



Co-cultivation of cyanobacteria for raising nursery of chrysanthemum using a hydroponic system

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Abstract

The present investigation aimed to utilize plant growth-promoting diazotrophic cyanobacteria as an option to raise the chrysanthemum varieties *Pusa Aditya* and *Jaya* in a nursery by co-culturing hydroponically. Fresh stem cuttings of chrysanthemum were planted in BG 11 medium (–N) which was inoculated to log-phase cultures of *Anabaena torulosa* (BF1), *Anabaena doliolum* (BF4), and *Anabaena laxa* (RPAN8) individually. Analyses of chrysanthemum growth and biometric/biochemical parameters after 30 days of co-cultivation revealed that co-culturing treatments performed significantly better, as compared with BG11 medium alone. *Anabaena laxa* brought about an increment of 27–40% in IAA production in the root tissues of both varieties grown in hydroponics. Quantification of biofilm formation on roots (measured as OD₅₅₀) illustrated a two- to four-fold increment in the co-culture treatments. PEP carboxylase activity was significantly enhanced in root and shoot tissues of cuttings in *Jaya*, and the medium chlorophyll enhanced by several folds in both varieties. Significant increases in root dry biomass were recorded, which positively correlated with root protein ($r = 0.992$) in *Pusa Aditya*, illustrating the superiority of co-culturing as a promising option for nursery propagation. The economic benefits of BG 11 medium (without combined N) as a novel growth medium for growing the cyanobacterium and raising chrysanthemum nursery, in co-culturing mode, were also highlighted. *Anabaena torulosa* (BF1) performed well in both *Pusa Aditya* and *Jaya*, while *Anabaena laxa* (RPAN8) was significantly superior in *Jaya*. Future research is focused towards integration of such novel and cheap organic inputs in maintaining disease-free and nutrient-enriched plants in long-term experiments up to flowering stage.

Keywords Biomass · Cyanobacteria · Chrysanthemum · Chlorophyll · Medium · Nutrients

Introduction

Ornamentals, such as chrysanthemum (commonly known as “mum”), are nursery propagated mainly through soil-less cultivation, and hydroponic culture is often used besides soil-less media (cocopeat, perlite, etc.) in greenhouse production

(Handreck and Black 1994). The production of chrysanthemum and other ornamentals requires an optimum mix of macro- and micronutrients, and several soil-less systems and nutrient film techniques have been developed, which can lead to higher productivity, as compared with the use of soil (De Visser and Hendrix 1987). Soil-less cultivation is gaining importance as it is easier to control both the physical and chemical properties of the growth environment and substrate/medium can be selected having negligible chemical activity (Barbosa et al. 2000). Chrysanthemum plants being heavy feeders require heavy doses of N and comparatively lower requirements of P (Yoon et al. 2000). Viyachaia et al. (2015) advocated the benefits of production of cut chrysanthemum produced using two soil-less systems with coconut peat. Alternatively, hydroponics also has several advantages including lower chances of soil-borne pathogens, and provides a safe alternative to soil disinfection. Additionally, hydroponic cultivation facilitates an even distribution of nutrients and water to the plants, thereby reducing wastage and simulating ideal growing conditions.

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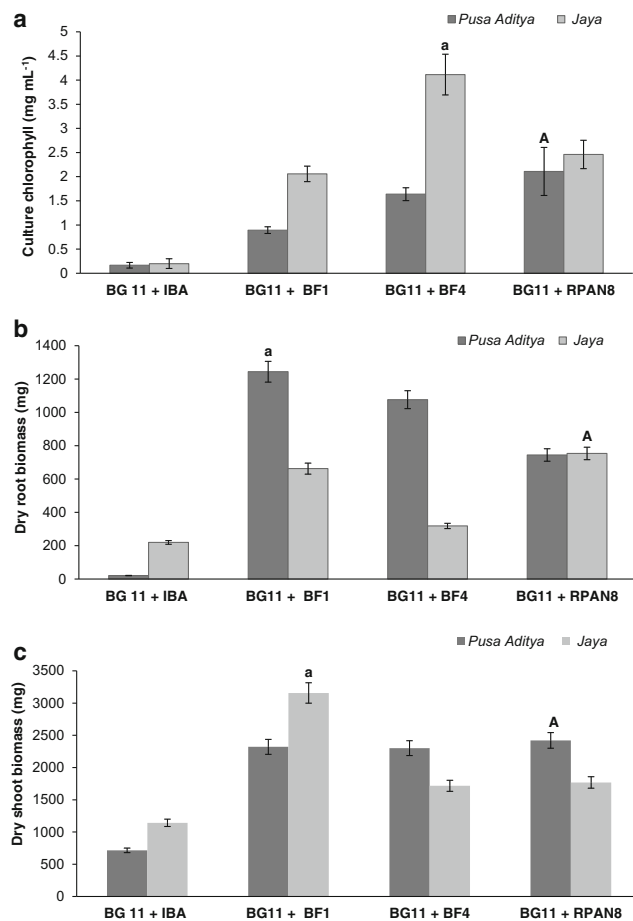


Fig. 1 (a) Estimation of chlorophyll in BG11 medium and (b, c) dry biomass in roots and shoots, respectively, of the chrysanthemum varieties *Pusa Aditya* and *Jaya* in 30-day-old nursery. Error bars denote standard deviations and superscripts denote the highest values among the treatments at $P < 0.05$. The highest ranking treatments in both varieties *Pusa Aditya* and *Jaya* are denoted as A and a, respectively, in the graphs. Abbreviations for treatments: IBA, indole butyric acid; BF1, *Anabaena torulosa*; BF4, *Anabaena doliolum*; RPAN8, *Anabaena laxa*

The use of photosynthetic prokaryotes such as microalgae, particularly cyanobacteria, can be a promising option for providing nutrients in an environment-friendly way, as several are known to fix atmospheric nitrogen and CO₂ and produce phytohormones and other bioactive metabolites. Such molecules elicit defense responses to biotic and abiotic stresses, and improve nutrient availability and plant health (Misra and Kaushik 1989; Gupta et al. 2013; Bharti et al. 2017; Bao et al. 2018; Renuka et al. 2018). Not only are they being used widely in agriculture as biofertilizers and biocontrol agents, they also play a critical role in bioremediation and industrial applications as sources of pigments, biocolloids, etc. Their ecological role in the sustenance of the biosphere

as nutrient-recycling and pollution abatement options is also being increasingly explored (Bao et al. 2018).

