

Development of cyanobacterium-based biofilms and their in vitro evaluation for agriculturally useful traits

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Abstract The ability of cyanobacteria to be useful as matrices for agriculturally important bacteria was evaluated. Biofilms were generated with the selected strain *Anabaena torulosa* after co-culturing with *Azotobacter chroococcum*, *Pseudomonas striata*, *Serratia marcescens*, and *Mesorhizobium ciceri*. The biochemical attributes were compared with individual bacterial and cyanobacterial cultures. The biofilms were characterized in terms of proteins, chlorophyll, IAA production, acetylene-reducing activity, phosphate solubilization, and antagonism towards selected phytopathogenic fungi. An enhancement in the population counts was recorded in *A. torulosa*–*S. marcescens* and *A. torulosa*–*P. striata* biofilms. The *A. torulosa*–*A. chroococcum* and *A. torulosa*–*M. ciceri* biofilms were also able to utilize new saccharides as compared to the individual cultures. Such novel biofilms with agriculturally useful traits can provide additional advantages including the broader spectrum of activity and the presence or formation of biologically active compounds; they also suggest the way to effective inoculants for sustainable and environment friendly agriculture.

Abbreviations

ARA	Acetylene-reducing activity
CD	Critical difference
CRYEMA	Congo red yeast extract mannitol agar
IAA	3-Indole-acetic acid
IARI	Indian Agricultural Research Institute
ITCC	Indian Type Culture Collection
NA	Nutrient agar

NB	Nutrient broth
ONPG	4-Nitrophenyl beta-D-galactopyranoside
PGPR	Plant growth promoting rhizobacteria
SEM	Standard error of means

Cyanobacteria have been widely employed as inoculants for enhancing soil fertility and improving soil structure, besides enhancing crop yields (Mandal et al. 1999), especially in rice crop and more recently in wheat crop and vegetables (Venkataraman 1981; Karthikeyan et al. 2007). Cyanobacterial growth in flooded rice soil has been reported to influence soil structure, availability of nutrients, besides stimulating or inhibiting growth of higher plants and microorganisms associated with them in their immediate environment (Whitton 2000).

The extracellular mucilage or polysaccharide layer of cyanobacteria is considered a nutrient rich environment for the growth of associated bacteria (both photosynthetic and/or non-photosynthetic types); however, it has been mainly evaluated as a source of polysaccharides. Co-cultivation of cyanobacteria with plants has also been undertaken (Rai and Bergman 2002; Svircev et al. 1997). Loose associations between free-living cyanobacteria and other macro and microorganisms in the form of biofilms are widespread in natural habitats (Whitton et al. 1988; Rasmussen and Johansson 2002). Such associations or biofilms are known to play a critical role in colonization in soil/plant surfaces besides being essential for their widespread adaptation to diverse ecological niche. However, they not have been much exploited in agriculture.

Plant growth promoting rhizobacteria (PGPR) represents one of the components of soil which could be effectively

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utilized for sustainable agricultural production by enhanced growth and disease suppression. A definite paucity of information exists regarding cyanobacterial strains which can form tight associations, with PGPR, especially agriculturally important microorganisms such as *Rhizobium*, *Azotobacter*, *Pseudomonas* which are used as inoculants for important crops. Our efforts would be directed towards developing novel agriculturally useful biofilms using cyanobacteria as matrices and co-inoculating with well-characterized plant growth promoting microbes involved in nutrient mobilization and/or biocontrol as partners in the association.

Materials and methods

Growth and maintenance of the strains A set of ten cyanobacterial and four bacterial strains were obtained from the germplasm of our Institute. *Serratia marcescens* (L11) was grown and maintained in nutrient broth (NB) and on nutrient agar (NA), *Mesorhizobium ciceri* (IC4059) in Congo Red-yeast extract–mannitol agar (CRYEMA) medium (Fred et al. 1932), *Azotobacter chroococcum* (W5) in Jensen (1954) medium and *Pseudomonas striata* (M27) in Pikovskaya (1948) medium. The exponential-phase cultures (1 day), incubated at $30 \pm 2^\circ\text{C}$ in a shaker, were utilized. Potato-dextrose agar medium was used for the growth and maintenance of the fungal cultures, incubation being done at 28°C in an incubator.

Cyanobacterial cultures were axenized by standard procedures and screened for the ability to form biofilms. The property of adhesion to cell surfaces was tested by hydrophobicity test using paraffin oil (both light and heavy), hexane, and engine oil (Rosenberg et al. 1980). The selected strain *Anabaena torulosa* (BF1) was used after its repeated subculturing in N_2 -free BG11 media (Stanier et al. 1971) and was grown under the light–dark cycle (16:8 h) under white light ($50\text{--}55 \mu\text{mol photons/m}^2/\text{s}$) and at $28 \pm 2^\circ\text{C}$ in N_2 -free BG11 medium in Haffkine flasks.

