizoctonia*-infected plots at the research station of CICR at Sirsa, Haryana, India (29.25°N; 74.30°E; altitude 202 m above mean sea level; mean annual rainfall is 323 mm) to study the biocontrol potential of different formulations in cotton. The physico-chemical characteristics of soil and other parameters at the initial stage of crop at Sirsa were as follows: soil type – Ustic Haplocambids, available N – 30–40 kg ha^{-1}, available P – 3.1 kg ha^{-1}; soil type was deep coarse loamy and sandy soils; EC – 0.3 dS m^{-1}, organic carbon – 0.19%, pH – 8.44. The temperature of the area during the crop growth ranged from 40–45 °C in June to 36–38 °C in August. The traditional local cotton variety CISA 310 (*Gossypium arboreum*) was selected as the test variety, which is a commercial variety released for the north zone of India. The experimental design was arranged as a RBD, including seven treatments comprising three controls – commercial formulation; uninoculated carrier (used as base for the microbial inoculants) and recommended chemical fungicide. All treatments included three replicates in the plot size of 40 m^2, and a blank dose of Diammonium phosphate (DAP) at the rate of 50 kg $acre^{-1}$ and zinc sulphate at the rate of 10 kg $acre^{-1}$ were applied in all the plots. The details of treatments are as follows: T1: *Anabaena* sp. RPAN8 T2: *Calothrix* sp., T3: *Anabaena–T. viride* biofilm, T4: *Anabaena–B. subtilis* biofilm, T5: Vitavax (containing Carboxin 75% WP; Dhanuka Agritech Ltd) applied at the rate of 300 mg for 150 g seeds was used as the recommended chemical control treatment as a soil drench; T6: *Trichoderma viride* commercial formulation (1% WP at the rate of 1.5 g for 150 g^{-1} seeds) was used as a recommended biological control agent and T7: uninoculated carrier. *Trichoderma* formulation was obtained from Indore Biotech Inputs and Research Pvt. Ltd, Indore. Sufficient humidity conditions required for efficient disease establishment and development of fungal mycelia were maintained.

Analyses of soil physico-chemical and biological parameters

Soil analyses were undertaken by collecting soil cores (0–20-cm depth) using a soil auger from 10 sites in each plot, and the composite sample was used as a replicate for each treatment for analyses at 45 days after sowing (DAS). A total of three such replicates were used for all the analyses.

Chlorophyll estimation of photosynthetic biomass in cyanobacterial cultures was estimated by the procedure of Mackinney (1941) using a spectrophotometer (Thermo Fisher UV VIS double beam Halo-DB-205 model) in methanolic extracts. Soil

chlorophyll was assayed using soil samples in test tubes with Dimethyl sulfoxide (DMSO) and acetone (1:1) added at the rate of 4 mL g^{-1} soil. The contents were thoroughly shaken and incubated for 48–96 h in dark at room temperature. Intermittent shaking was done after every 24 h to extract chlorophyll completely. The data were recorded at 663, 645, 630, 775 nm with triplicate samples, and chlorophyll *a* concentration was determined (Nayak *et al.*, 2004) using a spectrophotometer. Microbial biomass carbon (MBC) was estimated by the method of Nunan *et al.* (1998) using aliquots of potassium sulfate (K_2SO_4) extracts through dichromate digestion. MBC was calculated after back titration with ferrous ammonium sulphate and recorded using the following equation: Biomass C = 2.64 × CE, where CE = (organic C from fumigated soil) – (organic C from unfumigated soil). Microbial biomass C was expressed as μg C g^{-1} soil. Dehydrogenase activity was used as an index of microbial activity, and assayed using soil (6 g), incubated with triphenyl tetrachloride (3%) and absorbance taken at 485 nm. The values were expressed as μg of triphenyl formazan (TPF) g^{-1} soil d^{-1}. Available nitrogen in soil was estimated using the alkaline permanganate method. The procedure involves distilling the soil with alkaline potassium permanganate solution and determination of ammonium liberated, which serves as an index of available nitrogen status. Soil available P was extracted from triplicate soil samples with 0.5 M sodium bicarbonate ($NaHCO_3$) and measured. All the measurements were done using the optimized protocols as given in Swarnalakshmi *et al.* (2013). Three replicates were used for analyses.

For estimating the number of nematodes in different treatments, extraction of nematodes from soil samples was undertaken. Cobb's sieving and decanting method along with the modified Baermann funnel technique (Cobb, 1918) was followed. Soil samples from five points were taken from each replication of treatment. All the samples were thoroughly mixed and soil lumps were broken; and 250 g of soil was drawn from homogenous mixtures for processing. The nematodes extracted in water suspension after 48 h were examined in three 1-ml aliquots under Stereo-binocular microscope, and plant parasitic nematode species were identified using standard keys (Nickel, 1991).

Activity of hydrolytic and defence enzymes in plant roots and shoots

Fresh plant samples of roots and shoots at 45 DAS were taken in triplicate for analysis. Chitosanase, endoglucanase (β-1, 3-glucanase and β-1, 4-glucanase) activities were assayed spectrophotometrically using glycol chitosan, laminarin and carboxy methyl cellulose respectively, as substrate, as given in Prasanna *et al.* (2013a, b, c). One unit of chitosanase activity was defined as 1 μmol of glucosamine released min^{-1} ml^{-1} culture filtrate under assay conditions. Under assay conditions, one unit of endoglucanase activity represents 1 μmol of glucose liberated min^{-1} g fresh $weight^{-1}$ root or shoot $tissues^{-1}$. The activity of defence enzyme – polyphenol oxidase (PPO) was assayed with slight modifications as given earlier (Prasanna *et al.*, 2013b). Peroxidase activity (PO) was assayed using plant extracts and measured spectrophotometrically at 470 nm in which 7.5 μL guaiacol (50 mM in the mixture) and 792 μL Tris HCl

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

buffer (0.05 M, pH 6.0) were added. The reaction was initiated by adding 100 μL 0.6 M hydrogen peroxide. A blank consisting of guaiacol, Tris HCl buffer and hydrogen peroxide was used to set at 100% absorbance. The enzyme activity was expressed as change in absorbance min^{-1} g^{-1} fresh weight.

In order to visualize the colonization of cyanobacterial cells/filaments/bacteria/fungi/biofilms, pieces of root tissues (0.5–1 cm in triplicate from each treatment) from Sirsa farm were washed with 0.1 M phosphate buffer (pH 6.9) for 1 h at room temperature. This was followed by fixing the samples in 2.5% (w/v) glutaraldehyde and 2.5% paraformaldehyde (w/v) prepared in 0.1 M phosphate buffer (pH 6.9) for 24 h at room temperature for scanning electron microscopy. The samples were then rinsed twice in phosphate buffer, and placed in 1% (w/v) osmium tetraoxide (OsO_4) for 24 h in dark. The fixed cells were dehydrated by progressive processing using increasing concentrations of acetone (30–100% v/v). The samples were then embedded in LR white resin and polymerized at a temperature of 60 °C, dried in a critical point dryer, followed by coating with palladium for 12 min in an atmosphere saturated with CO_2 at a temperature of 40 °C under a pressure of 70 atm. Such samples, taken in triplicate, were then visualized using scanning electron microscope (Evo Maio Zeiss, Germany).

Disease incidence in Nagpur

Incidences of wilt and bacterial leaf blight (BLB) were recorded in all the plots after 8, 12 and 16 weeks. For measurements on wilting, data was recorded on all the plants, i.e. two plants per hill × 60, and calculated for 120 plants and represented as % wilting. For BLB, percentage incidence ((number of plants infected/total number of plants observed) × 100) was calculated and observations were taken for 10 plants. The number of bolls $plant^{-1}$ was calculated by taking five plants from each replication and the mean calculated for each replication.

Yield parameters

Yield was calculated in terms of the number of cotton bolls per treatment after first pickings (number of bolls/plant) followed by bolls in a total of five plants for each replication. The mean was calculated for each treatment. Also, yield as kg seed cotton for each plot was recorded at the end of the experiment, which was calculated on the basis of three replications per treatment.

Statistical analyses

The triplicate sets of data for various parameters were statistically analysed by ANOVA using SPSS package. Duncan's Multiple Range Test (DMRT) was employed to compare the mean performances of different treatments for the specific parameters under study and the critical difference between treatments, calculated at 5% level of significance, denoted as CD (probability of 0.05) and Standard Error of Means (SEM) in tables. Correlation analyses were undertaken using Microsoft Excel package, and standard deviations (SD), depicting deviations from mean values among replicates,

Table 1. Effects of microbial formulations on soil parameters and yield of cotton crop at CICR, Nagpur.