Several cyanobacteria are proficient in growing in both aquatic and soil, or soil-less media; co-cultivation with members of several kingdoms—Pteridophyta and Gymnosperms—and members of Angiosperms such as wheat and rice is well established (Gantar et al. 1991). Survey of literature indicates their ability to flourish with rice and wheat in aqueous media and help in enhancing plant growth by improving availability of nutrients and colonizing roots (Jaiswal et al. 2008; Karthikeyan et al. 2009; Babu et al. 2015; Bidyarani et al. 2015; Baglieri et al. 2016; Chittapun et al. 2018; Barone et al. 2019). They also possess the potential to colonize a large number of substrates, including living tissues and inert materials, and co-exist with other microalgae, making them useful options in diverse applications in the environment and, more recently, in aquaponics (Addy et al. 2017; Zhang et al. 2017; Patel et al. 2019).

Natural beneficial microflora prevalent in the rhizosphere are directly involved in the suppression of diseases, thereby limit pathogenic attacks on roots (Tu et al. 1999). One of the primary amendments for soil-less cultivation of cuttings is the use of growth stimulants, such as IBA (indole butyric acid), which is known to stimulate cell division in the ray cells between the primary bundles; this leads to improved root initiation and increased uniformity of rooting (Elhaak et al. 2015). It is now widely recognized that inoculation of plant growth-promoting bacteria (PGPB) can provide similar effects as growth stimulants, as recently reviewed by Ruzzi and Aroca (2015). In our earlier work with inoculants as beneficial options for chrysanthemum, the plant growth-promoting and nutrient-enriching properties of cyanobacterial formulations were illustrated (Kanchan et al. 2019; Prasanna et al. 2016). Hence, the present study was undertaken to evaluate the promise of co-cultivation of cyanobacteria with chrysanthemum for generating healthy and vigorous plants during nursery propagation, with N savings.

Material and methods

Growth and maintenance of cyanobacterial strains

Three cyanobacterial cultures as *Anabaena torulosa* (BF1), *Anabaena doliolum* (BF4), and *Anabaena laxa* (RPAN8) were cultured using standard medium, BG 11, excluding nitrogen source (Stanier et al. 1971) in Haffkine flasks. They were maintained and grown under light-dark cycle (16:8 h), using white light (50–55 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 25 ± 2 °C for 3 weeks.

Table 1 Analysis of plant growth parameters, R:S ratio (root:shoot), and IAA in hydroponically co-cultured cyanobacteria, along with cuttings of *Pusa Aditya* and *Jaya*

Treatment	Chlorophyll (mg g ⁻¹ leaves)	Total proteins (mg g ⁻¹ shoot tissues)	R:S ratio	IAA (μg g ⁻¹ root tissues)
<i>Pusa Aditya</i>				
BG11 + PA + IBA	1.49	1.52	0.84	28.28
BG11 + PA + BF1	1.90	2.06	1.25	25.88
BG11 + PA + BF4	1.34	1.47	0.58	29.74
BG11 + PA + RPAN8	1.47	1.42	0.91	24.31
CD (0.05)	0.33	0.06	NS	0.43
<i>Jaya</i>				
BG11 + J + IBA	1.21	1.40	0.22	22.43
BG11 + J + BF1	1.05	1.49	0.46	19.24
BG11 + J + BF4	1.75	1.37	0.22	21.33
BG11 + J + RPAN8	1.23	1.48	0.52	28.70
CD (0.05)	0.35	0.04	0.22	0.57

PA, *Pusa Aditya*; J, *Jaya*; IBA, indole butyric acid; BF1, *Anabaena torulosa*; BF4, *Anabaena doliolum*; RPAN8, *Anabaena laxa*

Experimental layout and plant material

Hydroponic cultivation of chrysanthemum nursery was established in the glasshouse belonging to the National Phytotron Facility, located at ICAR - Indian Agricultural Research Institute (IARI), New Delhi – 110,012 (latitude 28° 106 38' N, longitude 77° 107 12' E, and altitude 228.4 m) between November and December 2017. The ambient atmospheric conditions were as follows: average temperature of 28 °C and relative humidity of 70–80%.

Plastic pots 4 in. in size were filled with 250 mL sterile N-free BG11 medium (Stanier et al. 1971) and inoculated with cyanobacterial cultures at the rate of 2 μg chlorophyll mL⁻¹ medium in each pot, except control pots. Fresh cuttings of *C. morifolium* varieties *Pusa Aditya* and *Jaya* (apical stem with 2–3 leaves) were collected from the Floriculture field on the day of experimental setup. They were dipped in indole butyric acid solution (500 ppm) for 5 min and air-dried. Following air-drying, three cuttings of each variety were placed in holes made in the circular thermocol lids kept over the surface of

Fig. 2 Rooting pattern in chrysanthemum varieties *Pusa Aditya* and *Jaya* cuttings, co-cultured with cyanobacteria through hydroponic mode of cultivation. *Pusa Aditya* grown in BG11 medium: without inoculation (a), with *Anabaena torulosa* (b), *Anabaena doliolum* (c), *Anabaena laxa* (d). *Jaya* grown in BG11: without inoculation (e), with *Anabaena torulosa* (f), *Anabaena doliolum* (g), *Anabaena laxa* (h)



