Saudi Journal of Biological Sciences 27 (2020) 2325–2332

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Biological soil crust (BSC) is an effective biofertilizer on *Vigna mungo* (L.)

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ARTICLE INFO

Article history:
Received 23 February 2020
Revised 8 April 2020
Accepted 13 April 2020
Available online 27 April 2020

Keywords:
Biological soil crust
Indole 3-butyric acid
Nitrogenase activity
Biofertilizer
Cyanobacteria

ABSTRACT

Biological soil crusts (BSC) (Cyanobacteria) play an important role in the soil nitrogen fixation and soil stabilization. However, limited researches were carried out about the diversity and distribution of Bio crust in sacred forests. The study aims to identify the distribution of cyanobacteria in biological soil crusts from different sacred groves of Ariyalur and Pudukottai, Tamil Nadu. We identified following microbes of *Microcoleus, Scytonema, Anabaena* and *Nostoc* in biological soil crust. A surface experiment was conducted for the efficacy of biological soil crusts on crops seedling growths. The efficacy was assessed by measuring the root & shoot length, dry & fresh weight of the plant, total chlorophyll, protein & carbohydrate content, with reducing sugar. The plant growth was higher in biocrust inoculated pot than the control. In corresponding nitrogenase activity was determined by the acetylene reduction assay, and phytohormones documentation was executed by the high-performance liquid chromatography. The results indicating that the biological soil crusts are the promising factors influencing the plant growth by plant growth-stimulating auxins and nitrogenase activity.

1. Introduction

Biological soil crust (BSC) is one of the major components of the sacred grove forests. The cyanobacterial species-rich in Biological soil crust, which is beneficial for fixing atmospheric nitrogen (N) in the soil, retain moisture, soil adherence and preventing erosion by extracellular polymorphic substances (Xie et al., 2007). These biological soil crusts have flourished in monsoon season and it becomes drily wrinkled in the summer season. In living conditions, the BSCs cyanobacteria excrete plant-growth-promoting hormones and plant growth regulator substances (PGRs) like vitamins, amino acids and sugars (Paul and Nair, 2008). These polymorphic substances are the most important factors, that have to stimulate the growth of the plants (Rastogi and Sinha, 2009). This biological soil crust holds much amount of macro and micronutrient in dried form. The BSCs dry matter contributes to organic matter, which improves soil fertility, maintains the optimum level of NPK and improves the water holding capacity (Román-Fernández et al., 2018). The BSCs member converts complex nutrients that are readily absorbed by plants.

The BSCs inoculum in the soil is a suitable tool for soil restoration (Rossi et al., 2017). In modern years the soil has been contaminated by extensive use of chemical fertilizer and pesticides. Since the 1990s Farmer's death in India states the national crisis of farmer's suicide, regularly by consumption of pesticides, for unpaid loans from banks and NBFCs to procure expensive seeds and fertilizers, being sold by foreign companies (Schurman, 2013). Recently the agriculture depends on chemical fertilizers for higher yield which diminishes the soil microbiota restored soil fertility (Singh et al., 2016). These chemical fertilizers are a higher cost that affects the farmers financially. Hence, these biological soil crusts are found to be a positive contribution to soil fertility by increasing crop yield deprived of environmental, water or soil pollution (Rossi et al., 2017). Nitrogen-fixing and

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.04.022
1319-562X/

Phosphorus solubilizing cyanobacteria play an important role in nitrogen mobilization and phosphorus solubilization for the benefit of plant growth (Adessi et al., 2018).

Most of the crop soil has a natural population of filamentous cyanobacteria members which provide favorable nutrient availability (Weber et al., 2015), and moisture for seed germination and plant growth. Abundance and diversity of cyanobacteria in sacred grove soil BSCs have been reported. These all features indicate that the biological soil crust directly involved in plant growth and spread the plant vegetation in sacred groves forest (Vinoth et al., 2017). The cyanobacteria dominated Biological soil crust chosen for the study had formerly shown many plant-growth-promoting hormones like indole 3-acetic acid, IAA; indole 3-propionic acid, IPA and indole 3-butyric acid, IBA. These BSCs cyanobacteria are good P-solubilizer and N-fixers in a live condition and rich amounts of nutrients consisted of dry nature (Obana et al., 2007).

Soil inoculant rich in cyanobacteria members significantly increases the yield of many crop plants in the cultivated field. The cyanobacterium in the form of biological soil crust is a good beneficiary for the growth of crop plant and soil restoration. An important genus such as *Nostoc* and *Anabaena* has been used as a biofertilizer for crop cultivation (Shariatmadari et al., 2013).

Vigna mungo (L.) (Black gram) belongs to the Fabaceae family is a chief pulse crop cultivated in more than 50 countries. Black gram has a high amount of proteins, potassium, calcium and amino acids. Black gram has amino acids found in cereals which play a vital role in the diets of Nepalese and Indian people (Nitin et al., 2012). India is the biggest black gram, making country, but few works have been reported on black gram crops relative to growth and yield enhancement via plant growth-promoting organisms. Moreover, research on using Biological soil crust organisms as an inoculant for crop production is not performed. By keeping these realities in mind, the present study was framed to analyze the efficacy of the Biological soil crust as inoculant on the growth of *V. mungo* plants.