Treatments	% Germination	Available N (kg ha^{-1})	Dehydrogenase activity (μg TPF g^{-1} soil d^{-1})	Yield in (kg) of seed cotton plot^{-1}
T1: 50% N + PK + IARI *Azotobacter* inoculant	92^{e} ± 7.5	156.2^{f,g} ± 8.8	4.4^{c} ± 0.23	3.4^{a,b} ± 0.5
T2: 50% N + PK + *Anabaena* sp. (CW1)-based formulation	89^{g} ± 1.9	172.4^{d} ± 4.0	5.3^{a} ± 0.05	4.0^{a} ± 0.9
T3: 50% N + PK + *Providencia* sp.-based formulation	92^{f} ± 5.1	283.7^{a} ± 1.2	5.4^{a} ± 0.06	3.9^{a} ± 0.4
T4: 50% N + PK + *Anabaena* sp. (CW1) *Providencia* sp.-based formulation	96^{c} ± 3.8	227.7^{b} ± 2.7	4.9^{b} ± 0.28	3.7^{a,b} ± 0.4
T5: 50% N + PK + *Anabaena–Azotobacter* biofilm-based formulation	96^{c} ± 9.5	182.1^{c} ± 6.0	4.0^{d,e} ± 0.01	3.2^{b} ± 0.1
T6: 50% N + PK + *Anabaena–Pseudomonas* sp. Biofilm-based formulation	99^{a} ± 7.6	161.3^{e,f} ± 1.5	3.8^{e} ± 0.01	3.5^{a,b} ± 0.4
T7: 50% N + PK + *Anabaena–Nostoc* consortium-based formulation	98^{b} ± 5.4	189.6^{c} ± 2.7	4.5^{c} ± 0.01	3.8^{a,b} ± 0.3
T8: 50% N + PK + *Anabaena* sp. (RPAN59)-based formulation	92^{f} ± 1.0	166.4^{d,e} ± 8.5	3.9^{d,e} ± 0.19	3.9^{a} ± 0.1
T9: 50% N + PK + *Calothrix* sp.-based formulation	92^{f} ± 1.0	152.6^{g} ± 2.9	4.5^{c} ± 0.01	3.5^{a,b} ± 0.1
T10: 50% N + PK	90^{e} ± 1.9	96.7^{i} ± 4.5	3.4^{f} ± 0.11	3.7^{a,b} ± 0.4
T11: Recommended NPK	95^{d} ± 4.4	123.7^{h} ± 1.5	4.2^{c,d} ± 0.01	3.6^{a,b} ± 0.1
SEM	0.04	2.8	0.1	0.2
CD (5%)	0.1	7.8	0.3	0.6

are denoted as error bars in figures. The alphabets given as superscripts in tables and as lower case alphabets in figures denote ranking of treatments based on Duncan's multiple range test. All plant and soil parameters were taken at mid-crop stage in both field experiments.

RESULTS

Field experiment at Nagpur

The proportion of germination in the field ranged from 89 to 99% (Table 1). The highest values was recorded in treatment T6 (*Anabaena–Pseudomonas* biofilm formulation) followed by T7 (*Anabaena–Nostoc* consortia). In terms of seed vigour index (data not shown), T9 (*Calothrix* sp.-based formulation) and T6 (*Anabaena–Pseudomonas* biofilm-based formulation) recorded the highest values, which were 20–40% higher than the controls (T10, T11). At mid-crop stage, treatment T8 (RPAN 59 *Anabaena* sp.) recorded the highest values of fresh weight of 17.8 g, followed by T4 (*Anabaena–Providencia* biofilm), T9 (*Calothrix* sp.) and T5 (*Anabaena–Azotobacter* biofilm)

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

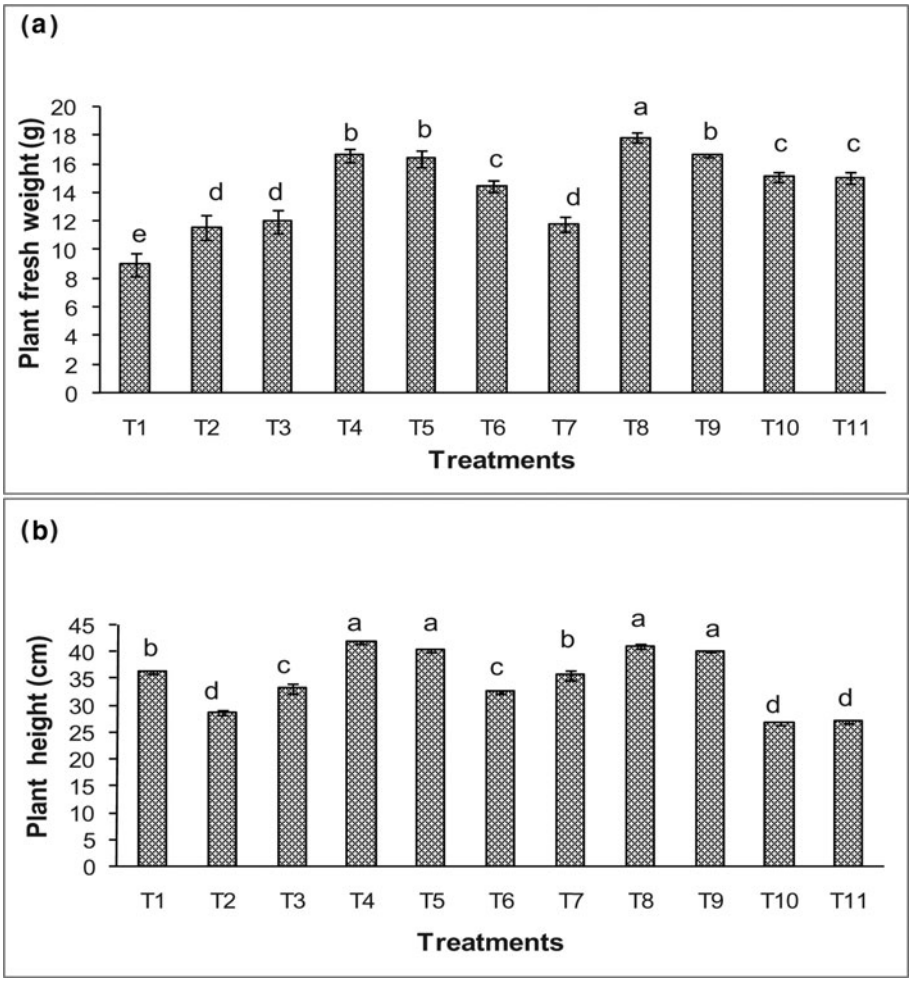

Figure 1. Influence of microbial formulations on plant biometric attributes at mid-crop stage of cotton crop at CICR, Nagpur. (a) Fresh weight (g); (b) Plant height (cm). Standard deviation (SD) values are denoted as error bars.

respectively (Fig. 1a), which were significantly higher than chemical controls – T10 and T11. The plant height values ranged from 26.6 to 41.7 cm (Fig. 1b). Treatments T4 (*Anabaena–Providencia*), T5 (*Anabaena–Azotobacter*), T8 (RPAN 59 *Anabaena* sp.) and T9 (*Calothrix* sp.) showed the highest values of plant height, which were statistically at par.