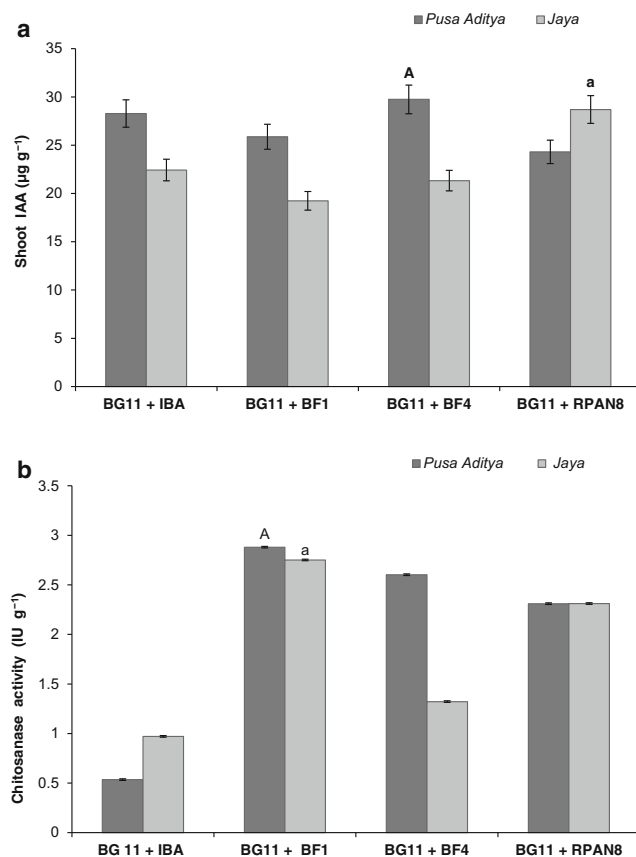


Fig. 3 Assay of (a) indole acetic acid production in the shoots and (b) chitosanase activity in the roots of chrysanthemum in BG11 medium. Error bars denote standard deviations and superscripts denote the highest values among the treatments at $P < 0.05$. The highest ranking among treatments in both varieties is denoted as A and a in the graphs. Abbreviations for treatments: IBA, indole butyric acid; BF1, *Anabaena torulosa*; BF4, *Anabaena doliolum*; RPAN8, *Anabaena laxa*

each pot, such that cuttings were dipped in medium solution. The experiment was set up following a completely randomized design, with three replicates for each treatment, and monitored at regular intervals for a period of 30 days.

Microbiological analyses

Chlorophyll content of the hydroponic medium was measured as an index for photosynthetic biomass, both in treatment and control pots. Ten milliliter of suspension was taken, and centrifuged at $9000\times g$ for 10 min. Ten milliliter of 95% methanol was added to the pellet and kept in a water bath at 70°C for 30 min. Extracted pigment was measured spectrophotometrically at 650 nm and 665 nm (Mackinney 1941).

Microscopic analyses of roots of *Pusa Aditya* and *Jaya* were done using a phase-contrast light microscope (with attached Zeiss Model Axio Scope) without staining, at $\times 20$ and $\times 40$ magnifications to illustrate the colonization of cyanobacterial filaments.

Plant analyses

Hydroponically grown nursery samples were analyzed for root, shoot and total biomass, in terms of fresh and dry weight. Lengths of roots and shoots were measured separately using a centimeter scale, while for evaluating their dry biomass, plant samples were kept in an oven at 60°C and incubated until constant weight was achieved. Leaf chlorophyll *a* and *b* were analyzed by adding 10 mL of dimethyl sulfoxide (DMSO) to 100 mg fresh weight of leaves (Hiscox and Israelstam 1979). Absorbance of extracted pigments was measured at 480, 510, 645, and 663 nm. Calculations were done using the equations given by Arnon (1949).

$$\text{Chlorophyll } a \text{ (mg g}^{-1} \text{ Fwt)} = \frac{\{(12.7 \times \text{OD}_{663}) - (2.69 \times \text{OD}_{645})\} \times V}{1000 \times W}$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1} \text{ Fwt)} = \frac{\{(22.9 \times \text{OD}_{645}) - (4.68 \times \text{OD}_{663})\} \times V}{1000 \times W}$$

where:

V volume of chlorophyll extracted in DMSO (mL)

W fresh weight of the leaf tissue (g)

IAA in root and shoot samples was estimated following Gordon and Paleg (1957). Fresh plant tissues (0.5 g fresh weight) were ground using 2 mL of methanol and centrifuged at $8000\times g$ for 10 min. One milliliter of acidified filtrate (with orthophosphoric acid) was added to 2 mL of reagent (containing 35% perchloric acid and 2% 0.5 M FeCl_3), and the filtrate was incubated in the dark at 30°C for 1 h. The color intensity was recorded spectrophotometrically at 535 nm.

Chrysanthemum roots were gently washed with distilled water and 100 mg of tissue was soaked in 2 mL of 0.1% crystal violet for 15 min. Excess stain was then decanted and roots were washed with tap water. Thereafter, destaining of roots with 4 mL of 30% acetic acid was followed for 10 min. The color intensity of destained solution was determined spectrophotometrically at 550 nm to measure biofilm formation around root surface (O'Toole 2011).

Phosphoenol pyruvate carboxylase (EC 4.1.1.31) activity was assayed following the method given by Wu and Wedding (1985). Fresh leaves/root tissues (1 g) were finely ground in 1.5 mL extraction buffer containing 0.1 M Tris-HCl pH 7.8, 0.01 M MgCl_2 , 0.5 M sucrose, 0.05 M dithiothreitol, and 1 g of PVP. Following centrifugation at $10,000\times g$ for 15 min, 100 μL of tissue extract was taken as substrate in incubation buffer (containing 0.1 M Tris-HCl pH 7.8, 0.01 M MgCl_2 , 0.05 M NaHCO_3 , 1 mM PEP- Na_3 , and 0.1 mM NADH). Absorbance was taken at 340 nm and enzyme activity expressed as micromoles oxidized NADH mg^{-1} protein min^{-1} .