2. Materials and methods

2.1. Collection of BSC samples

Biological soil crusts were collected from top soil at different sacred groves sites of Ariyalur and Pudukottai districts during early summer. BSCs under microscopic examination, observed with different morphology were collected separately and labeled properly. The collected samples were analyzed at the Department of Botany, Jamal Mohamed College, Tiruchirappalli, India.

2.2. Extraction of plant growth promoters

A known quantity of the lyophilized BSC sample was extracted with methanol 80 mL and water 20 mL in an ICS Ultrasonic Cleaner. The extract was then centrifuged at 6000 rpm for 10 min at the temperature of 15 °C. The supernatant was filtered through a PTFE syringe filter with pore size 0.45 μm and then concentrated to 500 to 1000 μL in a nitrogen evaporator. The Chromatography was performed in high-performance liquid chromatography (HPLC) system C18 column. The UV maxima of auxins IAA, IPA and IBA, observed at 225 nm with excitation 280 nm and emission wavelengths 360 nm in the fluorescence detector, respectively. The standards and extracts were passed into the reverse phase column and compared using retention time (Shariatmadari et al., 2011).

2.3. Acetylene reduction assay

The crust was hydrated for 24 h after wetting it to be homogenized. The 0.5 μL of the BSC cyanobacteria sample was taken and transferred to the sterile testing vials sealed with rubber cork. The 10% of the air in the head phase of each vial was exchanged with pure acetylene gas (99.8%) using an airtight syringe. Then the vials were kept for 30 min to allow the reduction of acetylene gas to ethylene. After 30 min a 5 μL gas mixture was aspiratedfor gas chromatography analysis (Turner and Gibson, 1980)

2.4. Biological soil crusts as biofertilizer

Plastic pots of uniform size (height-10 cm, dia-10 cm) were filled with the required amount of garden soil for plant growth. A uniform and known quantity of powdered biological soil crusts were added to the surface of the soil in the pots. Pots without biological soil crusts served as control. All the pots were sprayed with water regularly for three days. On the fourth day, 20 healthy seeds of *V. mungo*, were sown in each pot. The seed was procured from the Tamil Nadu, Government Agriculture Department, Tiruchirappalli, India. The pots were kept in the garden and watered regularly. Plant growth parameters were analyzed on the 14th day after sowing seeds.

2.5. Seed germination

The percentage of seed germination was analyzed in triplicates of 100 seeds using pot culture as defined in ISTA guidelines. During the germination test was performed at atmospheric temperature. In the germination test, germination counts were documented for normal seedlings on the fifth and the eighth day. The percentage of seed germination (G%) was determined by the following formula.

$$G\% = \frac{100 \times A}{N}$$

A = Number of seeds found germinated
N = Total number of seeds used in the germination test.

2.6. Root length of seedlings

The length of the main root measurement was taken from root tip to stem of the plant at last harvest point, in centimeters (cm).

2.7. Shoot length of seedlings

The length of the main stem measurement was taken from shoot collar to tip of the plant at last harvest point, in centimeters (cm).

2.8. Seedling fresh weight

After harvest, the fresh weight of *V. mungo* (Shoots and roots) was evaluated in a gram (g).

2.9. Seedling dry weight

After harvest, the dry weight of *V. mungo* (Shoots and roots) was evaluated in gram (g) at 70 °C for 48 h after drying in an oven until a constant weight was reached.

2.10. Seed vigor index

The seed vigor index was studied using the succeeding formula.

SVI = Germination percentage *x* Seedling length

2.11. Absolute growth rate (AGR)

AGR was calculated by using the below-mentioned formula $AGR = \frac{h2-h1}{t2-t1}$cm day^{-1} Where that h1 and h2 are the height of the plant atthe time of t1 and t2.

2.12. Relative growth rate (RGR)

RGR was calculated by using the formula given below $RGR = \frac{W2-W1}{t2-t1} gg^{-1}d^{-1}$ Where that W1 and W2 are the dry weight of whole-plant at the time of t1 and t2 respectively.

2.13. Estimation of biochemical parameters of plants

The biochemical parameter such as total chlorophyll (Mackinney, 1941), sugar (Sadasivam and Manickam, 1996) and protein (Lowry et al., 1951) were analyzed.

2.14. Statistical analysis

The statistical analysis was performed with the SPSS-16 version. The outcomes are presented as mean ± SE (Standard error) and data from the different biological soil crust inoculant and control were related by Duncan's multiple range test ($p < 0.05$). Graphs were plotted using Microsoft Office Excel 2010 to represent morphological and biochemical parameters.

3. Results

Sacred grove forests are the most important biome, which has been conserved the plants for many years. Sacred grove forests are protected ecosystems that signify a promising site for the evolution of numerous microorganisms like cyanobacteria in the form of biological soil crust.