In order to determine microbial growth and establishment of inoculated cyanobacteria in the field, soil chlorophyll from the rhizosphere environment of all the treatments was measured using soil cores (Fig. 2a). Treatment T9 (*Calothrix* sp.-based formulation) showed the highest values of 3.05 μg g^{-1} soil, followed by T7 (*Anabaena–Nostoc* consortia-based formulation). The MBC values ranged from 102.1 to 1228.9 μg C g^{-1} soil (Fig. 2b). The highest MBC values was recorded in T9 (*Calothrix* sp.-based

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

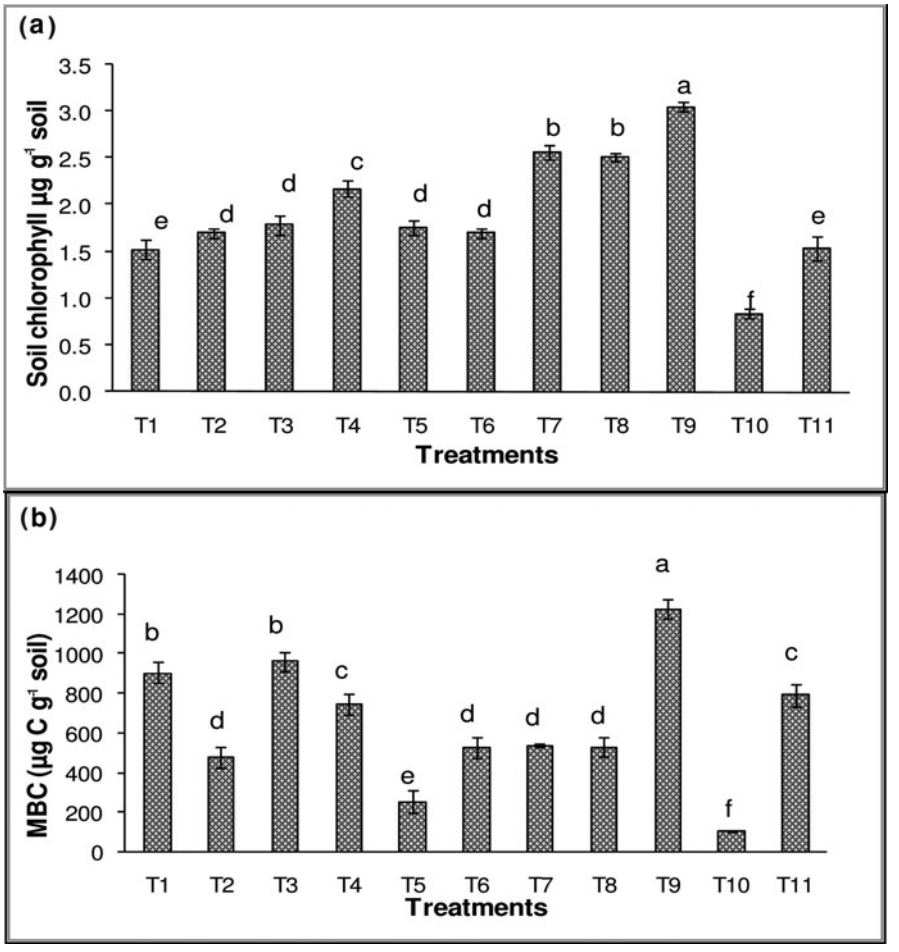

Figure 2. Evaluation of soil microbiological parameters at mid-crop stage of cotton crop at CICR, Nagpur. (a) Soil chlorophyll; (b) Microbial biomass carbon (MBC). Error bars denote values of standard deviation (SD).

formulation), which was followed by T3 (*Providencia* sp.-based formulation) and T1 (IARI *Azotobacter* inoculant) respectively. Significantly higher values of dehydrogenase activity (5.4, 5.3 μg TPF g soil^{-1} d^{-1}) were observed in T2 (*Anabaena* sp.-based formulation) and T3 (*Providencia* sp.-based formulation) followed by T4 (*Anabaena–Providencia* sp.-based formulation), i.e. 4.97 μg TPF g soil^{-1} d^{-1} (Table 1). The available N in soil values ranged from 96.7 to 283.7 kg ha^{-1} (Table 1). Treatment T3 (*Providencia*) showed the highest values of available N, followed by T4 (*Anabaena–Providencia*). T5 and T7 recorded statistically par values. All treatments showed significantly higher values as compared with controls – T10 and T11. Available P values ranged from 4 to 8.7 mg kg^{-1} soil and treatment T6 recorded the highest values, but no significant differences were observed.

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

The PPO activity ranged from 0.001 to 0.039 IU min^{-1} g^{-1} fresh weight. Although no significant differences were observed (data not shown), treatments T8 (RPAN 59 *Anabaena* sp.-based formulation), T3 (*Providencia* sp-based formulation) and T2 (*Anabaena* sp-based formulation) exhibited higher values. In the present study, the number of cotton balls per plant (from the first pickings) ranged from 17 to 21 bolls per plant (data not shown). Treatment T1 (IARI *Azotobacter* inoculant) showed the highest values, followed by T8 (*Anabaena* sp. RPAN59-based formulation), but no significant differences were recorded. In terms of yield (measured as kg of seed cotton/plot) and the means calculated on the basis of three replications, treatment T2 (*Anabaena* sp. CW1-based formulation) followed by T3 (*Providencia* sp.-based formulation) recorded higher values (Table 1). However, no significant differences were recorded among treatments.

The number of plants showing wilting as an index was recorded for 120 plants/treatment and expressed as percentage mortality, which ranged from 7.5 to 11.4% (data not shown as treatments did not show significant differences). T6 (*Anabaena* sp.–*Pseudomonas* sp. biofilm-based formulation) and T11 (recommended NPK control) showed the lowest mortality values of 7.5%, which were statistically at par. The BLB incidence was recorded at intervals of 8, 12 and 16 weeks (data not shown); values ranged from 0 to 23.3% at 8 weeks and reduced to the highest values of 20% after 12 weeks and 16.7% after 16 weeks. T5 (*Anabaena* sp.–*Azotobacter* biofilm-based formulation) outperformed all other treatments in recording 0% incidence up to 16 weeks. T9 (*Calothrix* sp.), T10 (50% N + PK control) and T11 (recommended NPK control) showed no disease incidence at 8 weeks stage; however, it recorded 6.7% at 16 weeks after sowing. Since the values were not statistically significant at each interval, these have not been included in the tables and figures.

The population of nematodes was counted in terms of commonly observed major nematode genera (including population of plant parasitic nematodes and free living nematodes) in cotton fields (Table 2). The population count of *Hoplolaimus columbus* per kg soil ranged from 16 to 76, with T7 (*Anabaena* + *Nostoc* sp. consortia) showing the lowest values of 12. T11 (recommended dose of NPK fertilizers) showed the highest count of nematodes of 76. Population counts of *Rotylenchulus reniformis* showed 40–50% lower values in T4 (*Anabaena–Providencia*). The count of *Pratylenchus goodeyi* was lowest in T9 (*Calothrix* sp.) and control T10 with 2–5 folds higher population in other treatments. The percentage decrease in parasitic nematode population in the field ranged from 22 to 41% and T3 (*Providencia* sp.) and T7 (*Anabaena* sp. CW1) recorded the highest values of 41 and 39% respectively. A significant negative correlation ($r = 0.6$) between the population of plant parasitic nematodes and microbiological activity of roots was recorded. The percentage increase in the population of free living nematodes in soil as compared with control, T11, ranged from 0 to 60%. Treatments receiving biofilmed formulations, T6 (*Anabaena–Pseudomonas*) and T5 (*Anabaena–Azotobacter*), recorded the greatest increase in the population of free living nematodes in soil. It was interesting to note a significant decrease in parasitic nematode population in the field ranging from 51.2 to 73.2%, especially with T1 (IARI *Azotobacter* inoculant) and T7 (*Anabaena–Nostoc* consortia-based formulation).

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

Table 2. Effect of inoculants on nematode population at CICR, Nagpur in cotton crop.

Treatments	Population of selected nematodes (per kg soil)			% decrease in parasitic nematode population*	% increase in free living nematode population*
	Hoplolaimus columbus	*Rotylenchulus reniformis*	*Pratylenchus goodeyi*		
T1: 50% N + PK + IARI *Azotobacter* inoculant	$37^{b} \pm 5$	$44^{b} \pm 4$	$40^{a} \pm 4$	22	0
T2: 50% N + PK + *Anabaena* (CW1)-based formulation	$39^{b} \pm 6$	$36^{c} \pm 4$	$20^{b} \pm 4$	39	40
T3: 50% N + PK + *Providencia*-based formulation	$24^{c,d} \pm 4$	$48^{b} \pm 4$	$20^{b} \pm 4$	41	52
T4: 50% N + PK + *Anabaena* (CW1) *Providencia sp.*-based formulation	$28^{c} \pm 4$	$33^{c} \pm 5$	$44^{a} \pm 4$	33	40
T5: 50% N + PK + *Anabaena.–Azotobacter* biofilm-based formulation	$32^{b,c} \pm 4$	$68^{a} \pm 4$	$17^{b} \pm 5$	25	60
T6: 50% N + PK + *Anabaena–Pseudomonas* biofilm-based formulation	$28^{c} \pm 4$	$63^{a} \pm 6$	$16^{b} \pm 4$	32	66
T7: 50% N + PK + *Anabaena* + *Nostoc* consortia-based formulation	$16^{d} \pm 4$	$60^{a} \pm 4$	$44^{a} \pm 4$	23	43
T8: 50% N + PK + *Anabaena* (RPAN59)-based formulation	$39^{b} \pm 6$	$51^{b} \pm 2$	$20^{b} \pm 4$	31	57
T9: 50% N + PK + *Calothrix*-based formulation	$39^{b} \pm 6$	$67^{a} \pm 6$	$12^{b} \pm 4$	26	52
T10: 50% N + PK	$40^{b} \pm 4$	$64^{a} \pm 4$	$12^{b} \pm 4$	26	–20
T11: Recommended NPK	$76^{a} \pm 4$	$60^{a} \pm 4$	$20^{b} \pm 4$	–	–
SEM	3.03	2.88	2.67	–	–
CD (5%)	8.36	7.94	7.36	–	–

*In comparison with number in recommended NPK treatment.