Table 2 Analysis of root enzyme activities in hydroponically grown *Pusa Aditya* and *Jaya* co-cultured with cyanobacteria

Treatment	PAL (nmol trans-cinnamic acid mg ⁻¹ root protein h ⁻¹)	β,1-3 endoglucanase (IU mg ⁻¹ root protein)	PEP carboxylase (μmol oxidized NADH mg ⁻¹ root protein min ⁻¹)	Glutamine synthetase (γ-glutamyl hydroxamate g ⁻¹ tissue min ⁻¹)
<i>Pusa Aditya</i>				
BG11 + PA + IBA	0.08	1.92	9.83	1.49
BG11 + PA + BF1	0.99	2.65	6.74	1.90
BG11 + PA + BF4	0.74	1.99	7.57	1.34
BG11 + PA + RPAN8	0.64	1.89	3.23	1.47
CD (0.05)	0.07	0.04	0.08	0.15
<i>Jaya</i>				
BG 11 + J + IBA	0.45	2.28	3.39	1.21
BG11 + J + BF1	1.98	1.98	23.50	1.05
BG11 + J + BF4	0.18	1.57	13.31	1.75
BG11 + J + RPAN8	0.20	1.98	20.60	1.23
CD (0.05)	0.075	0.03	0.32	0.14

PA, *Pusa Aditya*; J, *Jaya*; IBA, indole butyric acid; BF1, *Anabaena torulosa*; BF4, *Anabaena doliolum*; RPAN8, *Anabaena laxa*

Glutamine synthetase (EC 6.3.1.2) activity was measured by the method of Shapiro and Stadtman (1970). A total of 0.5 g of root tissue was extracted using 1.5 mL of buffer containing 2-imidazole HCl (50 mM, pH 7.0), 5 mM MgCl₂, and 10 mM sodium glutamine. The extract was centrifuged at 10,000×g for 10 min. One hundred microliters of enzyme extract was added to 1 mL of assay mixture (containing 0.1 M MnCl₂, 0.1 M glutamate, pH 7.0; 1.0 M disodium hydrogen arsenate, pH 7.0; 0.01 M ADP, pH 7.0; 2.0 M hydroxylamine HCl, 2.0 M sodium hydroxide; 50 mM imidazole HCl buffer, pH 7.0) and incubated at 37 °C for 30 min. Absorbance was recorded at 540 nm and enzyme activity was expressed in μmol γ-glutamyl hydroxamate produced g⁻¹ tissue min⁻¹.

Assays of hydrolytic enzymes β-1,3-endoglucanase (EC 3.2.1.39) and chitosanase (EC 3.2.1.99) were performed using substrates, laminarin, and glycol chitosan, respectively, as described in Prasanna et al. (2008). For performing these assays, 2 g of plant tissue was macerated using 10 mL buffer (0.1 M phosphate buffer, pH 7.5). These activities were expressed as one unit of chitosanase activity being representative of μmol glucosamine released min⁻¹ g⁻¹ fresh weight, following standard assay conditions. One unit of endoglucanase activity was representative of 1 μmol of glucose liberated min⁻¹ g⁻¹ fresh weight under standard assay conditions. Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) activity was determined following the method of Whetten and Sederoff (1992). One hundred microliters of plant tissue filtrate (1 g extracted in 5 mL of 0.1 M phosphate buffer, pH 7.2) was added to 600 μL of 0.2% L-phenylalanine and incubated at 30 °C for 60 min, followed by addition of 2 N HCl to stop the reaction. A total of 1.5 mL toluene was then added in reaction mixture,

vortexed for 20 s, and centrifuged at 10,000×g for 5 min. Absorbance of separated upper phase was taken at 290 nm, and activity was expressed as nmol trans-cinnamic acid mg⁻¹ protein min⁻¹.

Total phenol content was determined following the protocol given by Singleton et al. (1999), using 1 g tissue and macerating in 5 mL buffer (0.1 M phosphate buffer, pH 7.2). A total of 0.5 mL of filtrate obtained from plant tissue macerated was diluted with 1 mL of distilled water. To this, 0.5 mL of Folin-Ciocalteu reagent (diluted with water in 1:1 ratio) and 1 mL of Na₂CO₃ were added and incubated in boiling water for 1 min. After cooling at room temperature, distilled water was added to make up the volume to 5 mL. Absorbance was then measured at 660 nm and amount of total phenol in samples was expressed as mg of caffeic acid equivalent (CAE) g⁻¹ fresh weight. Total protein in plant root extract (1 g in 5 mL of 0.1 M phosphate buffer) was determined by Lowry method (Lowry et al. 1951). A total of 0.5 mL of filtrate was added to a tube containing 4.5 mL of reagent I (containing 2% Na₂CO₃ in 0.1 N NaOH, 1% sodium potassium tartrate, and 0.5% CuSO₄ in 48:1:1 ratio) and incubated for 10 min at room temperature. After this, 0.5 mL of reagent II (Folin-Ciocalteu reagent and distilled water in 1:1 ratio) was added and incubated under dark conditions for 30 min. The intensity of the blue color obtained was measured spectrophotometrically at 660 nm and protein content was calculated using standard curve of bovine serum albumin (BSA).

Statistical analyses

Mean values of triplicates were calculated using Microsoft Excel including standard deviation (SD) values as depicted