Microscopic analysis The associations between the cyanobacterium and specific bacterium was evaluated using phase contrast light microscope attached with Canon Power Shot S50 Digital Camera and Canon utilities Remote Capture ver. 2.7.2.16 software. The specimens were observed under 4X, 10X and 100X magnifications, with or without Gram staining.

In vitro fungicidal assays were undertaken against selected phytopathogenic fungi (*Pythium debaryanum* Indian type culture collection ITCC 95, *Fusarium oxysporum* ITCC 195, *F. oxysporum* f.sp. *lycopersici* ITCC 4998, *Rhizoctonia solani* ITCC 4576, *Fusarium solani* ITCC 1984) obtained from the Division of Plant Pathology (Indian Agricultural Research Institute, IARI New Delhi). Fifty microliters of filtrate of cyanobacterium or bacteria or piece of biofilm

($10 \times 10 \text{ mm}$) were used for analyses by inoculating sterile 5-mm discs and placed on the lawn of respective fungal strains.

Biochemical characterization of strains Fifty microliters of homogenized cultures of the cyanobacterial strain, biofilms and four bacterial strains were inoculated in the wells of plates of HiCarbohydrate Kit (HiMedia Laboratories, India). For cyanobacteria and the biofilms, incubation in 16:8 (light-to-dark cycles) and for bacterial strains incubation at 30°C was undertaken. Colorimetric observation was recorded up to 2 days and evaluated according to the manufacturer's instructions.

Experimental set up Optimization of inoculum rate of the two partners was undertaken by using 1–5% cyanobacterial cultures *vis-à-vis* 1–5% exponential-phase bacterial cultures; *A. torulosa* inoculated at the rate of 5% in BG11 medium was found to be optimum. After 4 days of growth under optimum conditions of temperature and illumination, the overnight-grown cultures of the bacteria were inoculated at 2% (v/v) inoculum level. Incubation was done under the optimum conditions of light and temperature for the cyanobacterium. Samples were removed periodically and examined under microscope to monitor the colonization of cyanobacterial filaments by the bacterial cultures. Biochemical characterization was undertaken in terms of chlorophyll and proteins (Herbert et al. 1971; Prasanna et al. 2010) after 3 and 6 weeks (Table 1).

Acetylene reduction activity Gas chromatographic quantification of ethylene formed was utilized as an index of N_2 fixation. Commercially available ethylene was utilized for quantification and vials with an equivalent volume of water served as controls (Hardy et al. 1973). Measurement of acetylene-reducing activity (ARA) was done after 3 and

Table 1 Arrangement of the experiments

Treatment	Culture(s)	Medium
T0	<i>A. torulosa</i>	BG11
T1A	<i>A. chroococcum</i>	BG11
T2A	<i>A. chroococcum</i>	Jensen 1954
T3A	<i>A. chroococcum</i> + <i>A. torulosa</i>	BG11
T1P	<i>P. striata</i>	BG11
T2P	<i>P. striata</i>	Pikovskaya 1948
T3P	<i>P. striata</i> + <i>A. torulosa</i>	BG11
T1S	<i>S. marcescens</i>	BG11
T2S	<i>S. marcescens</i>	NA
T3S	<i>S. marcescens</i> + <i>A. torulosa</i>	BG11
T1R	<i>M. ciceri</i>	BG11
T2R	<i>M. ciceri</i>	CRYEMA
T3R	<i>M. ciceri</i> + <i>A. torulosa</i>	BG11

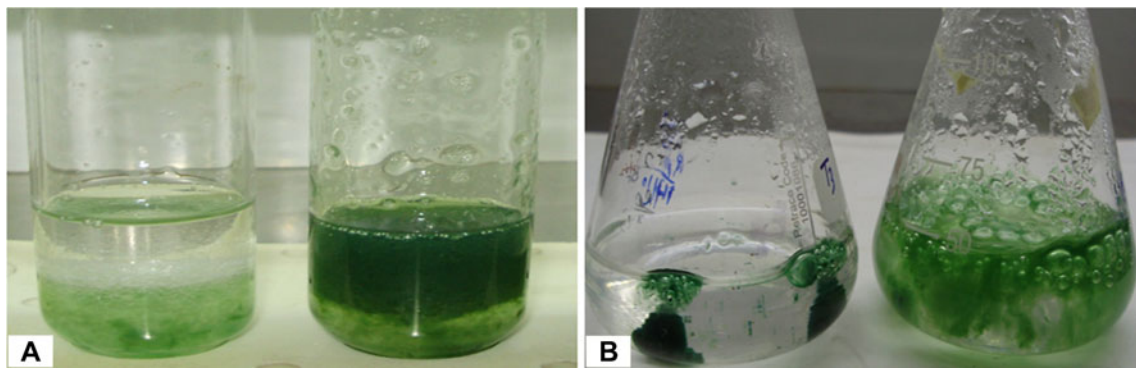


Fig. 1 **a** Cell surface hydrophobicity test using paraffin oil (*left*) and engine oil (*right*) of *A. torulosa*; **b** growth of biofilm in flasks after 3 and 6 days of incubation

6 weeks. Ten percent (v/v) acetylene was injected after removal of equivalent amount of air and incubation was done in light for 1.5 h. The ARA values were expressed as nanomoles ethylene produced per milliliter; all the measurements were taken in triplicate.