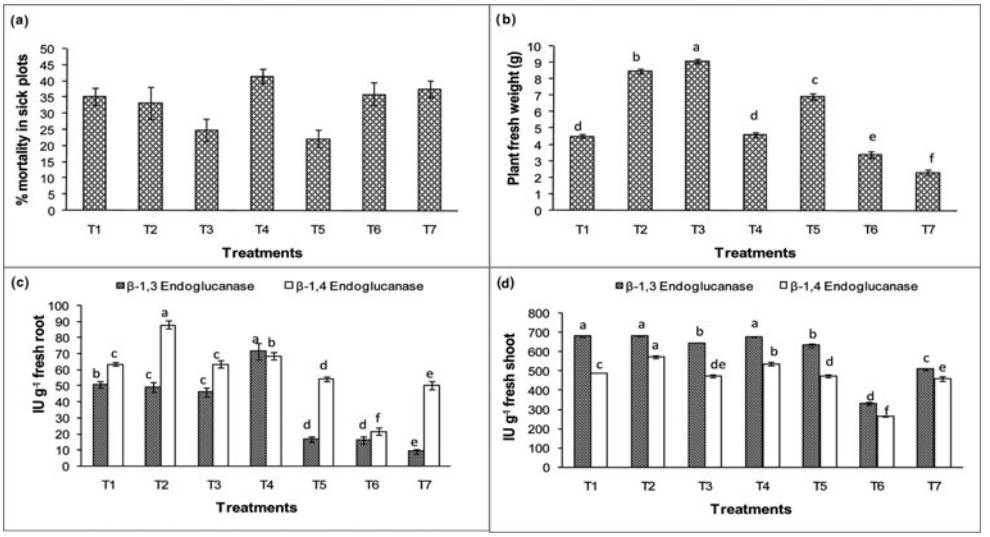

Figure 3. Evaluation of microbial formulations on cotton crop at CICR farm, Sirsa in terms of (a) % mortality in sick plots; (b) fresh weight (g); (c) β-1,3 and β-1,4 endoglucanase activity in roots; (d) shoots of cotton plants at CICR farm, Sirsa. Error bars denote values of standard deviation (SD).

Field experiment at Sirsa

The mortality values ranged from 37.5 to 22.2%. Although T5 (Vitavax formulation) showed the lowest mortality rate of 22.2%, application of T3 (*Anabaena–T. viride* biofilm) recording only 24.8% mortality proved promising (Fig. 3a). Treatment T3 (*Anabaena–T. viride* biofilm) recorded the highest fresh weight of 9.09 g followed by T2 (*Calothrix* sp.) and T5 (Vitavax-based formulation), which 8.46 and 6.93 g respectively (Fig. 3b). These three treatments recorded values which were significantly higher than biological control (T6). The lowest values were observed in T7 (uninoculated carrier). As a prelude to the use of formulations-coated seeds in field, the percentage mortality/germination of formulation-coated seeds was recorded under laboratory conditions. In the water agar medium, germination values ranged from 50.8 to 85.5% with the highest values in treatment T4 (*Anabaena–B. subtilis* biofilm-based formulation), followed by T3 (*Anabaena–T. viride* biofilm). The seed vigour index was comparatively higher in the seedlings of formulation-coated seeds from treatments T1, T2 and T3 in both the water agar and field soil-enriched media (data not shown). In water agar medium, *Calothrix* sp. formulation (T2) showed the highest values, followed by T1 – IARI *Azotobacter* inoculant and T3 (*Anabaena–T. viride* biofilm). In addition, *R. solani*-inoculated PDA medium was employed and mortality values of 46.4 to 89.1% were recorded. The CFU of bacteria, cyanobacteria, *Trichoderma* in different formulation coated seeds were ranged at 10^5, 10^2 and 10^5 per seed respectively.

As an index of accretion of photosynthetic biomass and an indirect indicator of establishment of inoculated cyanobacteria in soil, the soil chlorophyll values were measured, which ranged from 0.08 to 0.32 μg g^{-1} soil and showed a positive correlation ($r = 0.6$; $df = 6$) with fresh weight of plants. Treatment T2 (*Calothrix*

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

sp.-based formulation) showed the highest values, whereas T1 (*Anabaena laxa* RPAN8-based formulation) and T3 (*Anabaena–T. viride* biofilm-based formulation) also recorded significantly higher values than controls – T6 and T7 (0.06 and 0.08 μg g^{-1} soil) (Table 3). The microbial biomass carbon ranged from 35.8 to 255.7 μg C g^{-1} soil. The highest MBC values were recorded in T2 (*Calothrix* sp.-based formulation), which was followed by T4 (*Anabaena–B. subtilis* biofilm-based formulation) recording 166.28 μg C g^{-1} soil. The dehydrogenase values ranged from 4.9 to 9.2 μg TPF g^{-1} soil d^{-1}. Significantly higher values of 9.2 and 9.0 μg TPF g^{-1} soil d^{-1} were observed in T1 (*Anabaena laxa* RPAN8-based formulation) and T4 (*Anabaena–B. subtilis* biofilm-based formulation) followed by T2 (*Calothrix* sp.-based formulation), i.e. 8.8 μg TPF g^{-1} soil d^{-1}); however, the treatments did not show any significant differences (Table 3).

Peroxidase activity in root and shoot tissues ranged from 7.00 to 100.5 IU min^{-1} g^{-1} fresh weight (Table 3). Treatment T1 (*Anabaena laxa* RPAN8-based formulation) showed the highest values of 100.5 IU min^{-1} g^{-1} fresh weight, followed by T6 (*Trichoderma* commercial formulation), i.e. 75.5 IU min^{-1} g^{-1} fresh weight. In shoot tissues also, T1 (*Anabaena laxa* RPAN8-based formulation) showed the highest values of 44.6 IU min^{-1} g^{-1} fresh weight, followed by T4. The uninoculated carrier showed the lowest values for both of these parameters. Reports on the use of *Bradyrhizobium–Penicillium* biofilm combination have shown that such biofilms exhibit better growth, nitrogen-fixing ability and colonization abilities than their monocultures (Jayasinghearachchi and Seneviratne, 2004). Significant correlation of plant parameters with peroxidase activity, mortality of cotton plants with MBC and defence enzyme activity with mortality was also observed in this study ($r = 0.6–0.8$; $df = 6$).

The activity of pathogenesis-related enzymes showed significant differences among treatments and between roots and shoot tissues (Figs. 3c, d). β 1,4 endoglucanase activity in root tissues showed significant differences among treatments with values ranging from 21.59 to 87.85 IU g^{-1} fresh tissue. Treatment T2 (*Calothrix* sp.) showed the highest values of 87.85 IU g^{-1} root tissues, followed by 68.4 IU g^{-1} in T4 (*Anabaena–B. subtilis* biofilm), which were significantly higher than T7 (uninoculated carrier), i.e. 50.18 IU g^{-1}. A similar trend was also recorded in shoot tissues, but 5–8-fold higher values of 571.7 and 537.1 IU g^{-1} fresh tissues were recorded in these two treatments (T2 and T4). Endoglucanase activity in root and shoot tissues that ranged from 8.99 to 680.6 IU g^{-1} fresh tissue showed significant differences. In terms of root and shoot tissues, T4 (*Anabaena–B. subtilis* biofilm), T2 and T1 recorded the highest values. However, the activity of shoot tissues was almost 10 folds higher than in roots (21–87 IU g^{-1} in roots to 506.7–677.7 IU g^{-1} in shoots). The activity of endoglucanase of roots and shoots showed a highly positive correlation with MBC of soil ($r = 0.87$; $df = 6$) and also with soil chlorophyll and plant fresh weight ($r = 0.6, 0.64$; $df = 6$). Chitosanase activity in root and shoot tissues ranged from 14.6 to 782.40 IU g^{-1} fresh tissues (Table 3). In terms of root tissues, T3 (*Anabaena–T. viride* biofilm-based formulation), and T1 (*Anabaena laxa* RPAN8-based formulation) showed the highest values of 54.8 and 50.1 IU g^{-1} fresh weight respectively. In case of shoots, T4

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

Table 3. Influence of inoculants on plant tissues and soil microbiological parameters at CICR Farm, Sirsa.