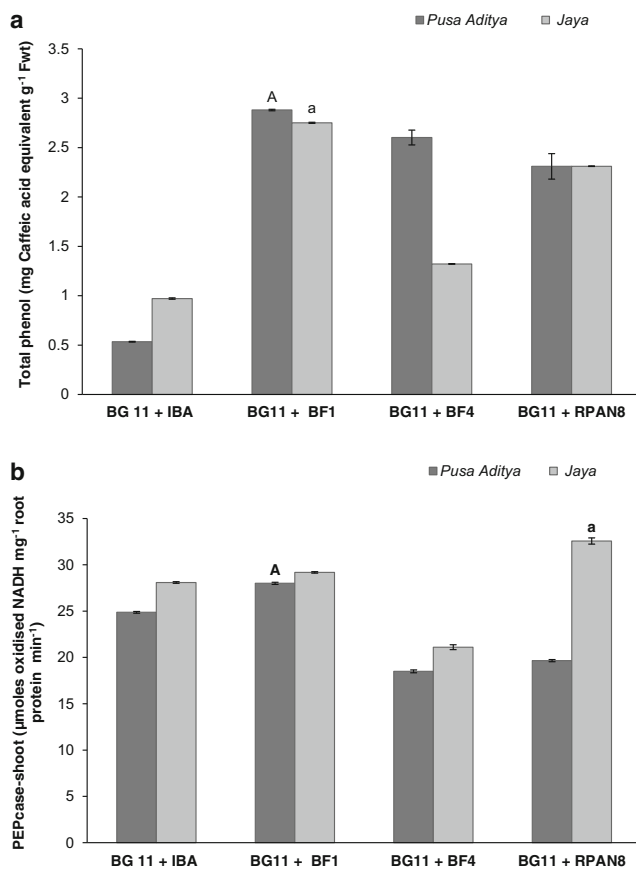


Fig. 4 (a) Analysis of total phenolics in the roots and (b) phosphoenolpyruvate carboxylase activity in the shoots of chrysanthemum cuttings grown in BG11 medium. Error bars denote standard deviations and superscripts denote the highest values among the treatments at $P < 0.05$. The highest ranking among treatments in both varieties—*Pusa Aditya* and *Jaya*—are denoted as A and a, respectively, in the graphs. Abbreviations for treatments: IBA, indole butyric acid; BF1, *Anabaena torulosa*; BF4, *Anabaena doliolum*; RPAN8, *Anabaena laxa*

in graphs (error bars). Statistical analyses were done with statistical package WASP 2 (Web Agri Stat Package, Indian Council of Agricultural Research, India) to obtain critical differences (C.D.) at a probability level of 0.05. Correlations were calculated using XLSTAT program at P value of 5%. In the graphs and tables, IBA refers to indole butyric acid, and BG 11 denotes liquid medium used for hydroponic co-cultivation of both cyanobacteria and chrysanthemum, while BF1, BF4, and RPAN8 represent the cyanobacterial cultures (*Anabaena torulosa*, *Anabaena doliolum*, and *Anabaena laxa*, respectively).

Results

Growth response of cyanobacteria in co-culturing experiment

Estimation of photosynthetic biomass (chlorophyll content) in the aqueous solution was undertaken to evaluate the growth of

the cyanobacterial strains in the hydroponic medium. Chlorophyll accumulation was highest in treatment containing RPAN8 in *Pusa Aditya* and BF4 in *Jaya*, with 11.6- and 19.5-fold increases respectively (Fig. 1a). Leaf chlorophyll measurements showed 27% and 44% increments in *Pusa Aditya* (BF1) and *Jaya* (BF4), respectively (Table 1). Dry biomass in root was found to be highest in *Pusa Aditya* which has a 67.5-fold increment in dry biomass, having maximum value with treatment BF1 (55.6-fold) while the treatments with *Jaya* resulted in lower values for root biomass. Increase in shoot dry biomass was lesser as observed in both varieties, where RPAN8 brought a 2.39-fold increase, while BF1 showed a 1.76-fold increase (Fig. 1b, c).

Early rooting initiation and more dense appearance were observed in the co-culturing treatment with cyanobacteria in both the varieties. Co-culturing with cyanobacteria brought about significant differences in the rooting pattern, when compared with controls in both varieties (Fig. 2), besides showing correlation with shoot protein (0.956) and R:S ratio (0.961) (Supplementary Tables 1, 2). In addition to increase in R:S ratio, the inoculated treatments also exhibited early initiation of secondary roots in both varieties, as observed visually. The enhanced rooting supported higher root:shoot ratio, with treatment using BF1, recording values of 1.2 in *Pusa Aditya* and 0.52 with RPAN8 in *Jaya* when compared with their controls (0.8 and 0.22 respectively) (Table 1). R:S values were significantly higher with respect to control in *Pusa Aditya*, with two-fold higher values recorded in BF1 and RPAN8 treatments, as compared with control in *Jaya*. The R:S ratio was highly correlated with leaf chlorophyll ($r = 0.961$) in *Pusa Aditya* and with root dry biomass in *Jaya* ($r = 0.990$) (Supplementary Tables 1, 2).

BF1 brought significant increments of 35.5% in *Pusa Aditya* and 6.2% in *Jaya* in shoot protein (Table 1), which were also highly correlated with leaf chlorophyll ($r = 0.956$) and β ,1-3 endoglucanase activity ($r = 0.988$). Positive correlation was observed for root dry biomass in *Pusa Aditya* for plant protein (root, $r = 0.992$; shoot, $r = 0.962$) and with R:S ratio ($r = 0.990$) in *Jaya* (Supplementary Tables 1, 2).

Biochemical activities in the plant tissues

Cyanobacterial co-culturing enhanced root and shoot IAA in chrysanthemum cuttings grown in hydroponics. Inoculation of *Anabaena laxa* (RPAN8) significantly enhanced IAA production in roots and shoots of chrysanthemum; in relation to root IAA, enhancements of 64% and 14.3% were observed with *Pusa Aditya* and *Jaya*, respectively (Table 1; Fig. 3a), while a 28% increase in shoot IAA was observed with *Jaya*, and *Pusa Aditya* having lower values with BF4 (Fig. 3a). IAA production in *Jaya* was also correlated with root phenol content ($r = 0.988$) and chitinase activity ($r = 0.972$) (Supplementary Tables 1, 2).

Analyses of plant defense and hydrolytic enzyme activities illustrated elicitation of polyphenylalanine lyase (PAL),