In the present study, typical soil samples were obtained from the sacred grove forest in the Ariyalur and Pudukottai area of Tamil Nadu. In total, 3 types of biological soil crust were observed from the selected sacred grove forest (Fig. 1).

3.1. Total species inventory of cyanobacteria in BSC from Ariyalur district

A maximum of 79 species of cyanobacteria representing 13 genera was identified in the BSC collected from the sacred groves of Ariyalur, in which three species were members of Chroococcaceae, 42 Oscillatoriaceae, seven Nostocaceae, and 25 Scytonemtaceae. A total of 72 species of cyanobacteria representing 18 genera was identified in the BSCs from Pudukkotai district sacred groves, of which eight species belonged to the members of Chroococcaceae, 48 Oscillatoriaceae, three Microchaetaceae, four Nostocaceae, eight Scytonemtaceae and one Stigonemataceae (Fig. 2 & Table 1). Table 1 shows the cyanobacteria genera in BSC collected from Ariyalur and Pudukottai district, Tamil Nadu.

The efficacy of the Biological soil crust as bioinoculant for *V. mungo* plant growth was tried. Comparative analysis disclosed that there is a remarkable variance between inoculated pot and controls pot, mainly in morphological as well as biological parameters such as total chlorophyll, protein, and carbohydrate of *V. mungo*.

Fig. 1. Appearance of biological soil crusts in sacred groves forest. A) Blackish crusts in the dry soil of Ariyalur, B) Blackish crusts in the dry soil of Pudukkottai, C) Green coloured crusts in the soil of Ariyalur, D) Green coloured crusts in the soil of Pudukkottai, E) Blackish-brown crusts in the soil of Ariyalur, F) Blackish-brown crusts in the soil of Pudukkottai.

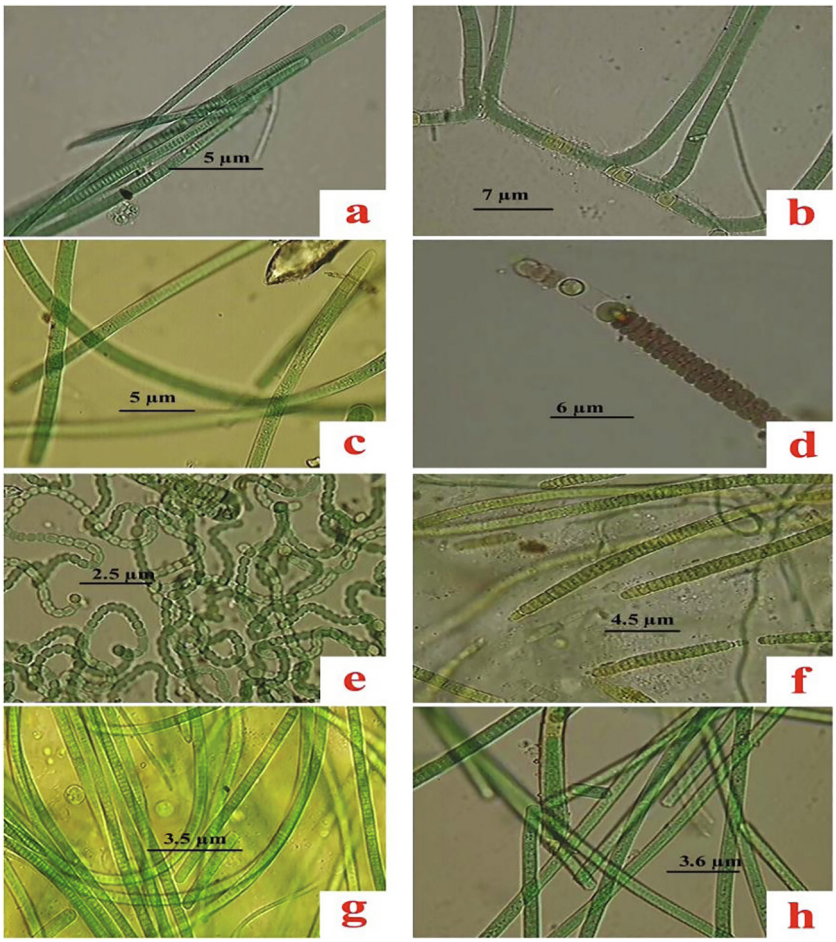

Fig. 2. Photomicrographs of some species of BSC forming cyanobacteria. a) *Oscillatoria subbrevis,* b) *Scytonema hofmanni,* c) *Lyngbya cryptovaginata,* d) *Anabaena khannae,* e) *Nostoc calcicola,* f) *Oscillatoria formosa,* g) *Oscillatoria limosa,* h) *Oscillatoriaj asorvensis.*

Table 1
Contribution of cyanobacteria genera in BSC from the sacred groves of Ariyalur and Pudukottai district, Tamil Nadu.