Treatments	Chitosanase (IU g^{-1} fresh weight)		Peroxidase activity (IU g^{-1} fresh weight)		Soil chlorophyll (μg g^{-1} soil)	Microbial biomass carbon (μg C g^{-1} soil)
	Root	Shoot	Root	Shoot		
T1: *Anabaena laxa* (RPAN8)-based formulation	$50.1^{a} \pm 2.4$	$729.5^{c} \pm 8.0$	$100.5^{a} \pm 3.8$	$44.6^{a} \pm 0.1$	$0.23^{b} \pm 0.004$	$139.5^{c} \pm 19.3$
T2: *Calothrix*-based formulation	$23.8^{c} \pm 4.7$	$762.7^{b} \pm 21.8$	$31.6^{d} \pm 1.6$	$27.1^{c} \pm 0.1$	$0.32^{a} \pm 0.022$	$255.7^{a} \pm 8.2$
T3: *Anabaena–T. viride* biofilm-based formulation	$54.8^{a} \pm 5.0$	$506.5^{d} \pm 3.2$	$29.3^{d} \pm 3.2$	$11.5^{e} \pm 0.3$	$0.19^{c} \pm 0.003$	$144.8^{c} \pm 9.3$
T4: *Anabaena–B. subtilis* biofilm-based formulation	$17.1^{d} \pm 4.8$	$782.4^{a} \pm 2.9$	$41.1^{c} \pm 6.4$	$38.0^{b} \pm 4.6$	$0.12^{d} \pm 0.011$	$166.3^{b} \pm 14.2$
T5: Vitavax-based formulation	$36.6^{b} \pm 4.5$	$351.5^{e} \pm 3.7$	$30.4^{d} \pm 1.0$	$9.3^{ef} \pm 0.1$	$0.08^{e} \pm 0.014$	$50.1^{d} \pm 3.1$
T6: *Trichoderma*-based formulation	$31.1^{b} \pm 4.3$	$284.3^{f} \pm 1.5$	$75.5^{b} \pm 8.5$	$19.4^{d} \pm 0.1$	$0.06^{e} \pm 0.007$	$48.3^{d} \pm 14.2$
T7: Uninoculated carrier	$14.6^{d} \pm 3.6$	$343.1^{e} \pm 1.0$	$18.6^{e} \pm 6.3$	$7.0^{f} \pm 0.02$	$0.08^{e} \pm 0.005$	$35.8^{d} \pm 8.2$
SEM	2.5	5.2	2.9	1.0	0.01	6.9
CD (5%)	6.8	14.4	8.1	2.8	0.03	19.1

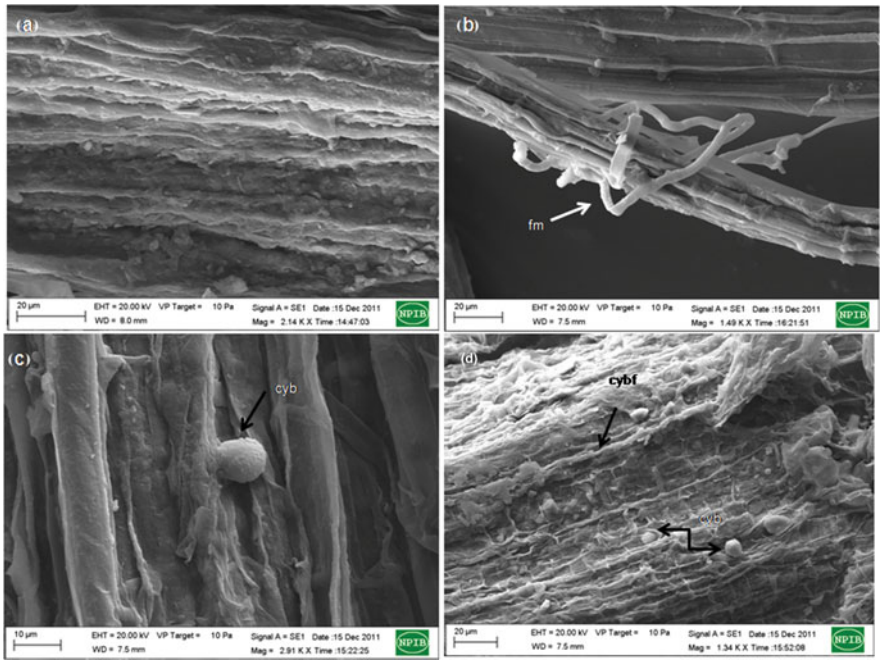

Figure 4. Scanning electron micrographs showing colonization of roots by mycelia/filaments and biofilms in cotton plants. (Left–right): (a) Healthy root tissues; (b) Fungal mycelia/filaments (fm) on the surface of roots; (c) Biofilms (cyb) on the root surface (comprising cyanobacterial filaments with attached bacteria–*Anabaena-bacillus* biofilms); (d) Cyanobacterial strain *Anabaena–Trichoderma* biofilms growing as thick filamentous structures (cybf) and compact mucilaginous balls (cyb) in the root tissues of biofilm-treated samples.

(*Anabaena–B. subtilis* biofilm-based formulation) showed the highest values followed by T2 (*Calothrix* sp.-based formulation), i.e. 782.4 IU g^{-1} and 762.7 IU g^{-1} respectively.

In the present experiment, the microbial formulations tested exhibited significant differences, ranging from 10–50% increase in terms of fresh weight and nitrogen availability to the crop. The measurement of soil chlorophyll from the rhizosphere environment revealed the promise of *Calothrix/Anabaena*-based formulations (T8/T7).

The colonisation of roots of cotton plants was evaluated using scanning electron microscopy (Fig. 4). The degraded state of root tissues and fungal mycelia is visible in control – untreated plants (Figs. 4a, b), while Figs. 4c, d revealed biofilm-like growth (mucilaginous ball-like structure, representing cyanobacterial filaments, embedded with bacterial colonies on the surface of roots) and the presence of *Anabaena–Trichoderma* biofilms (cybf) growing as thick filaments (Fig. 4d). Cells/biofilms of inoculated organisms were recorded in 80% of the samples observed, with differences in degree of colonisation varying from 60–70% among the replicates of each of the treatments (T1–T4).

Comparative performance of treatments at Nagpur, in terms of their performance based on all the parameters evaluated, revealed that T3 (*Providencia*), T8 (RPAN 59 *Anabaena* sp.-based formulation) and T9 (*Calothrix* sp.) were the top ranking strains

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

Table 4. Comparative performances of treatments (top three ranks) in term of microbiological and plant parameters in cotton crop at CICR, Nagpur.

Parameters/treatments		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Plant fresh wt.				+		+			+			
Plant height					+	+			+	+		
% Germination		+	+		+		+	+	+			
Cotton ball no. per plant		+	+	+	+	+	+	+	+	+	+	+
PPO	Root		+	+					+			
	Shoot	+				+	+			+		
MBC		+		+						+		
Dehydrogenase			+	+	+			+				
Soil chlorophyll										+		
Available N				+	+			+				
% reduction in plant parasitic nematodes			+	+	+							
% increase in free-living nematodes						+	+		+			
Wilting				+					+	+		
Total		5	5	8	6	5	4	5	7	6	1	1

(Table 4). In Sirsa, *Calothrix* sp. (T2) was the top ranked strain, followed by *Anabaena–Bacillus subtilis* biofilmed formulation (T4) and *Anabaena laxa* (T1) (Table 5). The *Anabaena–T. viride* biofilm application performed best, with mortality values 11.1% lower than commercial *Trichoderma* formulation, but 2.6% higher as compared with the Vitavax treatment.