3-Indole-acetic acid (IAA) production by biofilms vis-à-vis microbial partners The amount of IAA was quantified spectrophotometrically by measuring the intensity of pink color at 530 nm using calibration curve of standard IAA stock solution (10–100 µg/mL) prepared in 50% ethanol (Gordon and Weber 1951).

Phosphate solubilization by biofilms vis-à-vis microbial partners This was undertaken to check whether the cyanobacteria or bacteria and biofilm can solubilize phosphate using Pikovskaya (1948) medium. A loopful of culture was added to the plate and the plates were incubated at 34–37°C. The plates were checked for the formation of clear zone after 24, 48, and 56 h.

Saccharide production by biofilms vis-à-vis microbial partners For this, 1 mL of cyanobacterial strain, growing individually or as biofilm (after co-inoculation with bacterial strains) was taken, and 4 mL of Anthrone reagent was added to it and mixed thoroughly. The tubes were kept

in a water bath in 100°C for 10 min. After color development (emerald green), the absorbance was read at 620 nm and the amount of sugars calculated from the standard curve of known amount of sugars and expressed as micrograms of sugar per milliliter of culture (Spiro 1966).

Statistical analyses The data, recorded in triplicates for the growth and biochemical parameters in selected treatments were subjected to ANOVA (Analysis of Variance) in accordance with the experimental design (completely randomized block design) using SPSS.10 statistical package to quantify and evaluate the source of variation. The alphabets, given as superscripts in the tables denote ranking of strains based on Duncan's Multiple range test. Values denoted by the same superscripts in a column are not statistically different, i.e., do not exhibit significant differences. Standard deviations are depicted in the graphs and critical difference (CD) and standard error of means (SEM) values were calculated at a probability of 0.05, as given in the tables.

Results

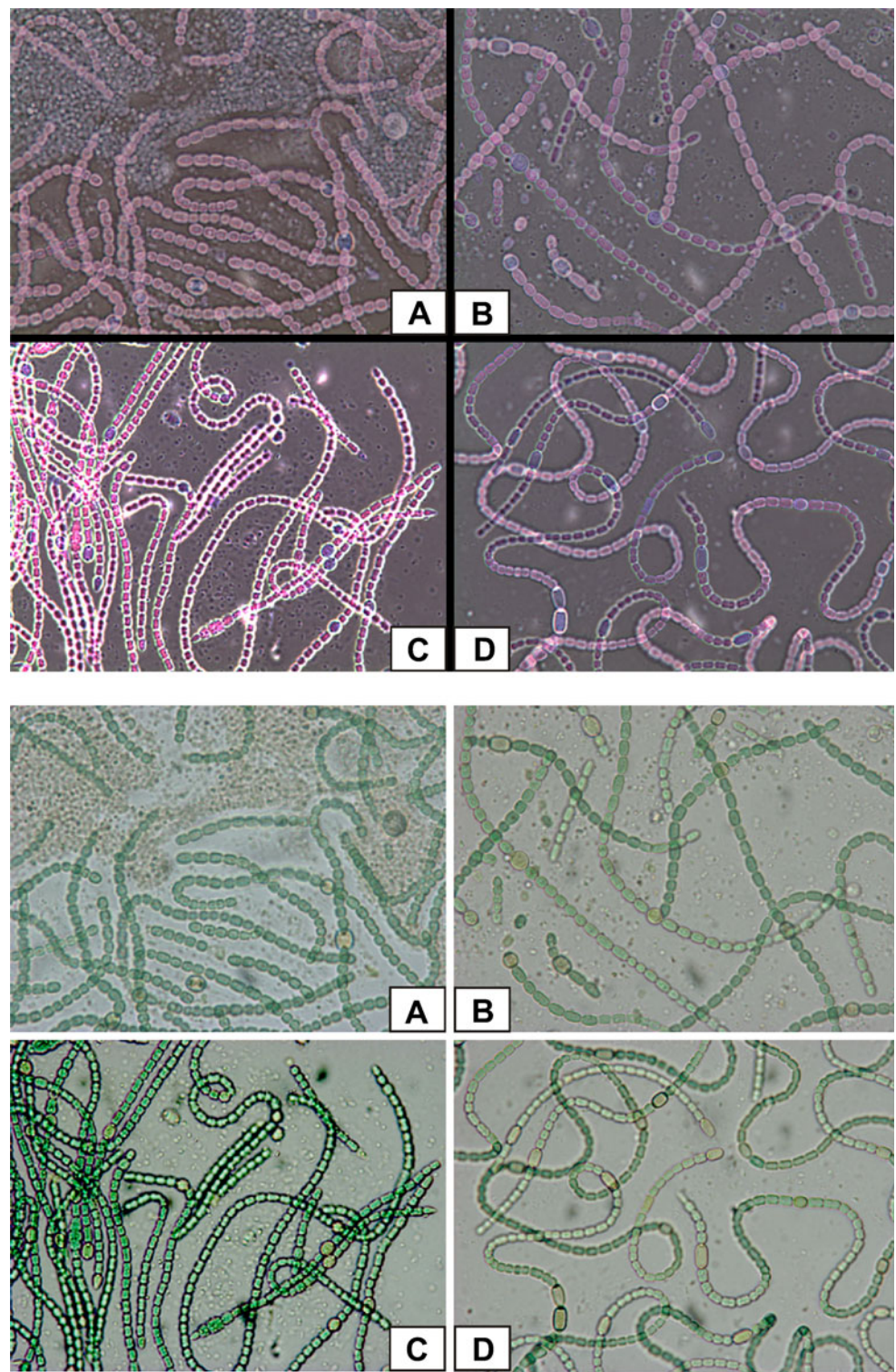
A set of ten cyanobacterial strains available in the germplasm were screened in terms of formation of biofilms