S. No.	Cyanobacterial species	Ariyalur	Pudukkottai
	Microcystis		+
	Chroococcus	+	+
	Gloeocapsa	+	
	Synechococcus	+	
	Aphanocapsa		+
	Arthrospira	+	
	Oscillatoria	+	+
	Phormidium	+	+
	Lyngbya sp.	+	+
	Schizothrix		+
	Symloca		+
	Microcoleus	+	+
	Hydrocoleum		+
	Microchaete		+
	Anabaena	+	+
	Nostoc sp.	+	+
	Aulosira		+
	Plectonema	+	+
	Scytonema	+	+
	Tolyporthrix	+	+
	Haplosiphon		+

3.2. Plant growth promoters in BSCs

The HPLC chromatograms of biological soil crust samples raised in optimal conditions are shown in Fig. 3A. The fluorescence detector equipped HPLC revealed the existence of three endogenous auxins comprising IAA, IBA, and IPA in biological soil crusts collected both from Ariyalur and Pudukkottai districts. The HPLC chromatogram for the standard auxin is shown in Fig. 3B.

3.3. Acetylene reduction assay

Nitrogenase activity was higher in BSCs obtained from the Pudukkottai district and lesser in Ariyalur district. The value of nitrogenase activity was 55.007 nmol. $ml^{-1}h^{-1}$ in BSCs from Pudukkottai and 45.020 nmol. $ml^{-1}h^{-1}$ in Ariyalur (Fig. 4).

3.4. Effect of biological soil crusts on plant growth

The comparative analysis showed that there is a significant difference between biological soil crust inoculated pot culture and controls, exclusively in vegetative growth features (Fig. 5).

A twofold increase in plant root length over the control documented in *V. mungo* inoculated with biological soil crusts collected both from Ariyalur and Pudukkottai district (Fig. 6.1. A). However, a small development in shoot size than the control was noticed in *V. mungo* inoculated with biological soil crusts from Ariyalur but, the shoot length of *V. mungo* inoculated with the BSC from Pudukkottai district was on par with the control (Fig. 6.1.B).

Seedling fresh weight (Fig. 6.2.A) and dry weight (Fig. 6.2.B) improved by twofold over the control in *V. mungo* inoculated with biological soil crusts of Ariyalur and Pudukkottai district. The seedling fresh weight and dry weight of *V. mungo* on the 14th

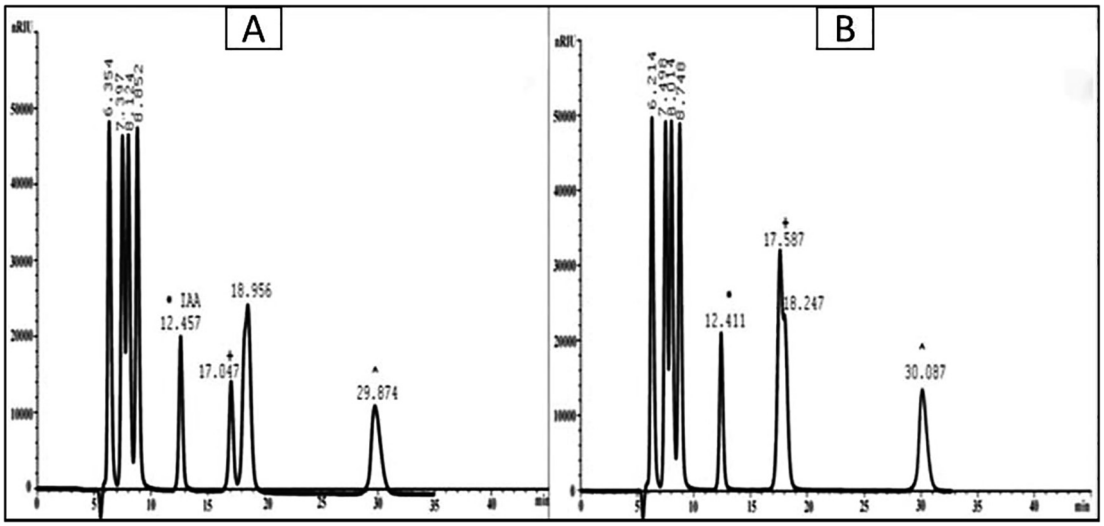

Fig. 3a. High-performance liquid chromatography (HPLC) chromatograms of the ultrasonicated samples for the presence of plant growth-promoting substances in biological soil crusts collected from Ariyalur (A) and Pudukkottai (B) districts.

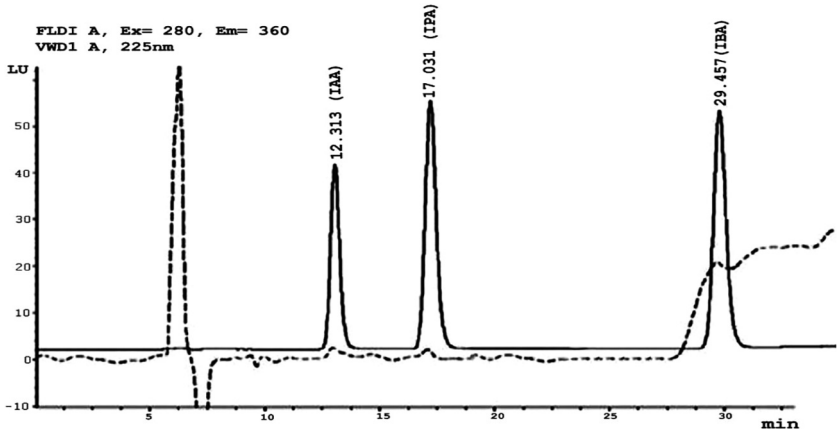

Fig. 3b. HPLC chromatograms of a 250 ng mL^{-1} standard of three auxins (IAA, IPA and IBA).