DISCUSSION

Cyanobacteria represent a major component of diverse ecosystems; however, they have been mainly utilized as inoculants for rice (Prasanna *et al.*, 2012; Roger *et al.*, 1993; Venkataraman, 1981), and in recent times in wheat and other crops, as both consortia or biofilms (Chaudhary *et al.*, 2012; Karthikeyan *et al.*, 2007; Manjunath *et al.*, 2011; Nain *et al.*, 2010; Prasanna *et al.*, 2013b; Rana *et al.*, 2012). They can grow as biofilms in soil and aquatic bodies, playing a significant role in soil aggregation and metal binding/biosorption (Caire *et al.*, 1990). Use of biofilms as inoculants is a recently evolved concept, which attempts to simulate microbial communities, and is attracting attention because they can colonise and persist in diverse niches (Prasanna *et al.*, 2013a, b; Seneviratne *et al.*, 2009; Swarnalakshmi *et al.*, 2013). Seneviratne *et al.* (2009) found significant increase in the growth of rice and dry weight of plants through the inoculation of biofilmed fertilizers as compared to the conventional method of monoculture inoculation.

Evaluation of microbiological parameters at Nagpur revealed that the highest values for MBC and soil chlorophyll were recorded in treatment T9 (*Calothrix* sp.-based formulation), which highlights the effective colonization of this cyanobacterium (based on soil chlorophyll as an index) and associated C enrichment through photosynthesis leading to higher MBC. This also resulted in higher yields, which were statistically at

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

Table 5. Comparative performance of inoculants in terms of microbiological and plant parameters in cotton crop grown in *Rhizoctonia* sick plots at CICR Farm, Sirsa.

Parameters/Treatments		*Anabaena laxa* (RPAN8)	*Calothrix sp.*	*Anabaena–T. viride* biofilm	*Anabaena–B. subtilis* biofilm	Vitavax	*Trichoderma* commercial formulation	Uninoculated carrier
β-1,3 Endoglucanase	Root	+	+		+			
	Shoot	+	+		+	+		
Chitosanase	Root	+		+		+		
	Shoot	+	+		+			
β-1,4 Endoglucanase	Root		+	+	+			
	Shoot	+	+		+			
% Mortality			+	+		+		
MBC			+	+	+			
Dehydrogenase		+	+		+			
PO	Root	+			+		+	
	Shoot	+	+		+			
Soil chlorophyll		+	+	+				
Plant fresh wt.			+	+		+		
Total		9	11	6	9	4	1	0

par with fertilizer controls, T10 and T11, illustrating an environment and resource friendly strategy for this cash crop. Plant growth promotion mediated by microbes has been mainly attributed to an increase in the mobilization of insoluble nutrients and subsequent enhancement of nutrient uptake by plants, or enhanced colonisation and production of plant growth regulators or allelochemicals (Bashan, 1998; Kulik, 1995). This can be visually observed as enhanced plant biomass or plant height. Several researchers (Ahmad *et al*., 2008; Khan, 2002; Soomro *et al*., 2005; Taohua and Haipeng, 2006) suggested that plant height was among the factors positively correlated with increase in seed cotton yield and may contribute up to 70% of the total variability for seed cotton yield. A comparative evaluation of treatments at Nagpur, in terms of their performance based on all the parameters evaluated, revealed that T3 (*Providencia*), T8 (RPAN 59 *Anabaena* sp.-based formulation) and T9 (*Calothrix* sp.) were the top ranking strains.

Cotton is one of the most important commercial crops playing a key role in economic and social affairs globally. Despite decades of warning, the inappropriate usage of fertilizers and pesticides has led to not only environmental pollution but also to development of more than 500 resistant pathogens. The problem of pests has led to the advent of transgenics in cotton; however, little attention is being paid to organic practices for improving the tolerance of cotton crop to abiotic/biotic stress. Therefore, there is a definite need to employ a mix of traditional and organic agricultural practices to sustain soil fertility and crop productivity, keeping in view the environmental health and safety issues. The present study is a step forward to evaluate promising cyanobacterial strains and biofilmed preparations for their efficacy in nutrient mobilization and biocontrol of phytopathogenic fungi in cotton crop in two different agro-ecological locations.

Many rhizobacteria have been recorded as antagonists of plant parasitic nematodes (Tian *et al*., 2007). Khan *et al*., (2005) recorded the nematicidal potential of *Microcoleus* sp. and *Oscillatoria chlorina* against the root-knot nematode *Meloidogyne arenaria* in tomato plants. However, there are no reports using *Anabaena*-based formulations. This genus is well recognised as a rich source of neurotoxins and hydrolytic enzymes (Prasanna *et al*., 2008, 2010), which may be playing a role in the decrease of nematode population in soil. Decrease in parasitic nematode population ranging from 22 to 41%, especially in T7 (*Anabaena* sp. CW1), was noteworthy. It was interesting to note a significant decrease in parasitic nematode population in the field ranging from 51.2 to 73.2%, especially with T1 (IARI *Azotobacter* inoculant) and T7 (*Anabaena–Nostoc* consortia-based formulation). The colonisation of cotton by *Rotylenchulus* is known to elicit induction of enzymes involved in Systemic Acquired Resistance (SAR) in cotton crop (Aryal *et al*., 2011). Aerobic endospore-forming bacteria, belonging mainly to *Bacillus subtilis* and *Pseudomonas* spp., are able to reduce plant parasitic nematode population (Sikora, 1992), and *Anabaena–Pseudomonas* sp. proved promising in our study. The mechanisms of antagonistic action of rhizobacteria, including *Pseudomonas*, towards plant parasitic nematodes include the production of antibiotics/metabolic byproducts, enzymes and induction of systemic resistance (Aryal *et al*., 2011; Kloepper *et al*., 1991; Sikora, 1992). Some species of *Pseudomonas* have been recorded as highly aggressive colonizers

of the rhizosphere of various crop plant pathogens such as nematodes. A significant negative correlation ($r = 0.6$) between the population of plant parasitic nematodes and microbiological activity of roots was also recorded. A concurrent increase was observed in the population of free living nematodes in soil, especially in the treatments receiving biofilmed formulations – T6 (*Anabaena–Pseudomonas*) and T5 (*Anabaena–Azotobacter*). Cyanobacteria are also known to produce a variety of volatiles and their biofilms can attract nematodes, besides providing shelter to several nematode species (Höckelmann *et al.*, 2004; Moens and Vincx 1998). This is supported by the observations on increase in the population of the free living nematode population in soil, which play a key role in nutrient cycling, revealing the utility of our formulation as a suitable green option. It has been often cited (Santoyo *et al.*, 2012) and well researched that *Pseudomonas* and *Bacillus* represent the most commonly employed PGP and biocontrol agents because of their ubiquity as soil inhabitants in diverse cropping systems, and therefore play a major role in the development of disease suppressive soils globally. A metabolite of *Calothrix* sp. has been earlier identified as a novel substituted benzoic acid (Natarajan *et al.*, 2012), which may indirectly enhance microbial activity by controlling the proliferation of soil borne phytopathogenic fungi. In the present study, plant growth promotion in terms of fresh weight or height of plants was significantly enhanced through the use of microbial formulations. These strains have shown tremendous promise when evaluated for their PGP and biocontrol potential (Karthikeyan *et al.*, 2007; Manjunath *et al.*, 2010) earlier in wheat and tomato crops respectively.

Most of the known biocontrol agents bring about induced systemic resistance (ISR), fortifying the physical and mechanical strength of cell wall and changing the physiological and biochemical reaction of host leading to synthesis of defence chemicals against pathogens. Defence reaction occurs due to synthesis of defence and PR-enzymes such as chitosanase, β-1, 3 endoglucanase, PAL, PO, phenolics and phytoalexins (Chen *et al.*, 2000; Kloepper *et al.*, 1991), which can indirectly lead to enhanced plant growth, improved yields and quality of produce, besides enriching soil fertility. The ability of an inoculated strain to establish a significant population size along the root system is a key determinant of its PGP ability or antagonism against soil borne pathogens. The ecological environment at Sirsa poses a serious challenge for growing the crop due to heavy infestation of fungi, including *Rhizoctonia* and other associated phytopathogenic fungi. At Sirsa, the microbiological and plant-related assays revealed significant interactions of inoculated organisms and plant in sick plots, leading to lower or comparable levels of mortality as observed with commercial chemical or biocontrol formulations. Our earlier studies have shown that *Anabaena*, *Calothrix* sp. and biofilmed formulations exhibit fungicidal activity, which could be correlated with the production of hydrolytic enzymes, and homologues for chitosanase/endoglucanase were identified in these strains (Gupta *et al.*, 2010, 2012; Natarajan *et al.*, 2012; Prasanna *et al.*, 2008, 2010, 2011, 2013b). Synergy among partners in the biofilmed formulations illustrated the potential of *Anabaena* as a matrix which can prove to be more viable inoculation option for organic agriculture practices (Prasanna *et al.*, 2013a, c). Biofilms observed on plant roots are known to protect colonization sites and act as a sink for nutrients in rhizosphere, thereby reducing the

https://doi.org/10.1017/S0014479714000143 Published online by Cambridge University Press

availability of root exudates, nutritional elements for pathogen stimulation or their subsequent colonization on roots (Weller and Thomashow, 1993). Observations on enhanced nutrient uptake and availability are similar to the results recorded in legumes and wheat crop, in which the application of cyanobacterial biofilmed inoculants had shown a significant enhancement in soil nutrient status, microbiological and plant parameters (Prasanna *et al*., 2013c; Swarnalakshmi *et al*., 2013).

