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A study on two potential BGA isolates *Cylindrospermum majus* and *Nostoc muscorum* of Assam, North-East India

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ABSTRACT

A study was conducted on two BGA isolates, Cylindrospermum majus and Nostoc muscorum of Assam North-East India. For which their growth behavior, nutrient requirement and biomass production through out the year and seasonal influence on them were thoroughly studied. The parameters mostly included biomass productivity, chlorophyll-a and total chlorophyll content, total N-content, packed cell volume (PCV), indole-acetic acid (IAA) production and seasonal growth. Out of the two, Nostoc muscorum was the better strain in terms of biomass (0.128mg/100ml), total chlorophyll content (0.42mg/ml), total N content (2.62%), PCV (1.57 ml pellet ml^{-1}), IAA content (4 ppm) and as a result of composite culture of two BGA isolates in field condition, non sterile soil was identified as most suitable combination when taken as substrate. Both the strain showed their best growth during the summer season.

Keyword: Biomass, N-content, substrate, biofertilizer

INTRODUCTION

Cyanobacteria are employed in agriculture as biofertilizers and soil conditioners. The majority of cyanobacteria are capable of fixing atmospheric nitrogen and are effectively used as biofertilizers ([1];[2];[3]). Algalization benefit crop plants through excreting part of the biologically fixed nitrogen, secreting growth promoting substances and different types of secondary metabolites; adding appreciable amounts of organic matter into the soil, solubilizing insoluble phosphates and improving the physical and chemical nature of the soil [4]. The technology for mass cultivation of cyanobacteria was developed by G.S. Venkataraman [5].

Previous studies reported that cyanobacteria improve soil structure by increasing soil aggregation, soil aeration, water holding capacity, therefore, its application is useful for the

reclamation of soils ([6];[7];[8]). The results indicate that these cyanobacteria brought about considerable aggregation of loose unbound soil particles. These algae play an important role in improving the physical texture of the soil and thus help in checking soil erosion to a certain extent [9]. In recent years, biofertilizers have emerged as promising components of the integrated nutrient supply system in Indian agriculture. Among biofertilizers benefiting the cereal crop production are *Azotobacter, Azospirillum*, cyanobacteria, *Azoll]a*, P-solublising microorganisms and mycorrhizae. The beneficial effects of biofertilizers are addition of nitrogen, increased soil organic matter and soil aggregation [10]. The role of biofertilizers in sustainable agriculture recorded special significance, particularly in the present context of high cost of chemical fertilizers [11]. The production and application of 13 biofertilizers to leguminous plants, oilseeds, rice, millets and forest nursery plants are very common in India ([11];[12]). Chemical fertilizers are expensive, they disturb the equilibrium of agro-ecosystems and cause pollution to the environment. These problems may be avoided by the use of biofertilizers. The application of chemical-N fertilizers to farm crops does not seem to negotiate with the income of farming masses without which strides in agriculture production are not possible. Therefore, exploitation of new agricultural technologies such as the biological nitrogen fixation method has largely been hampered [13].

1.1 Cyanobacteria as biofertilizers and nitrogen fixers:

Nitrogen is an essential constituent of proteins, nucleic acids, chlorophylls, enzymes, and other physiological substances in green plants. Nitrogen is the macronutrient that is required in high amounts by plants, and its availability in the soil may change substantially in relatively short time intervals [14]. For rapid growth of all plants, nitrogen is probably the most common limiting factor. Hence, an adequate supply of nitrogen in agriculture is very important [15].

Cyanobacteria attract the attention of scientists due to their nitrogen fixing capacity, and hence their role in the maintenance of soil fertility is well documented [16]. A wide range of N2-fixing cyanobacteria exists in rice field ecosystems ([17];[18]). Nitrogen fixed by the symbiotic association of cyanobacteria (cyanobionts) is transferred to and used by various plant groups other than rice ([19];[20];[21]).

The species of cyanobacteria which are known to fix atmospheric nitrogen are classified into three groups (i) Heterocystous-aerobic forms, (ii) Aerobic unicellular forms and (iii) Non-heterocystous, filamentous, microaerophilic forms. Cyanobacteria that dominate a wide range of diverse environments are characterized by their tolerance to high temperatures, desiccation, pH, salinity, light intensity and nutrients ([22];[23]).