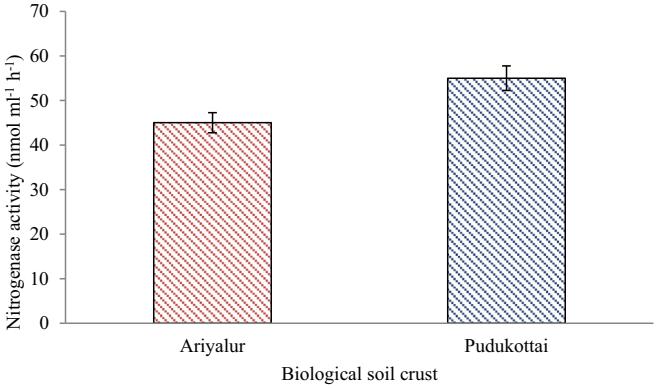

Fig. 4. Nitrogenase activity of BSCs collected from Ariyalur and Pudukkottai districts.

Fig. 5. Plant height and root length of control and treated plants Ariyalur (A) and Pudukkottai (B).

day as affected by BSC from Ariyalur (A) and Pudukkottai (B) districts.

The absolute growth rate increased by more than three times over the control in *V. mungo* inoculated with BSCs collected from Ariyalur sacred forest while a significant increase in the absolute growth rate over the control was recorded with BSCs from Pudukkottai sacred grooves (Fig. 6.3. A). The relative growth rate increased by three times over the control in *V. mungo* inoculated

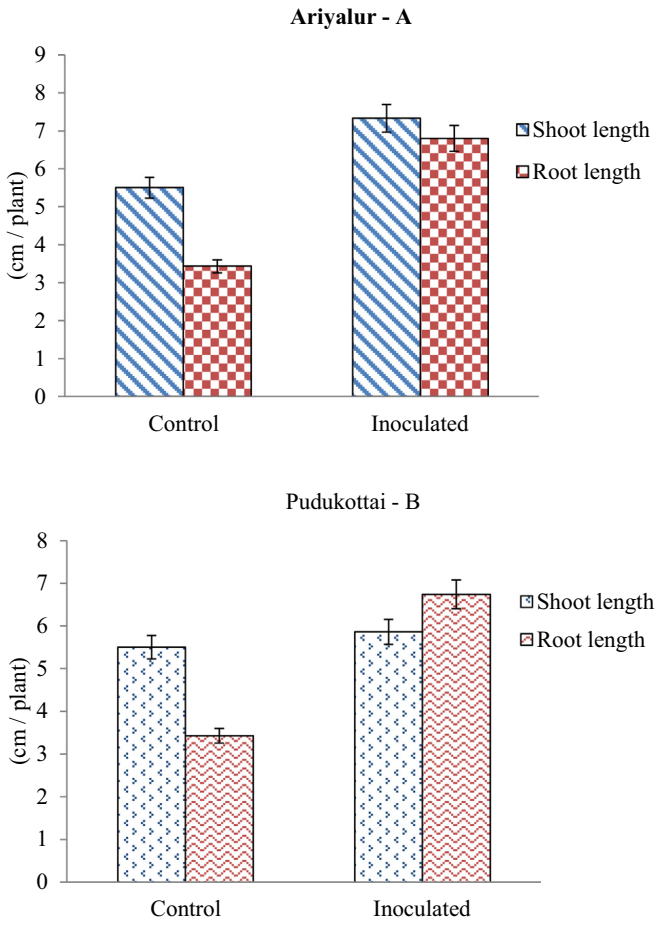

Fig. 6.1. Root length and shoot length of *Vigna mungo* (L) on the 14th day as affected by BSC from Ariyalur (A) and Pudukkottai (B) districts.