Nesbat and Slusarenko (1983) found that microbial inoculation leads to the generation of fibrillar material in which the entrapped bacteria continue to proliferate, leading to increase in the large clumps of bacterial cells, forming mat-like growth/biofilms over the surface of roots. A similar observation was recorded in our study, as the biofilms inoculated served as an optimal mode of attachment and a suitable surface for bacteria to grow, leading to effective colonization. Visual observations of field also supported the promise of *Anabaena–T. viride* formulation, which was confirmed by SEM observations. The presence of biofilms and coiled filaments of cyanobacteria/mycelia of *Trichoderma* vis-a-vis control-infected roots established the colonisation of our inocula and their role in reducing disease severity.The colonization of roots by cyanobacteria has been recorded earlier in rice and wheat and correlated with plant growth promotion (Karthikeyan *et al*., 2009; Prasanna *et al*., 2009). Jetiyanon & Kloepper (2002) suggested that mixtures of plant growth promoting rhizobacteria (PGPR) can elicit induced disease resistance to a number of bacterial, fungal and viral diseases, thereby enhancing plant growth and reducing disease severity in a number of crops. As biofilms represent mixed communities proliferating in a diverse range of environments, the synergism among microbial partners in formulations is an important determinant for the efficacy of inoculation.

These investigations clearly demonstrated that the cyanobacterial formulations are effective and environmentally sustainable as easy to use inoculants even in field experiments for a crop such as cotton, in which fertilizers and pesticides are a major cost component for their large-scale cultivation. The use of biofilmed formulations provides a promising alternative, which avoids growing multiple strains and their maintenance. In future, these need to be tested in diverse agro-ecologies for their efficacy and potential in combating abiotic and biotic stress.

Acknowledgements. The authors are thankful to the Department of Biotechnology (DBT) and Indian Council of Agricultural Research – Application of Microorganisms in Agriculture and Allied Sectors (ICAR-AMAAS) projects for funding the research work. The authors are grateful to the Director, CICR, Nagpur, India for facilitating the conduct of field experiments at Nagpur and Sirsa, and providing necessary experimental facilities. The authors gratefully acknowledge the Division of Microbiology, IARI, New Delhi for providing necessary facilities to undertake this study.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0014479714000143.

REFERENCES

Ahmad, W., Khan, N. U., Muhammad, R. K., Praveen, A., Aiman, U. E., Samiullah, M. S. and Shah, S. A. (2008). Genetic variability and correlation analysis in upland cotton. *Sarhad Journal of Agriculture* 24:573–580

Aryal, K. S., Davis, F. R., Stevenson, L. K., Timper, P. and Ji, P. (2011). Influence of infection of cotton by *Rotylenchulus reniformis* and *Meloidogyne incognita* on the production of enzyme involved in systemic acquired resistance. *Journal of Nematology* 43:152–159.

Bashan, Y. (1998). Inoculants of plant growth promoting bacteria for use in agriculture. *Biotechnology Advances* 16:729–770.

Brussaard, L., Ruiter, P. C. and Brown, G. G. (2007). Soil biodiversity for agricultural sustainability. *Agriculture, Ecosystem and Environment* 121:233–244.

Caire, G. Z., Cano, M. S., Mule, M. C. Z. and Halperin, D. R. (1990). Antimycotic products from the cyanobacterium *Nostoc muscorum* against *Rhizoctonia solani*. *Phytopathology* 51:1–4.

Chaudhary, V., Prasanna, R., Nain, L., Dubey, S. C., Gupta, V., Singh, R., Jaggi, S. and Bhatnagar, A. K. (2012). Bioefficacy of novel cyanobacteria-amended formulations in suppressing damping off disease in tomato seedlings. *World Journal of Microbiology and Biotechnology* 28:3301–3310.

Chen, C., Belanger, R. R., Benhamaou, N. and Paulitz, T. (2000). Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiology and Molecular Plant Pathology* 56:13–23.

Cobb, N. A. (1918). Estimating the nematode population of soil. In *Agricultural Technical Circular*, 1–48. Washington, DC: Bureau of Plant Industry, US Department of Agriculture.

Dukare, A. S., Prasanna, R., Dubey, S. C., Chaudhary, V., Nain, L., Singh, R. and Saxena, A. K. (2011). Evaluating novel microbe amended composts as biocontrol agents in tomato. *Crop Protection* 30:436–442.

Gupta, V., Prasanna, R., Chaudhary, V. and Nain, L. (2012). Biochemical, structural and functional characterization of novel antifungal endoglucanases from *Anabaena laxa*. *Biocatalysis and Agricultural Biotechnology* 1:338–347.

Gupta, V., Prasanna, R., Natarajan, C., Srivastava, A. K. and Sharma, J. (2010). Identification, characterization and regulation of a novel antifungal chitosanase gene (cho) in *Anabaena* spp. *Applied and Environmental Microbiology* 76:2769–2777.

Höckelmann, C., Moens, T. and Juüttner, F. (2004). Odor compounds from cyanobacterial biofilms acting as attractants and repellents for free-living nematodes. *Limnologica Oceanographica* 49:1809–1819.

Jaiswal, P., Prasanna, R. and Singh, P. K. (2008). Cyanobacterial bioactive molecules – an overview of their toxic properties. *Canadian Journal of Microbiology* 54:701–717.

Jayasinghearachchi, H. S. and Seneviratne, G. (2004). A bradyrhizobial – *Penicillium* spp. biofilm with nitrogenase activity improves N2 fixing symbiosis of soybean. *Biology and Fertility of Soils* 40:432–434.

Jetiyanon, K. and Kloepper, J. W. (2002). Mixtures of plant growth promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biological Control* 24:285–291.

Karthikeyan, N., Prasanna, R., Lata, N. and Kaushik, B. D. (2007). Evaluating the potential of plant growth promoting cyanobacteria as inoculants for wheat. *European Journal of Soil Biology* 43:23–30.

Karthikeyan, N., Prasanna, R., Sood, A., Jaiswal, P., Nayak, S. and Kaushik, B. D. (2009). Physiological characterization and electron microscopic investigations of cyanobacteria associated with wheat rhizosphere. *Folia Microbiologica* 54:43–51.

Kaushik, B. D. (1987). *Laboratory Methods for Blue Green Algae*. New Delhi: Associated Publishing Company.

Khan, N. U. (2002). *Genetic Analysis, Combining Ability and Heterotic Studies for Yield, Its Components, Fibre and Oil Quality Traits in Upland Cotton* (*G. hirsitum L.*). PhD dissertation. Sindhi Agriculture University, Tandojam, Pakistan.

Khan, Z., Park, S. D., Shin, S. Y., Bae, S. G., Yeon, I. K. and Seo, Y. J. 2005. Management of *Meloidogyne incognita* on tomato by root-dip treatment in culture filtrate of the blue green alga, *Microcoleus vaginatus*. *Bioresource Technology* 96:1338–1341.

Kloepper, J. W., Zablokovicz, R. M., Tipping, E. M. and Lifshitz, R. (1991). Plant growth promotion mediated by bacterial rhizosphere colonizers. In *The Rhizosphere and Plant Growth*, 315–326 (Eds. D. L. Keister and P. B. Cregan). Amsterdam, Netherlands: Kluwer.

Kulik, M. M. (1995). The potential for using cyanobacteria (blue green algae) and algae in the biological control of plant pathogenic bacteria and fungi. *European Journal of Plant Pathology* 101:585–599.