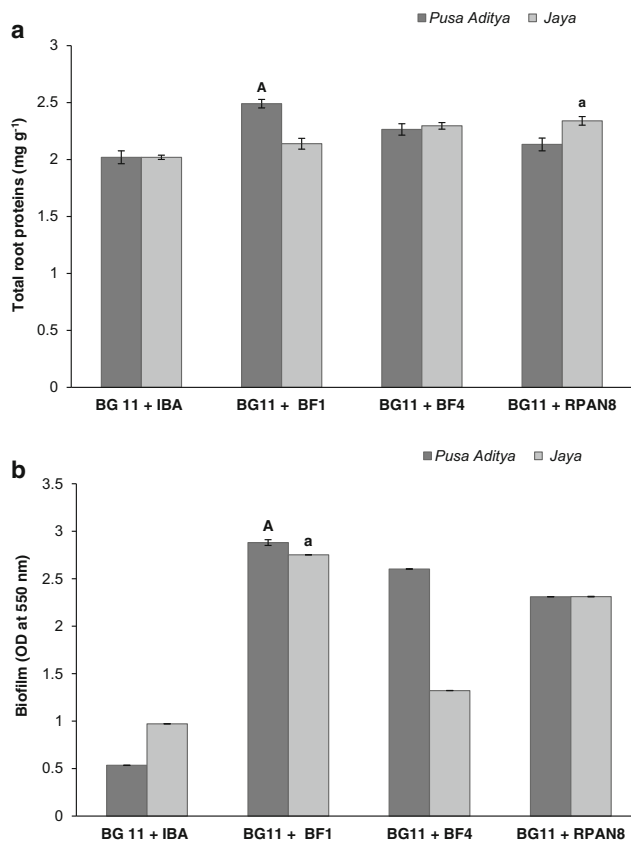


Fig. 5 (a) Estimation of total proteins in chrysanthemum root tissues. (b) Biofilm formation around the root surface in BG11 medium. Error bars denote standard deviations and superscripts denote the highest values among the treatments at $P < 0.05$. The highest ranking among treatments in both varieties is denoted as A and a in the graphs. Abbreviations for treatments: IBA, indole butyric acid; BF1, *Anabaena torulosa*; BF4, *Anabaena doliolum*; RPAN8, *Anabaena laxa*

chitinase, and β ,1-3 endoglucanase, when co-cultured with BF1 in *Pusa Aditya*, which brought 11.4- and 0.38-fold increases in PAL and β ,1-3 endoglucanase, respectively (Table 2) with a low level of stimulation of chitinase activity (Fig. 3b). Variety *Jaya* showed negligible activity for these enzymes, except a 0.14-fold increase in chitinase activity with RPAN8. Plant root and shoot enzymes were positively correlated with many physiological and biometric parameters evaluated in both the varieties (Supplementary Tables 1, 2).

Total phenolics in chrysanthemum root extract were also assessed, and the highest phenol content was observed in treatment containing BF1 in *Pusa Aditya* (35% increase) while in *Jaya* (13.5%) it was much lower (Fig. 4a). Plant enzymatic machinery was triggered in chrysanthemum by co-culturing with cyanobacteria. Photosynthetic assimilatory enzyme, phosphoenolpyruvate (PEP) carboxylase, activity in shoots was elevated by 12.6% in *Pusa Aditya* (through BF1) and 15.9% in *Jaya* (through RPAN8) (Fig. 4b). PEPCase activity in root was 6-fold higher in *Jaya* (BF1) while negligible in *Pusa Aditya* (Table 2). The activity of another

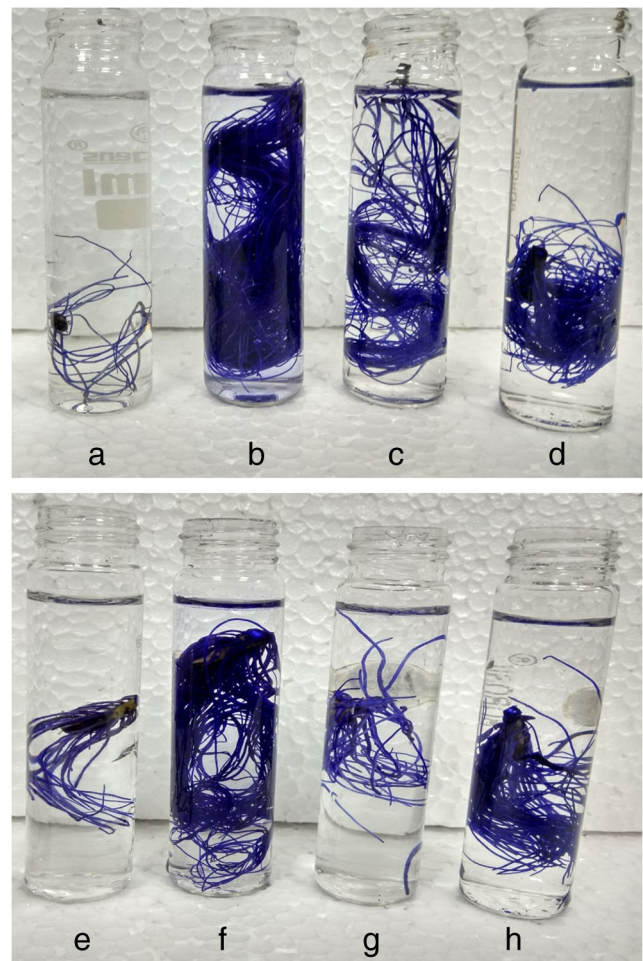


Fig. 6 Biofilm staining using crystal violet of the roots from the *Pusa Aditya* and *Jaya* cuttings, co-cultured with cyanobacteria through hydroponic mode of cultivation from 30-day-old nursery. *Pusa Aditya* grown in BG11 medium: without inoculation (a), with *Anabaena torulosa* (b), *Anabaena doliolum* (c), *Anabaena laxa* (d). *Jaya* grown in BG11: without inoculation (e), with *Anabaena torulosa* (f), *Anabaena doliolum* (g), *Anabaena laxa* (h)

assimilatory enzyme—glutamine synthetase activity in root extracts, essential in nitrogen metabolism—showed a marked increase of 87% with BF1 inoculum in *Jaya* while *Pusa Aditya* showed increase of 22% with RPAN8 (Table 2).

Total proteins in *Pusa Aditya* and *Jaya* were assayed in root tissues as an index of root colonization-derived benefits. BF1 treatment brought enhancement of 23.3% in *Pusa Aditya* while RPAN8 showed 15.8% increase in *Jaya* in terms of root protein (Fig. 5a).

Biofilm formation and root colonization by cyanobacteria

All the three cyanobacterial strains showed a greater fold increase in absorbance values for biofilm formation with the values of BF1 (4.38) > BF4 (3.86) > RPAN8 (3.31) in *Pusa Aditya* while

for *Jaya* the order was BF1 (1.83) > RPN8 (1.38) > BF4 (0.36). *Anabaena torulosa* (BF1) accounted for the highest absorbance in terms of biofilm formation around roots of both varieties (Figs. 5b and 6). Light microscopy showed short filaments of *Anabaena* in *Pusa Aditya* and *Jaya*. Filaments of *A. torulosa* (c, d); *A. doliolum* (e, f) and *A. laxa* (g, h) adhering to the surface of roots (Fig. 7).