Nitrogen fixing cyanobacteria are the dominant microflora in rice fields and are currently used as a supplement to chemical nitrogen fertilizers for rice cultivation in rice-growing countries, including India and Bangladesh. This technology suffers from serious drawbacks and its use at farm level is not gaining universal acceptance due to some major problems. Cyanobacteria possess plant hormone-like activity and thus they influence growth of rice through the release of these substances [24]. Many cyanobacteria are known to produce different types of secondary metabolites such as auxins, auxin like substances, gibberellin like substances, cytokinins and abscisic acid [25-28].

The success of any technology usually depends upon how much it costs and how simple it can be during operation and application. One of the biotechnological applications resulting from the development of a cyanobacterial biofertilizer program are the preparation and distribution of biofertilizers to farmers [29]. Polybag bottles, polyethylene and polypropylene sachets were used for distribution of liquid cyanobacterial cultures instead of the expensive glass flasks ([30];[31]). Nutrients fixed by cyanobacteria are made available mainly in the form of ammonia to rice plants through exudation, autolysis and microbial decomposition [32]. This biofertilizer technique is still limited. Recently, there are serious attempts to introduce large scale cyanobacterial culture and application.

Physiologically it plays the key role and has been considered as a yield limiting factor. However, increased cost of the fertilizer is becoming an economic constrains for the farmers of the developing countries like Bangladesh. Moreover, the continuous use of chemical fertilizers causes the ecological and biochemical imbalance in the rice field [33]. As a consequence, to over come this dual problems, the concepts of biofertilizers is recently being gaining momentum and is successfully practiced in rice field in many countries like India, China and Uganda. The significant contribution of blue-green algae as an alternative source of nitrogen particularly in the rice field has long past history [36]. The algalization technology has been reported to be successful to a great extent in India ([34];[35]).

MATERIALS AND METHODS

The strains *Cylindrospermum majus* and *Nostoc muscorum* were isolated from the rice fields of Kamrup district of Assam, North East of India. Serial dilution method was employed for purification of all the samples which were further pure cultured by agar plating method and transferring each colonies to BG11 liquid culture media. For the experiment culture racks having fluorescent lamps with required light intensity were used as a source of light. The light intensity was adjusted to 2500~3500 lux for all the culture flasks and 16 hrs of light and 8 hrs of dark cycles were repeated for growth of all the cultures. The temperature was adjusted to 25.C for all the flasks.

2.2 Determination of Biomass, Chlorophyll-a and total Chlorophyll Content

Each cyanobacterial culture was harvested at 10 days, 20 days, 30 days and 40 days. During each interval of time Biomass were collected by filtering the media through Whatman No 41 filter paper and finally weighed to record the biomass. In this investigation the final biomasses were recorded at specific time intervals of incubation at their stationary phases which showed maximum growth. Total biomass was calculated on the basis of weight which showed maximum biomass production and hence taken for further evaluation. The curve of chlorophyll-a and total chlorophyll content was estimated as an index of algal growth. The pigment was extracted and estimated by using cold extraction method [37] and expressed in mg/ml of fresh culture.

2.3 Determination of total N-content

After biomass determination of each BGA isolates, 0.1 gm of dry algal flakes were collected and total N-content was determined using Micro Kjeldahl method [38]. The biomass production and total N content were found varied among the BGA isolates.