MacKinney, G. (1941). Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry* 140:315–322.

Mandal, B. P. L., Vlek, G. and Mandal, L. N. (1999). Beneficial effect of blue green algae and *Azolla* excluding supplying nitrogen, on wetland rice fields: a review. *Biology and Fertility of Soils* 28:329–342.

Manjunath, M., Prasanna, R., Nain, L., Dureja, P., Singh, R., Kumar, A., Jaggi, S. and Kaushik, B. D. (2010). Biocontrol potential of cyanobacterial metabolites against damping off disease caused by *Pythium aphanidermatum* in solanaceous vegetables. *Archives of Phytopathology and Plant Protection* 43:666–677.

Manjunath, M., Prasanna, R., Sharma, P., Nain, L. and Singh, R. (2011). Developing PGPR consortia using novel genera *Providencia* and *Alcaligenes* along with cyanobacteria for wheat. *Archives of Agronomy and Soil Science* 57:873–887.

Moens, T. and Vincx, M. (1998). On the cultivation of free-living marine and estuarine nematodes. *Helgoländer Meeresunters* 52:115–139.

Mundt, S., Kreitlow, S., Nowotny, A. and Effmert, U. (2001). Biochemical and pharmacological investigations of selected cyanobacteria. *International Journal of Hygiene and Environmental Health* 203:327–334.

Nain, L., Rana, A., Joshi, M., Jadhav, S. D., Kumar, D., Shivay, Y. S., Paul, S. and Prasanna, R. 2010. Evaluation of synergistic effects of bacterial and cyanobacterial strains as biofertilizers for wheat. *Plant and Soil* 331:217–230.

Nakkeeran, S., Fernando, W. G. D. and Siddiqui, Z. A. (2005). Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and disease, In *PGPR: Biocontrol and Biofertilization*, 257–296 (Ed. Z. A. Siddiqui). Dordrecht, Netherlands: Springer.

Natarajan, C., Prasanna, R., Gupta, V., Dureja, P. and Lata, . (2012). Dissecting the fungicidal activity of *Calothrix elenkinii* using chemical analyses and microscopy. *Applied Biochemistry and Microbiology* 48:59–65.

Nayak, S., Prasanna, R., Pabby, A., Dominic, T. K. and Singh, P. K. (2004). Effect of urea and BGA – *Azolla* biofertilizers on nitrogen and chlorophyll accumulation in soil cores from rice fields. *Biology and Fertility of Soils* 40:67–72.

Nesbat, F. E. and Slusarenko, A. J. (1983). Ultrastructure of the interaction of cells of *Pseudomonas phaseolicola* with cell walls of a resistant and susceptible bean cultivar. *Phytopathologische Zeitschrift* 108:148–159.

Nickel, W.R. (1991). *Manual of Agricultural Nematology*. New York: Marcel Dekker.

Nunan, N., Morgan, M. A. and Herlihy, M. (1998). Ultraviolet absorbance (280 nm) of compounds released from soil during chloroform fumigation as an estimate of the microbial biomass. *Soil Biology and Biochemistry* 30:1599–1603.

Persello-Cartieaux, F., Nussaume, L. and Robaglia, C. (2003). Tales from the underground: molecular plant–rhizobacteria interactions. *Plant, Cell and Environment* 26:189–199.

Prasanna, R., Gupta, V., Natarajan, C. and Chaudhary, V. (2010). Allele mining for chitosanases and microcystin-like compounds in *Anabaena* strains. *World Journal of Microbiology and Biotechnology* 26:717–724.

Prasanna, R., Babu, S., Rana, A., Kabi, S. R., Chaudhary, V., Gupta, V., Kumar, A., Shivay, Y. S., Nain, L. and Pal, R. K. (2013a). Evaluating the establishment and agronomic proficiency of cyanobacterial consortia as organic options in wheat-rice cropping sequence. *Experimental Agriculture* 49:416–434.

Prasanna, R., Chaudhary, V., Gupta, V., Babu, S., Kumar, A., Shivay, Y. S. and Nain, L. (2013b). Cyanobacteria mediated plant growth promotion and bioprotection against *Fusarium* wilt in tomato. *European Journal of Plant Pathology* 13:337–353.

Prasanna, R., Jaiswal, P., Nayak, S., Sood, A. and Kaushik, B. D. (2009). Cyanobacterial diversity in the rhizosphere of rice and its ecological significance. *Indian Journal of Microbiology* 49:89–97.

Prasanna, R., Joshi, M., Rana, A., Shivay, Y. S. and Nain, L. (2012). Influence of co-inoculation of bacteria–cyanobacteria on crop yield and C-N sequestration in soil under rice crop. *World Journal of Microbiology and Biotechnology* 28:1223–1235.

Prasanna, R., Lata, , Tripathi, R., Gupta, V., Middha, S., Joshi, M., Ancha, R. and Kaushik, B. D. (2008). Evaluation of fungicidal activity of extracellular filtrates of cyanobacteria – possible role of hydrolytic enzymes. *Journal of Basic Microbiology* 48:186–194.

Prasanna, R., Pattnayak, S., Sugitha, T. C. K., Nain, L. and Saxena, A. K. (2011). Development of cyanobacterium based biofilms and their in vitro evaluation for agriculturally useful traits. *Folia Microbiologica* 56:49–58.

Prasanna, R., Triveni, S., Bidyarani, N., Babu, S., Yadav, K., Adak, A., Khetarpal, S., Pal, M., Shivay, Y.S. and Saxena, A. K. (2013c). Evaluating the efficacy of cyanobacterial formulations and biofilmed inoculants for leguminous crops. *Archives of Agronomy and Soil Science* 60:349–366.

Roger, P. A., Zimmerman, W. J. and Lumpkin, T. (1993). Microbiological management of wetland rice fields. In *Soil Microbial Ecology*, 417–455 (Ed. B. Metting) New York: Marcel Dekker.

Seneviratne, G., Thilakaratne, R. M. M. S., Jayasekara, A. P. D. A., Seneviratne, K. A. C. N., Padmathilake, K. R. and De Silva, M. S. D. L. (2009). Developing beneficial microbial biofilms on roots of non-legumes: a novel biofertilizing technique. In *Microbial Strategies for Crop Improvement* 51–62 (Eds. M. S. Khan, A. Zaidiand J. Musarrat). Berlin, Heidelberg: Springer-Verlag.

Sergeeva, E., Liaimer, A. and Bergman, B. (2002). Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. *Planta* 215:229–238.

Sikora, R. A. (1992). Management of the antagonistic potential in agriculture ecosystems for the biological control of plant parasitic nematodes. *Annual Reviews of Phytopathology* 30:245–270.

Singh, P. P., Shin, Y. C., Park, C. S. and Chung, Y. R. (1999). Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. *Phytopathology* 89:92–99.

Soomro, A. R., Kakar, R. G., Ali, H. and Abid, S. A. (2005). Comparison of yield and its components in some commercial cotton varieties. *Indus Journal of Plant Sciences* 4:545–552.

Stanier, R. Y., Kunnisawa, R., Mandel, M. and Cohen-Bazire, G. (1971). Purification and properties of unicellular blue green algae (Order: Chroococcales). *Bacteriological Reviews* 3:171–305.

Swarnalakshmi, K., Prasanna, R., Kumar, A., Pattnaik, S., Chakravarty, K., Shivay, Y. S., Singh, R. and Saxena, A. K. (2013). Evaluating the influence of novel cyanobacterial biofilmed biofertilizers on soil fertility and plant nutrition in wheat. *European Journal of Soil Biology* 55:105–116.

Taohua, Z. and Haipeng, Z. (2006). Comparative study on yield and main agri-characters of five hybrid coloured cotton varieties. *Journal of Anhui Agricultural University* 33:533–536.

Tian, B., Yang, J. and Zhang, K. Q. (2007). Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action, and future prospects. *FEMS Microbiology Ecology* 61:197–213.

Venkataraman, G. S. (1981). Blue green algae: a possible remedy to nitrogen scarcity. *Current Science* 50:253–256.

Weller, D. M. and Thomashow, L. S. (1993). Advances in rhizobacteria for biocontrol. *Current Opinion in Biotechnology* 4:306–311.

Zulpa, G., Zaccaro, M. C., Boccazzi, F., Parada, J. L. and Storni, M. (2003). Bioactivity of intra and extracellular substances from cyanobacteria and lactic acid bacteria on wood blue stain fungi. *Biological Control* 27:345–348.