Table 2 Protein, chlorophyll, and saccharides in *A. torulosa* (all in milligrams per liter)

Age, days	Protein	Chlorophyll	Saccharides	Age, days	Protein	Chlorophyll	Saccharides
3	327 ^M	0.72 ^I	10.5 ^L	30	426 ^D	2.41 ^C	24.7 ^D
6	3337 ^L	0.91 ^H	13.0 ^K	33	424 ^E	2.45 ^{BC}	26.0 ^{AB}
9	348 ^K	1.25 ^G	15.4 ^J	36	435 ^B	2.45 ^{BC}	25.7 ^{BC}
12	385 ^J	1.56 ^F	18.6 ^H	39	437 ^C	2.50 ^{BC}	26.5 ^A
15	394 ^I	1.82 ^E	22.5 ^E	42	434 ^B	3.04 ^A	20.5 ^G
18	401 ^H	2.21 ^D	25.5 ^{BC}	45	451 ^A	3.12 ^A	21.9 ^F
21	405 ^G	2.17 ^D	25.2 ^{CD}	SEM	0.141	0.009	0.049
24	409 ^F	2.53 ^B	26.0 ^{AB}	CD (5%)	0.389	0.024	0.135
27	434 ^{BC}	2.45 ^{BC}	26.0 ^{BC}				

The same letters in the same column indicate that values do not differ at indicated significance level.

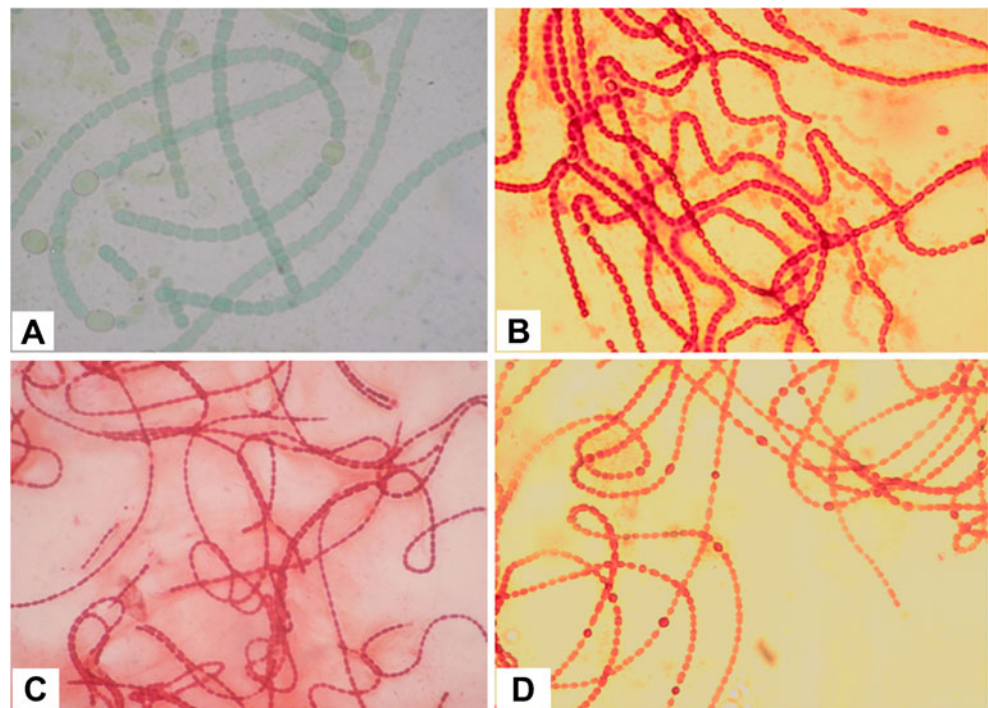
Fig. 2 Biofilms of *A. torulosa* with *A. chroococcum* (a), *P. striata* (b), *S. marcescens* (c), and *M. ciceri* (d) using negative (above) and positive (below) phase contrast microscopy (magnification $\times 40$)



by visual and microscopic analysis of biofilm morphology and hydrophobicity test (Fig. 1A). *A. torulosa* exhibited the most promising traits (data not shown). A gradual enhancement in all the growth attributes (chlorophyll and proteins) was observed up to 45 days, however, saccharides declined beyond 39 days (Table 2).