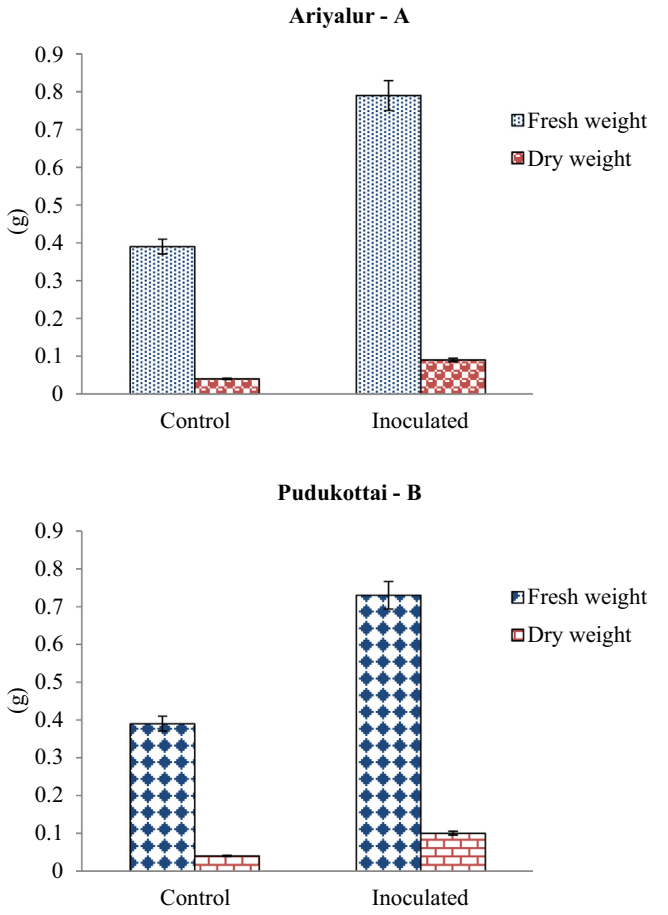

Fig. 6.2. Seedling fresh weight and dry weight of *Vigna mungo* (L) on the 14th day as affected by BSC from Ariyalur (A) and Pudukkottai (B) districts.

with BSCs collected from both Ariyalur and Pudukkottai district sacred forests (Fig. 6.3.B).

The seedling vigor index increased threefold and twofold over the control in *V. mungo* inoculated respectively, with biological soil crusts collected from Ariyalur and Pudukkottai district (Fig. 6.3.C).

Chlorophyll *a* content got doubled over the control in *V. mungo* inoculated with biological soil crusts collected from Ariyalur while, chlorophyll *b* and total chlorophyll showed a slight increase over the respective controls (Fig. 6.4.A).

Similarly, total protein content revealed a slight increase over the control as seen with chl. *b* and total chlorophyll, while the total sugar content exhibited a twofold increase over the control in *V. mungo* inoculated with biological soil crusts from Ariyalur (Fig. 6.4. A). In the case of Pudukkottai, *V. mungo* inoculated with biological crusts recorded a little increase of Chl.*a* content over the control. Total chlorophyll and chl.*b* in biological crust inoculated seedlings were more or less similar to their respective controls (Fig. 6.4. B) whereas the total sugar content increased by more than two times over the control (Fig. 6.4.B).

Comparative analysis displayed that there is a substantial difference between BSCs inoculated pot culture and control, exclusively in morphological parameters and biochemical parameters such as length, weight, Chlorophyll, sugarand protein ($P < 0.05$ Table 2). Besides, the results have shown that there is a noteworthy change in most factors in different plants inoculated with Ariyalur and Pudukottai's biological soil crust. However, the effect of biological soil crust is differing for all parts of diverse plants. The leaves numbers, root length and root weight were varying from controls.

4. Discussion

There are some rare reports about the study of biological soil crust of cyanobacteria in sacred groves forest Ariyalur and Pudukottai area of Tamil Nadu, India. Though *V. mungo* is the chief crop it was cultivated in the country. Representative soil samples were collected from sacred groves forest cultivation.

Among the biological soil crust collected, three types of crust (Blackish, Greenish, and Pinkish) were documented. Blackish and Pinkish color biological soil crust is found to be the dominant type in most sacred grove forests, whereas greenish were limited to sacred grove forests of Ariyalur and Pudukottai. Parallel results were stated by Vinoth et al. (2017) in the sacred grove forest of Ariyalur and Pudukottai, India. Soil crust cyanobacteria affected by solar radiation in drought conditions and plain soil produces photo protecting pigments (Vinoth et al., 2017). The black and pink soil crust in the sacred forest is due to the abundance of UV absorbing compounds Mycosporine-like amino acids and Scytonemin (Catenholz and Garcia-Pichel, 2000). This cyanobacterial crust has an impact on the growth of plants by releasing plant growth hormones and promoting the fertility of the soil.

Further, this study, disclosed that there is a substantial difference between the BSC's inoculatedplants and control. The vegetative growth parameters, especially the length of the plant and its weight were analyzed. A progressive effect of PGP (Plant growth promoters) on plant weight and the length was previously reported (Zahir et al., 2000). The outcomes of the present study disclosed that the growth parameters, for example, root length and