Discussion

Cyanobacteria have been widely applied as bio-inoculants in rice-based cultivation systems, which represent natural aqueous/hydroponic settings. In such environments, they proliferate and represent active colonizers not only in the floodwater/surface of aqueous column but also in the rhizosphere and on/around rice roots (Nilsson et al. 2002, 2005; Prasanna et al. 2009) under both in vitro and in vivo conditions. This niche behavior was explored for the use of cyanobacteria in the growth of chrysanthemum cuttings,

which revealed that the medium components along with root exudates of chrysanthemum supported the growth requirements of both the partners in the co-culturing experiment. The hydroponic mode has been investigated as a mode of priming plants such as those of rice and wheat with cyanobacteria earlier in our lab studies (Karthikeyan et al. 2009; Babu et al. 2015; Bidyarani et al. 2015). Svircev et al. (1997) also reported the promise of cultivation of cyanobacteria with different crops in hydroponic mode. This is supported by earlier studies on biofilm-forming abilities and its use as a matrix for developing synergistic formulations with agriculturally beneficial bacteria and fungi (Prasanna et al. 2011, 2016, 2018). The colonization of root tissues by cyanobacteria has been demonstrated earlier (Gantar et al. 1991; Jaiswal et al. 2008; Karthikeyan et al. 2009; Bidyarani et al. 2015), and as they readily form biofilms, the interaction becomes more effective. Cyanobacteria colonize the root tissues by excreting exopolymeric substances that help their adherence to roots, which was confirmed also by biofilm assay of chrysanthemum roots.

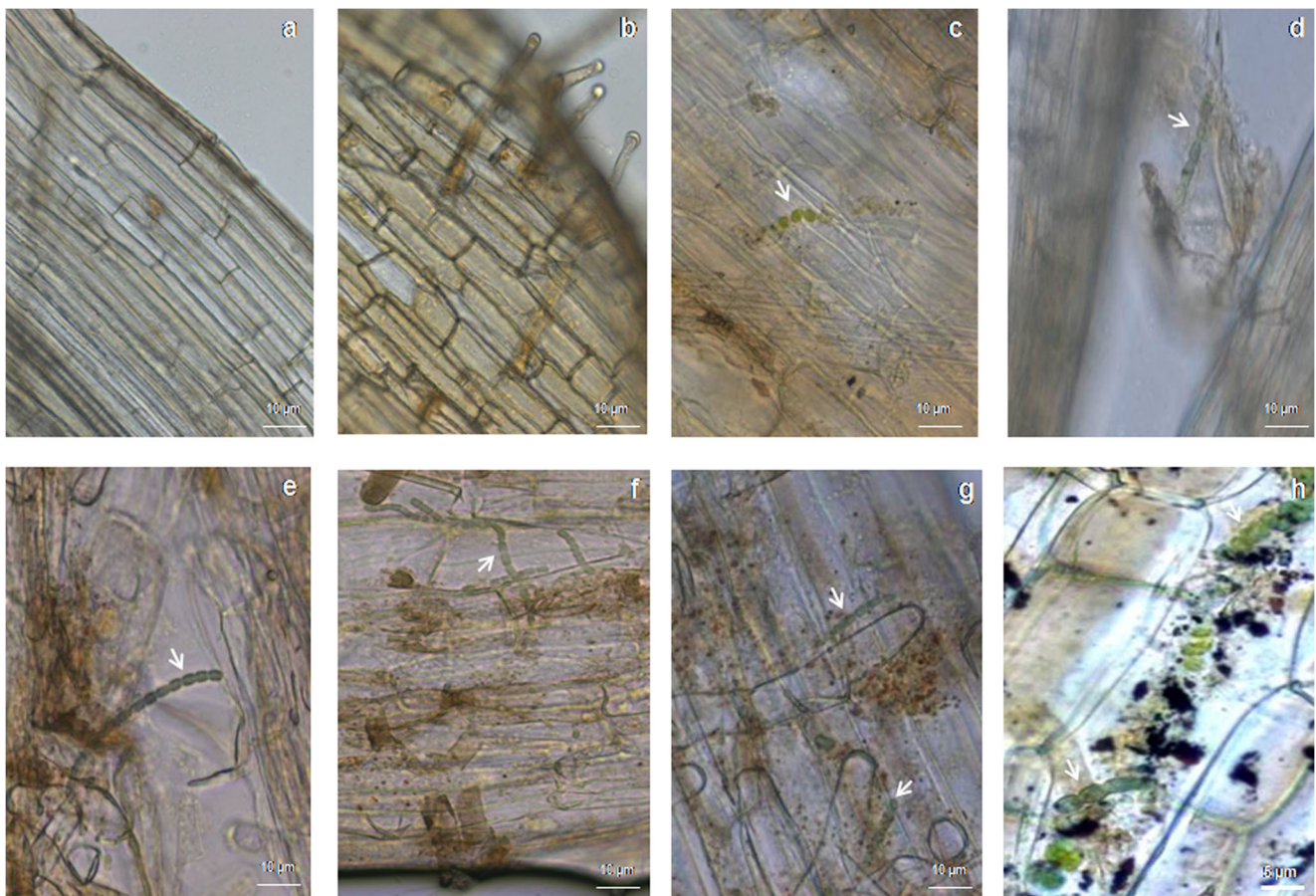


Fig. 7 Phase-contrast light microscopy photographs illustrating the colonization of *Anabaena* cultures around root tissues of chrysanthemum at $\times 20$ and $\times 40$ magnifications. Arrows depict 2–3-celled, short and long filaments of cyanobacteria surrounding roots; uninoculated samples of *Pusa*

Aditya (a) and *Jaya* (b) are included as controls. *Anabaena torulosa* in *Pusa Aditya* and *Jaya* (c and d, respectively); *Anabaena doliolum* in *Pusa Aditya* and *Jaya* (e and f, respectively); *Anabaena laxa* in *Pusa Aditya* and *Jaya* (g and h, respectively)