Co-inoculation simultaneously with bacterial and cyanobacterial cultures resulted in gradual disintegration of the biomass; therefore, the experiments were set up with 4-day-old cyanobacterial culture into which the bacterial cultures were inoculated individually. Four overnight-grown bacteria selected—*A. chroococcum*, *P. striata*, *S. marcescens*,

Fig. 3 **a** Biofilms of unstained filaments of *A. torulosa*; **b–d** Gram staining of *A. torulosa* with *A. chroococcum* (**b**), *P. striata* (**c**), and *M. ciceri* (**d**); magnification $\times 40$



and *M. ciceri*—were used. Microscopic analyses of the *A. torulosa*–*A. chroococcum* biofilm revealed the presence of *A. chroococcum* cells surrounding the filaments of *A. torulosa* forming an envelope-like structure (Fig. 2). Gram staining helped to further reveal the extent of aggregation of *A. chroococcum* cells with the outer surface of *A. torulosa* filaments (Figs 2 and 3). The cells of *P. striata* showed also the aggregating ability with the filaments of *A. torulosa* which was more random. *S. marcescens* and *M. ciceri* showed a lesser extent of aggregation with the filaments. A progressive increase in colonization was observed with time.

The plate counts of the bacterial cultures were taken before inoculation and monitored to evaluate the viability of cells (Table 3). Despite a 15–25% reduction in the number of cells after 1 week, the values remained almost static up to 6 weeks in all biofilms. The bacterial cultures also showed a similar level of reduction with time. The counts of 10-week-old biofilms evaluated as crushed (10 b) and intact biofilms (10 a), revealed that the values were higher than the individual cultures. A doubling of population was recorded in the 10-week-old biofilm of *A. torulosa*–*S. marcescens*. In all cases, the bacterial counts were similar at 6- and 10-week-old biofilm.

Biochemical and physiological attributes of the various treatments were followed after 1 week of co-inoculation; however, no significant changes could be observed (data not shown). Therefore, the biofilms were evaluated after 3 and 6 weeks and compared with the individual microbial parameters.

Saccharide content in general declined considerably in 6-week-old cultures. Highest values were recorded in the cyanobacterial strain grown alone in BG11 medium (Table 3).

Protein accumulation also showed a drastic reduction after 6 weeks as compared to 3-week-old cultures (Table 4). *S. marcescens* growing in NB, followed by *A. torulosa*–*S. marcescens*, recorded the highest values after 3 weeks. Chlorophyll values (index of photosynthetic biomass) recorded similar values after 3 and 6 weeks indicating the slower growth phase and viability of the cyanobacteria

Table 3 The log CFU/ml of inoculated bacteria at 0, 1, 6, and 10 weeks

Treatment	0	1	6	10 ^a	10 ^b
T1A	8.01	6.20	6.54	6.00	6.11
T2A	8.01	6.95	6.82	6.05	6.21
T3A	8.01	6.88	6.72	6.70	7.08
T1P	8.15	6.99	6.85	6.10	6.00
T2P	8.15	7.61	6.99	6.23	6.11
T3P	8.15	7.36	6.90	6.93	6.63
T1S	8.28	6.95	6.88	6.10	6.01
T2S	8.28	7.08	6.99	6.43	6.25
T3S	8.28	6.99	6.93	7.33	7.11
T1R	8.23	7.00	6.91	6.24	6.11
T2R	8.23	7.32	7.06	6.48	6.40
T3R	8.23	7.72	6.99	6.65	6.59

^a Intact biofilms

^b Crushed biofilms

Table 4 IAA, protein, saccharides, and chlorophyll after 3 and 6 weeks (all in milligrams/liter)