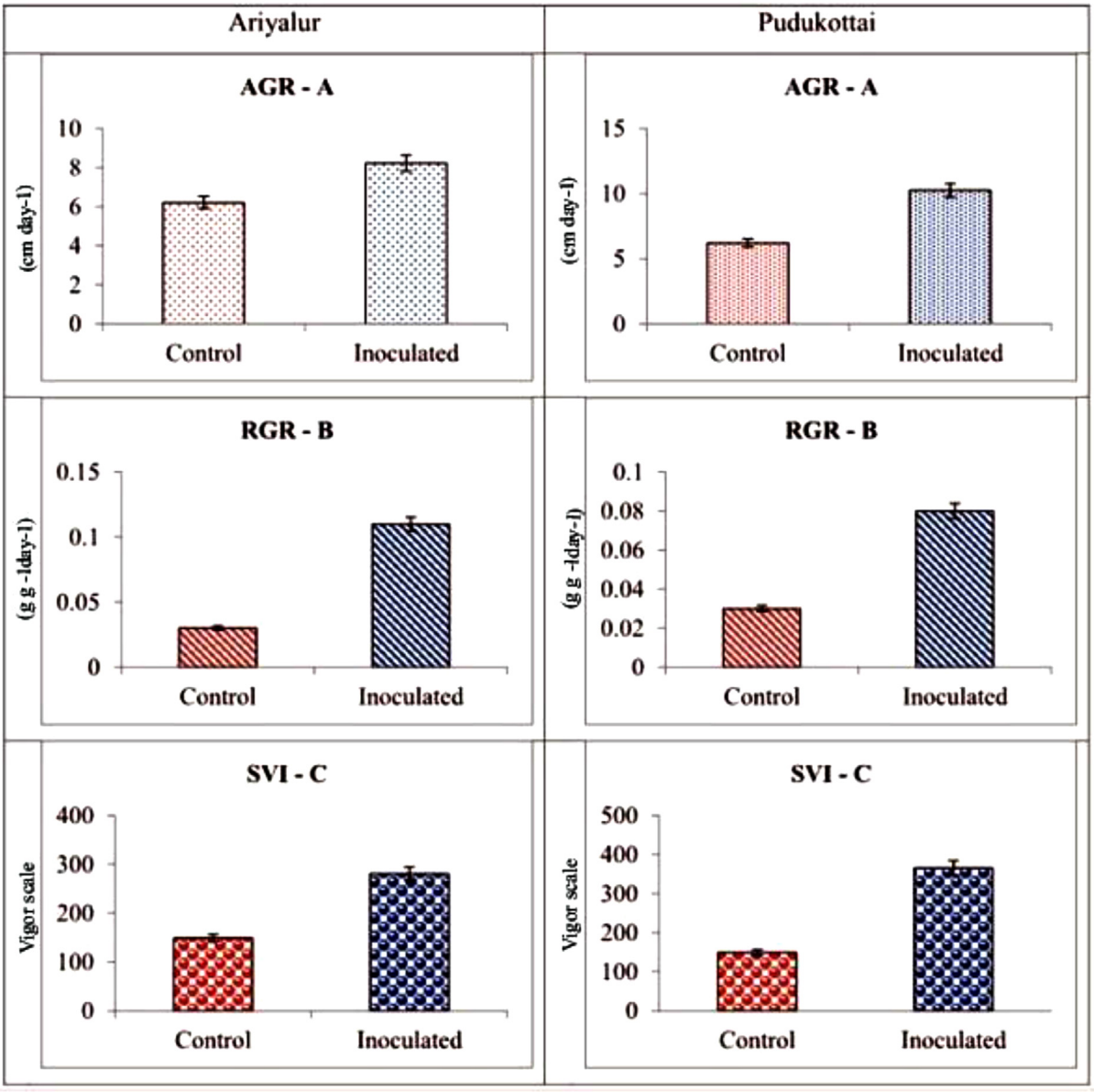

Fig. 6.3. Changes in the absolute growth rate (A), relative growth rate (B) and seedling vigor index (C) in *Vigna mungo* on the 14th day as influenced by BSC from Ariyalur and Pudukkottai districts.

weight, enlarged considerably, which increase uptake of nutrients and water from Biological soil crust. In the present study, biological soil crusts analyzed for the presence of growth-promoting substances with HPLC showed the presence of phytohormones such as IAA, IBA and IPA. Auxins are generally used to initiate root, particularly IBA, used as plant hormones for induction of root formation. Previously, multiple plant seedlings with IBA reported an increase in lateral root development (Khavar and Özcan, 2002; Mobli and Baninasab, 2009). Li et al. (2009) have also shown that phytohormones in rice seedlings can stimulation absorption and root elongation. Our results showed that improved rooting can be influenced by plant hormones like IAA and IBA.

Estimation of the BSC's N fixation rate revealed that these heterocystous cyanobacteria were capable of fixing atmospheric N in natural conditions which affect the plant growth (Nilsson et al., 2002). Nitrogenase activity was more in the biological crusts collected from the sacred groves of Pudukkottai, despite its lower diversity of heterocystous cyanobacteria than in Ariyalur. The increased acetylene reduction assay activity of Pudukkottai samples would probably be due to bacterial fixers.

To understand the possibility of biological soil crusts as biofertilizer (soil inoculants); the present study was performed with *V. mungo*. The plant growth parameters such as shoot and root length, fresh and dry weight of seeding, absolute growth rate, relative growth rate, seedling vigor index and biochemical contents such as chlorophyll, protein and sugar were more in *V. mungo* inoculated with biological soil crusts than their respective controls. This study is supported by the findings of Shariatmadari et al. (2013).