Hydroponics is gaining importance as a means of soil-less cultivation as it circumvents problems of soil-borne pathogens and nutrient deficiencies in different soil types (Liptay and Tu 2003). In the present investigation, BG 11 medium, without addition of N source (sodium nitrate), was used as nutrient solution/aqueous medium (Stanier et al. 1971), which is a general growth purpose medium for the cultivation of cyanobacteria. This medium supported the growth of chrysanthemum nursery, being quite similar in its composition to Hoagland's solution (except for absence of N source in BG 11 medium) and other commercial media used in hydroponics for various plant species (Hoagland and Arnon 1950). Hydroponic cultivation of chrysanthemum is often limited by nutrients and anaerobic conditions in the rooting region (Soffer and Burger 1988), as it affects the timing of rooting, rooting percentage, and number of roots. Co-culturing with cyanobacteria, which are oxygen-evolving phototrophs, can provide a continuous supply of desired oxygen gradients for the proliferation of roots and better growth of the cuttings, as observed in this investigation. Synergistic behavior of both partners illustrated was further analyzed in terms of biomass accretion and chemical profiles.

Increase in chlorophyll content is related to photosynthetic efficiency of the growing plant that is aided indirectly through cyanobacterial inoculation that furnish C and N metabolites in the periphery of plant roots. The positive effect of co-culturing with cyanobacteria was observed in terms of enhanced growth of chrysanthemum cuttings, with proliferation of root primordia. Early initiation of secondary roots in both varieties, as observed visually, has been earlier reported in strawberry by microalgal extracts (Mattner et al. 2018). The fresh cuttings itself possess some nutrients in the stem, and co-culturing with cyanobacteria can further provide carbohydrates, through photosynthesis, and facilitate supply of nitrogenous compounds (as result of nitrogen fixation) as well as phytohormone release such as IAA (Sergeeva et al. 2002; Shariatmadari et al. 2015), which help in enhancing root initiation, hair formation, and morphogenesis of root tissues. The hydrolytic and defense enzyme system may help in developing antagonism towards commonly encountered phytopathogens, such as *Pythium* in chrysanthemum (Liu et al. 2007) in hydroponic units. The property of cyanobacteria to induce/ elicit these enzymes makes them effective biocontrol agents, as reported for *A. laxa* (Prasanna et al. 2008). Biofilm formation around roots is a measure of the exopolysaccharide secretions by the cyanobacterial strain in the vicinity of plant roots, which also facilitates better interactions of the cyanobacterium with the roots. The comparative performance of cyanobacterial strains in hydroponic cultivation of chrysanthemum was analyzed for various parameters and *A. torulosa* was identified to be most promising as it performed equally well in both varieties and significantly better

in *Pusa Aditya*. *Anabaena laxa* was the top-ranked cyanobacterium as inoculant in *Jaya* (Supplementary Table 3).

Many defense-related pathways and precursor molecules for plant structures are generated with increase in phenolics (Siqueira et al. 1991) and it was interesting to note that they did not have deleterious effects on cyanobacterial metabolism. Increase in protein content also illustrates higher nitrogen content in plant which is made available by cyanobacterial nitrogen fixation. Liu et al. (2010) reported that nitrogen is essential for building plant biomass and the synthesis of enzymes in chrysanthemum. Andrews (1993) observed a decrease in dry weight ratios of shoot to root (S:R) as a result of N-mediated growth limitation. Andrews et al. (1999) evaluated the effects on this ratio in three crop species and concluded that the effect of macronutrients on plant metabolism is mediated through protein synthesis and growth, which in turn, in particular root differentiation, is often accomplished mainly by the availability of nitrogenous substrates. In this investigation, the nitrogen-fixing potential of cyanobacteria can represent a better substitute over chemical fertilization and provide a continuous supply facilitating better metabolic activities.

In the present experiment, cyanobacteria being oxygen-evolving phototrophs were able to help in early rooting and development of more roots, and influencing the timing of rooting, rooting percentage, number of roots, and root length. Selection of best growing media is based on several criteria, including optimal balance between water content and nutrient exchange, besides nutrient composition (Soffer and Burger 1988). Plant developmental stages can also be sometimes altered in hydroponic cultivation as observed in *Globba* spp. which flowered earlier when grown in hydroponic as compared to soil medium (Phantong et al. 2018).

Hydroponic farming practices also facilitate a stringent use of water and fits best in the theme “more crop per drop of water.” Hydroponics is additionally advantageous due to ease of sampling for biochemical and physiological studies, which can be made at different stages of growth and development as reported in various crops such as *Helianthus* (Soudek et al. 2006) and sugar beet (Barone et al. 2018), or in nutrient uptake by plants (Radzki et al. 2013). Besides, the cost incurred with the use of BG 11 medium as hydroponic medium was found to be low. The cost of 1000 L of BG 11 was Rs. 157.24 (equivalent to US\$ 2.21 as per currency exchange rates, February 2019), vis-a-vis commonly used nutritive solutions for hydroponics such as Hoagland and Arnon (Rs. 2028.69~US\$ 28.49), Castellane and Araujo (Rs. 2655.41~US\$ 37.29), and Furlani (Rs. 1402.52~US\$ 19.69) (as given in Melo and Dos Santos 2011). Therefore, the present medium can be supportive for low-income groups of progressive farmers to set up hydroponic farming units within a reasonable budget.

The hydroponic mode of co-culturing sustained the growth of chrysanthemum and cyanobacteria, highlighting the

superiority of co-culturing as a promising option for nursery raising, before planting in pots or field. The use of BG 11 medium, which is a novel option for chrysanthemum, proved to be a better nutrient mix for the proliferation of chrysanthemum cuttings through organic means, illustrating this option as an environmentally sustainable endeavor. Our future work is focused on evaluating the quality traits related to floral attributes from such soil-less nursery-grown plants.

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Compliance with ethical standards

The present investigation did not involve use or harm of animals nor the participation of humans. All authors gave consent for submission of the final manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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