Treatment	IAA		Protein		Saccharides		Chlorophyll	
	3	6	3	6	3	6	3	6
T0	1.98 ^G	0.86 ^I	335 ^I	62.0 ^E	26.8 ^H	16.3 ^G	0.64 ^A	0.41 ^C
T1A	4.98 ^E	3.55 ^D	91.4 ^K	23.3 ^L	3.75 ^J	2.02 ^I	–	–
T2A	33.0 ^A	8.06 ^A	615 ^E	103 ^A	2.77 ^{GH}	15.8 ^G	–	–
T3A	2.00 ^G	0.98 ^I	497 ^G	67.3 ^C	28.3 ^G	19.6 ^E	0.52 ^B	0.46 ^B
T1P	1.66 ^H	2.21 ^H	141 ^J	2.78 ^K	3.67 ^J	2.33 ^I	–	–
T2P	17.7 ^C	6.52 ^B	402 ^H	92.0 ^B	26.9 ^H	21.1 ^D	–	–
T3P	0.28 ^I	2.41 ^G	594 ^F	64.7 ^D	18.6 ^I	14.6 ^H	0.45 ^C	0.54 ^A
T1S	2.55 ^F	2.22 ^H	68.9 ^L	38.2 ^H	157 ^B	36.2 ^B	–	–
T2S	10.7 ^D	3.41 ^{DE}	795 ^A	56.7 ^G	46.2 ^F	18.6 ^F	–	–
T3S	2.20 ^G	3.30 ^E	705 ^B	64.6 ^D	162 ^A	37.4 ^A	0.47 ^{BC}	0.44 ^B
T1R	2.49 ^F	2.10 ^H	57.8 ^M	29.5 ^J	63.9 ^D	23.4 ^C	–	–
T2R	19.7 ^B	3.89 ^C	685 ^C	36.3 ^I	54.4 ^E	17.9 ^F	–	–
T3R	2.09 ^G	2.75 ^F	636 ^D	59.7 ^F	103 ^C	36.6 ^B	0.42 ^C	0.46 ^B
SEM	0.025	0.017	0.289	0.117	0.132	0.073	0.008	0.003
CD (5%)	0.069	0.046	0.798	0.323	0.364	0.201	0.022	0.008

See footnotes to Tables 1 and 2.

compared to bacteria. Evaluation of the biofilms for production of IAA revealed that *A. chroococcum* growing in Jensen (1954) medium recorded highest values after 3 and 6 weeks, followed closely by *P. striata* and *M. ciceri* growing in their respective media. Biofilms of *A. torulosa*–*P. striata* followed closely by *A. torulosa*–*S. marcescens* and *A. torulosa*–*M. ciceri* exhibited an increase in IAA after 6 weeks.

In the biofilms of *P. striata*, the ARA values doubled after 3–6 weeks, however, except for *A. torulosa*–*A. chroococcum*, all the other 6-week-old cultures showed lower ARA as compared to 3 weeks (Table 5 left). Highest

values were recorded in *A. torulosa*–*S. marcescens* biofilm after 3 weeks followed by *A. torulosa*–*M. ciceri* biofilm.

In phosphate solubilization, the *A. torulosa*–*A. chroococcum* combination gave a consistent zone up to 10 weeks (Table 5 right). *P. striata* growing in Pikovskaya (1948) medium showed the largest zone followed the same culture growing in BG11 medium. *A. torulosa* also exhibited phosphate solubilization as also the *S. marcescens* and *M. ciceri* growing in their respective media. Zones were absent after 10 weeks.

For the saccharide utilization see Table 6. *A. torulosa* was able to utilize lysine, ornithine, xylose, galactose,

Table 5 ARA as an index of N₂-fixing potential after 3 and 6 weeks (left) and phosphate solubilization after 1, 3, 6, and 10 weeks (right)

Treatment	ARA, nmol ethylene per mL		Phosphate solubilization ^a			
	3	6	1	3	6	10
T0	1.98	0.86	–	+	+	+
T1A	4.98	3.55	–	++	++	–
T2A	33.0	8.06	–	+++	+++	–
T3A	2.00	0.98	–	+	++	+
T1P	1.66	2.21	–	+	+	+
T2P	17.7	6.52	–	–	–	–
T3P	0.28	2.41	–	+	+	–
T1S	2.55	2.22	+	–	–	–
T2S	10.7	3.41	+	+	+	+
T3S	2.20	3.30	–	–	–	–
T1R	2.49	2.10	–	–	–	–
T2R	19.7	3.89	–	+	+	–
T3R	2.09	2.75	–	–	–	–
SEM	4.81	1.414				
CD (5%)	13.30	3.91				

^a Diameter of zone of solubilization: (+)—8–10 mm, (++)—11–15 mm, (+++)—16–18 mm

Table 6 Utilization of saccharides and other compounds of 10-week-old biofilm and original cyanobacterial and bacterial cultures^a