The comparative analysis of pot experiments exhibited a significant difference in morphological and biochemical growth factors, especially root growth parameters between the crusts inoculated pot and controls. Similarly a study by Thiet et al. (2014) revealed that algal biocrusts significantly influenced the growth of *Deschampsia flexuosa* (height 28.95 cm, vigor 5.29 cm and *Morella pensylvanica* (height 22.61 cm, vigor 18.36 cm) plants. When compared to controls, algal biocrusts and moss mats tended to increase

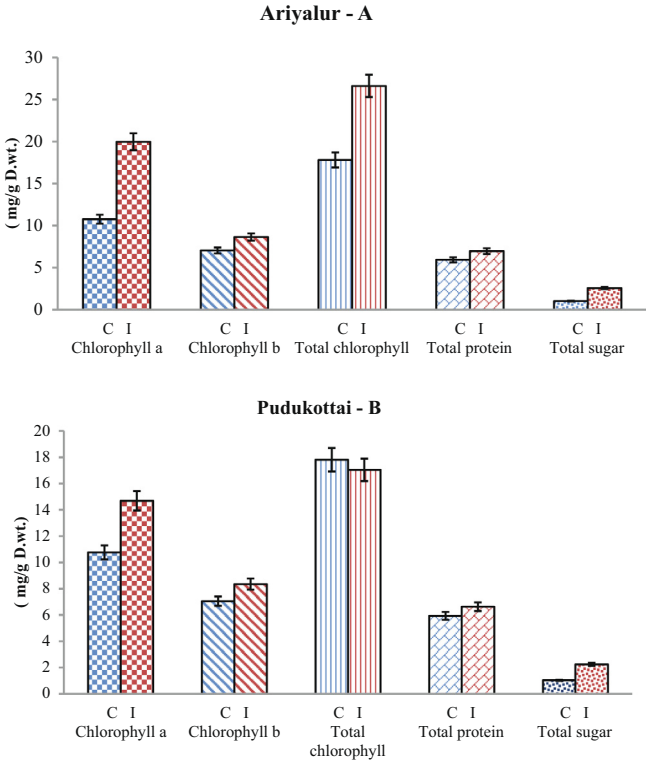

Fig. 6.4. Changes in biochemical contents in *Vigna mungo* (L) on the 14th day as affected by BSC from Ariyalur (A) and Pudukkottai (B) districts.

Table 2
Effect of biological soil crust on the growth of vegetable plants (Mean ± SE).

Growth parameter	Control	BSC of Ariyalur	BSC of Pudukottai
Shoot length (cm)	5.5 ± 0.11^{c}	7.33 ± 0.11^{a}	5.86 ± 0.10^{b}
Root length (cm)	3.43 ± 0.12^{c}	6.8 ± 0.05^{a}	6.74 ± 0.20^{b}
Fresh weight (g)	0.39 ± 0.00^{c}	0.79 ± 0.03^{a}	0.73 ± 0.04^{b}
Dry weight (g)	0.04 ± 0.01^{c}	0.09 ± 0.00^{b}	0.1 ± 0.00^{a}
Chlorophyll *a* (mg/g)	10.7 ± 0.01^{c}	19.9 ± 0.00^{a}	14.6 ± 0.03^{b}
Chlorophyll *b* (mg/g)	7.05 ± 0.01^{c}	8.64 ± 0.01^{a}	8.35 ± 0.01^{b}
Total chlorophyll (mg/g)	17.8 ± 0.01^{b}	26.6 ± 0.01^{a}	17.0 ± 0.01^{c}
Total protein (mg/g)	5.93 ± 0.00^{c}	6.97 ± 0.01^{a}	6.63 ± 0.00^{b}
Total sugar (mg/g)	1.03 ± 0.00^{c}	2.56 ± 0.00^{a}	2.24 ± 0.00^{b}

Means followed by the same letters within a column are not significantly different at $p \leq 0.05$. BSC = Biological soil crust.

seedling survivorship and vigor. A positive effect of biological soil crust on Chlorophyll, total sugar, total protein, shoot length, root length, fresh and dry weight of plants were previously reported (Pan et al., 1999; Zahiret al., 2000). The results of the current study exhibited the morphological and biochemical parameters in plants improved significantly, which could increase the uptake of nutrients and water from the soil.

5. Conclusion

The biological soil crust had numerous cyanobacteria such as unicellular, heterocystous and non-heterocystous forms. These biological soil crust cyanobacteria act as mediators for the incorporation of organic carbon and nitrogen through PGR secretion and nitrogenase activity, which increases nutrient availability and sustains the soil fertility. The BSC inoculated soil increases the *V. mungo* plant growth. These results proved that an ecosystem rich with biological soil crusts showed an enhanced population of cyanobacterial species which in return increased the growth of floral species in the soil.

Acknowledgments

The authors are thankful to the Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India for providing necessary facilities. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this research group NO (RG-1435-014).

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