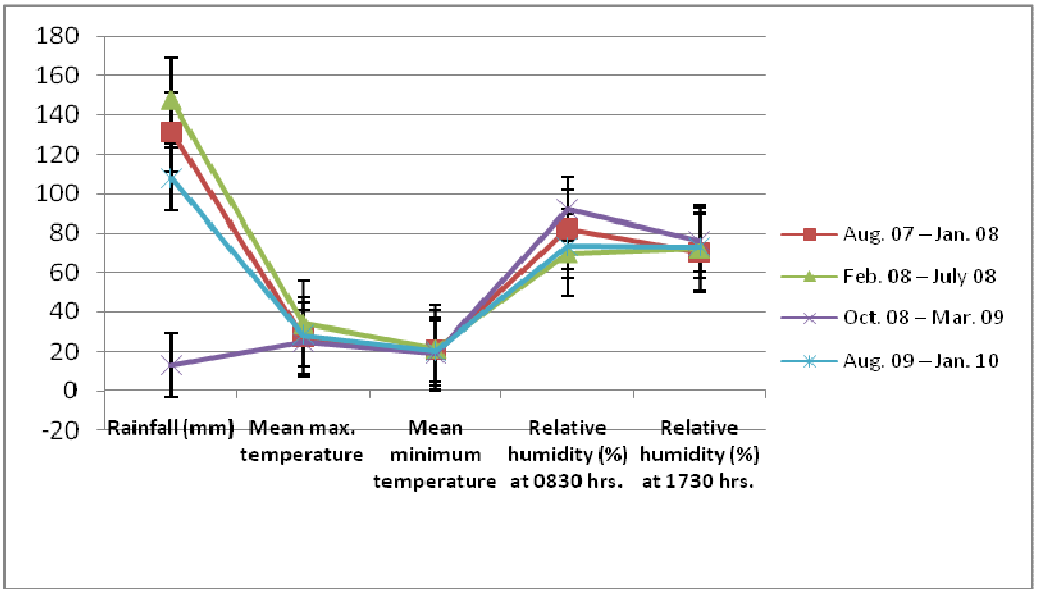
2.4 Determination of packed cell volume (PCV) as an index of growth

For determination of packed cell volume, a small amount of the sample (10 ml) was removed from the uniformly disposed suspension culture aseptically and centrifuged at 500 rpm for 15 minutes in 15 ml graduates centrifuged tubes. The packed cell volume is expressed as ml pellet ml^{-1}culture. The packed cell volume was taken at 4 consecutive interval of time in 10 days, 20 days at 30 days and 40 days.

2.5 Determination of Indole-acetic acid (IAA) like substance production:

IAA like substance production were determined with conical flasks in triplicates containing 50 ml of BG_{11} media for each culture were inoculated with 0.1 mg/ml isolates for 15days. Each culture flask was centrifuged at 10,000 rpm at 4^{o}C for 15 minutes and supernatant was acidified to pH 3 with 1 N HCL. The suspension were divided in two equal parts for determination of IAA like substances.One part of supernatant was extracted sequentially thrice with 10 ml of ethyl acetate at 30 minutes interval. The ethyl acetate fraction was allowed to evaporate to dryness. The residue was dissolved in 3 ml of absolute methanol and mixed with 4 ml of Fe–$HClO_4$ reagent (i.e. 1 ml of 0.5 M $FeCl_3$ and 50 ml of 35% $HClO_4$) 2-3 drops of orthophosphoric acid were added and incubated for 25 minute at room temperature. Lastly a standard curve was prepared using absorbance readings of standard IAA solution and used to determine concentration of the IAA like substance in the culture broths[39]

Figure-1: Meteorological Parameters were also recorded during the period of pot and field experiments (Source: India on Meteorological Department, Regional Meteorological Centre, Govt. of India)



2.6 In vivo studies on the production of BGA isolates in respect to the seasonal growth

A separate field experiments were carried out to observe the growth of BGA culture in different seasons throughout the year. For this experiment, a temporary bamboo culture bed was prepared. To assess the growth among the BGA isolates they were cultured in BG_{11} medium in poly bag measuring (12cm×12cm×18cm). 500 ml of BG_{11} media was poured in each poly bag and inoculated with a full loop of each strain of BGA in three replicates. The growth of culture was observed in the interval of 15 days, 21 days and 28 days. Multiple culture of BGA was also observed in same way considering three replications.

Meteorological Parameters were also recorded during the period of pot and field experiments which is showed in figure-1.

2.7 In vivo studies for mass multiplication of BGA based on sterile and non sterile paddy soils as substrate:

Sterile and non sterile paddy soils were considered and evaluated their chemical composition for the experiment. Table-1 describes the chemical characteristics of soil samples.