Substrate	<i>A. torulosa</i>	<i>A. chroococcum</i>	<i>A. chroococcum</i> + <i>A. torulosa</i>	<i>P. striata</i>	<i>P. striata</i> + <i>A. torulosa</i>	<i>S. marcescens</i>	<i>S. marcescens</i> + <i>A. torulosa</i>	<i>M. ciceri</i>	<i>M. ciceri</i> + <i>A. torulosa</i>
Glycerol	–	–	–	–	–	–	–	+	–
Ribitol	–	–	–	–	–	–	–	–	–
Xylitol	–	–	–	–	–	–	–	–	–
Ribose	+	–	–	+	–	–	–	+	+
D-Arabinose	–	–	–	–	–	–	–	–	–
L-Arabinose	+	–	+	+	–	–	–	+	–
DL-Arabinose	+	–	–	+	–	+	–	+	–
Xylose	+	–	–	+	+	+	–	+	+
Glucitol	–	+	–	–	–	+	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–
Galactitol	–	–	–	–	–	–	–	–	–
<i>myo</i> -Inositol	–	–	–	–	–	–	–	–	–
Glucose	–	–	+	–	–	–	–	–	–
Glucosamine	–	–	–	–	–	–	–	–	–
Methyl L-glucose	–	–	–	–	–	–	–	–	–
Sodium gluconate	–	–	–	–	–	–	–	–	–
Mannose	+	–	+	+	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–	–
Methyl D-mannoside	–	–	–	–	–	–	–	–	–
Galactose	–	–	–	+	–	+	–	+	–
ONPG	+	+	+	–	+	+	–	–	+
Fructose	–	–	–	–	–	–	–	–	+
Sorbose	–	–	–	–	–	–	–	–	–
Sucrose	+	–	–	–	–	–	+	–	+
Dextrose	+	–	–	+	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–	–
Maltose	–	–	–	–	–	–	–	–	–
Cellobiose	–	–	–	–	–	–	–	–	–
Melezitose	–	–	–	–	–	–	–	–	–
Melibiose	–	–	–	–	–	+	–	–	–
Raffinose	–	–	–	–	–	–	–	–	–
Trehalose	–	–	–	–	–	–	+	+	+
Salicin	–	–	–	–	–	–	–	–	–
Inulin	–	–	–	–	–	–	–	–	–
Urease presence	+	+	+	–	+	–	+	–	+
Citrate utilization	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	–	+	+	–	+	+
H ₂ S production	–	–	–	–	–	–	–	–	–
Lysine utilization	+	+	+	–	+	+	+	+	+
Malonate utilization	+	+	+	+	+	+	+	+	+
Nitrate reduction	–	–	–	–	–	–	–	–	–
Ornithine utilization	+	+	+	–	+	+	+	+	+
Phenylalanine deamination	+	+	+	–	+	–	+	–	+

^a (+)—positive result and/or change in color (recorded up to 2 days), (–)—negative result and/or no change in color

Table 7 Antagonistic activity of individual and co-inoculated cultures against selected fungi after 3 and 6 weeks^a

Treatment	<i>P. debaryanum</i>		<i>F. oxysporum</i>		<i>F. solani</i>		<i>F. lycopersicum</i>		<i>R. solani</i>	
	3	6	3	6	3	6	3	6	3	6
T0	–	–	–	–	–	–	–	–	–	–
T1A	+	+	–	–	+	+	–	–	–	–
T2A	+	+	–	–	+	+	–	–	–	–
T3A	–	–	–	–	–	–	–	–	–	–
T1P	–	+	–	–	+	+	–	–	–	–
T2P	+	+	–	–	++	+	+	+	–	–
T3P	–	+	–	–	+	+	–	–	–	–
T1S	+	+	–	–	+	+	–	–	–	–
T2S	+	+	–	–	+	+	–	–	–	–
T3S	+	+	–	–	+	–	–	–	–	–
T1R	+	+	–	–	+	+	–	–	–	–
T2R	+	+	–	–	+	+	–	–	–	–
T3R	–	–	–	–	–	–	–	–	–	–

^a (+), (++)—degree of inhibition, (–)—no inhibition

dextrose, sucrose, L-arabinose, mannose, ribose, and 4-nitrophenyl beta-D-galactopyranoside (ONPG) besides exhibiting urease activity to phenylalanine deamination. *A. chroococcum* and *S. marcescens* were only able to utilize glucitol. The *S. marcescens*–*A. torulosa* biofilm did not exhibit utilization of a number of substrates as compared to the individual cultures, such as xylose, galactose among others. The *A. torulosa*–*A. chroococcum* biofilm was able to utilize glucose, although the counterpart cultures had not exhibited this ability. Fructose utilization was shown by *M. ciceri*–*A. torulosa* while the partners individually did not exhibit utilization.

Among the bacteria, *P. striata* showed a broad spectrum of inhibition against *P. debaryanum*, *F. solani*, and *F. oxysporum* while the other three strains inhibited *P. debaryanum*, *F. solani* in both 3 and 6-week-old samples (Table 7). Maximum fungicidal activity was observed after 3 weeks, indicating that secondary metabolites are responsible for it. One- and 10-week-old biofilms did not show any inhibition.

Discussion

Cyanobacteria are a remarkable group of photosynthetic prokaryotes, comprising more than 150 genera and 2,000 species, which play diverse yet significant roles in aquatic and terrestrial ecosystems (Whitton 2000). Their major significance in agriculture as biofertilizers, which is of tremendous agronomic value in enhancing crop yields and sustaining soil health, especially in rice based cropping systems, is attributed to their capacity to

fix N₂ from the atmosphere and independence for C (Venkataraman 1981; Prasanna et al. 2009a). Extensive reviews exist on the role of cyanobacteria in rice fields, their geographic and regional distribution and taxonomic and molecular diversity (Mandal et al. 1999; Prasanna and Nayak 2007; Prasanna et al. 2009b).

Our investigation was aimed towards developing biofilms using a selected cyanobacterium as a matrix for co-inoculation with agriculturally useful microorganisms such as *M. ciceri*, *P. striata*, *S. marcescens*, and *A. chroococcum*. We suppose that the photosynthetic activity and production of extracellular polymeric substances by cyanobacteria aids them in associating with diverse members of the microbial, plant, and animal kingdom, which can be exploited as a carrier for inoculants.

Screening of a set of cyanobacterial strains revealed the potential of *A. torulosa* as a biofilm former. Our study using growth attributes and saccharides as indices revealed the viability and growth potential up to 5 weeks. The selected strain exhibits an extended growth phase, which can be a favorable attribute in terms of detainment of co-inoculated bacteria in the mucilaginous matrix.

Co-inoculation with four selected agriculturally useful microorganisms was followed. The presence of envelope of bacterial cells surrounding the cyanobacterial filaments was a good indicator of the promise of these biofilms and the positive role of the mucilage in improving the survival of inoculated bacteria. The higher plate counts of the biofilms showed that positive interactions existed among the cyanobacterium–bacterium, which was confirmed by microscopy. Although there was a dissimilar pattern of reduction in the populations of individual bacterial cultures,

which can be attributed to the intrinsic growth characteristics, the comparatively lower counts illustrated the loss of viability at a much earlier stage of incubation, as compared to biofilms.

The characterization of the biofilms after 3 and 6 weeks of co-inoculation vis-à-vis individual cyanobacterial and bacterial cultures revealed that N₂-fixing potential was much lower in the biofilms, as compared to individual cultures. The *P. striata* and *S. marcescens* strains exhibited N₂ fixation; very few of these strains are considered as legitimate N₂-fixers; on the other hand, Gyaneswar et al. (2001) have demonstrated that several strains classified as *Pseudomonas stutzeri* and *S. marcescens* do have the ability to fix N₂. The saccharide utilization revealed the broader spectrum of the biofilms as compared to the individual strains, confirming the additional benefits of the generated biofilms as inoculants in soil wherein, the ability to utilize a variety of substrates can provide a competitive advantage.

The ability of microorganisms to form phytohormone-like substances or biocidal compounds is also frequently useful in the production of commercial inoculants under controlled conditions. One of the direct evidence for production of phytohormones by PGPR is the production of IAA which is widespread among PGPR that inhabit the rhizosphere of crops. It has been estimated that 80% of bacteria isolated from the rhizosphere can produce IAA (Glick et al. 1999). Many cyanobacteria (such as *Anabaena*, *Nostoc*, *Cylindrospermum*, *Calothrix*, *Plectonema*) are also capable of synthesizing IAA (Sergeeva et al. 2002; Prasanna et al. 2009a, b, 2010). *Azotobacter* strains are known to exhibit high levels of IAA, as also observed by us, but a similar pattern was not observed in co-inoculated biofilm with cyanobacterium. On the other hand, *P. striata*, *M. ciceri*, and *S. marcescens* exhibited an enhancement in IAA production in 6-week-old biofilm over a 3-week-old one. This can be attributed to a positive interaction between the partners in the biofilm; such biofilms need to be tested in plant growth promotion studies.

Many bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Rhizobium*, *Bradyrhizobium*, and *Serratia* have shown potential for biocontrol both under in vivo and in vitro conditions, including suppression of soil-borne fungal pathogens (Glick et al. 1999; Arshad and Frankenberger 1998). Our biofilms developed with cyanobacterium–*P. striata* or *S. marcescens* showed biocidal activity towards the phytopathogenic fungi, as also the individual *P. striata* or *S. marcescens* strains. On the other hand, the *A. chroococcum* or *M. ciceri* exhibited fungicidal potential when growing alone but not in the biofilm.

Fluorescent pseudomonads have been implicated in the control of several soil-borne and wilt diseases in important crops such as wheat, rice and commercial fruits and

vegetables. The global biocontrol regulatory genes in *S. marcescens* and the diverse mechanisms exhibited by them may be significantly contributing to the bioactivity of biofilms in which they are associated. Among cyanobacteria, *Nostoc muscorum* and several *Anabaena* and *Calothrix* strains exhibit fungicidal activity against species of *Pythium*, *Fusarium*, and *Rhizoctonia* (Moon et al. 1992; Prasanna 2008; Radhakrishnan et al. 2009; Manjunath et al. 2010); however, the strain being used by us did not exhibit any fungicidal activity.

Our observations illustrate the positive effects of use of cyanobacteria as a carrier or matrix for improving the viability and agriculturally useful traits of bacterial strains. This would enable effective consortia of microorganisms with enhanced survival capability providing synergistic effects as plant growth promoting inoculants.

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