Table –1 : Chemical characteristics of prepared compost and soil sample

Sample	Chemical Composition			
	pH	%N	%O	%K
T_1 (Sterile Soil)	4.67	0.112	0.018	0.142
T_2 (Non sterile soil)	6.27	0.16	0.021	0.162

RESULTS AND DISCUSSION

The two strains of BGA namely *Cylindrospermum majus* and *Nostoc muscorum* were isolated by serial dilution method [40] and cultured in BG11 medium [41]. The BGA strain *Cylindrospermum majus* was dull blue green in colour, Thallus mucilaginous, trichome uniformly broad, 4-5µ; cells cylindrical, 5-6µ; heterocyst oblong, broader than the trichome; spores ellipsoidal, 10-15µ broad, epispore brownish with distinct papillae.

Similarly *Nostoc muscorum* shows characteristics of thallus gelatinous, irregularly expanded, attached by the lower surface, dull blue green colour; filaments densely entangled, sheath distinct only at the periphery; trichome 3-4µ broad; cells short barrel shaped; heterocyst spherical 5-6µ broad [42].

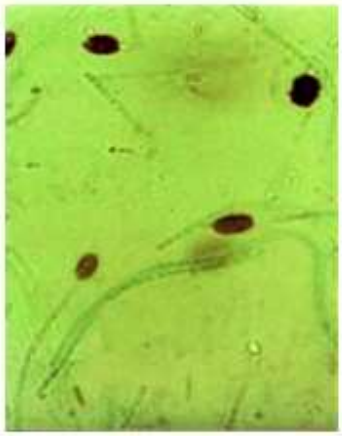
Both the BGA strain were grown on three selected media composition as described in table- 2.

Table –2: Growth of the Blue Green Algal Isolates on few selected culture media

BGA Isolates	BG_{11} Media (Nitrogen free)	Fogg's Media (Nitrogen free)	Chu-10 Media (Nitrogen free)
	Time (45 days) taken to develop into a visually recognizable growth(mg/100ml)		
Cylindrospermum majus	0.740±0.003	0.160±0.001	0.260±0.002
Nostoc muscorum	0.710±0.001	0.410±0.002	0.490±0.002

The results of the biomass study, chlorophyll-a and total chlorophyll, total N content and PCV of above mentioned two strains in four different days of interval are presented in table **3, 4, 5 & 6**. It was observed that among the two stains, *Nostoc muscorum* which showed highest amount of biomass (0.128 mg/100ml) as well as highest amount of chlorophyll a (0.67mg/ml) and total chlorophyll content (0.91mg/ml) and total N content (2.62%) at 40 days of growth in comparison to *Cylindrospermum majus.*

Photograph-1 : The microscopic photograph of *Nostoc muscorum* on BG_{11} media after one month of culture. (under 40X magnification)



Photograph-2 : The microscopic photograph of *Cylindrospermum majus* on BG_{11} media after one month of culture. (under 40X magnification)

Table – 3: Estimation of Biomass production of different BGA Isolates at four consecutive intervals of time periods

BGA Isolates	Biomass Production (mg/100ml) ± SE			
	Day 10	Day 20	Day 30	Day40
Cylindrospermum majus	0.023 ± 0.001	0.031 ± 0.001	0.0587 ± 0.002	0.072±0.001
Nostoc muscorum	0.038 ± 0.00	0.051 ± 0.002	0.087 ± 0.001	0.128±0.002

Table –4: Determination of Chlorophyll-a and total Chlorophyll of BGA Isolates (mg/ml) ± SE

BGA Isolates	Day 10		Day 20		Day 30		Day40	
	Chl-a (mg/ml ± SE)	Total Chl. (mg/ml ± SE)	Chl-a (mg/ml± SE)	Total Chl. (mg/ml± SE)	Chl-a (mg/ml±SE)	Total Chl. (mg/ml ± SE)	Chl-a (mg/ml±SE)	Total Chl. (mg/ml ± SE)
Cylindrospermum majus	0.042±0.001	0.089±0.002	0.072±0.00	0.092±0.003	0.13±0.002	0.219±0.004	0.20±0.00	0.33±0.0002
Nostoc muscorum	0.097±0.00	0.113±0.01	0.123±0.00	0.162±0.01	0.246±0.00	0.30±0.001	0.32±0.00	0.42±0.004

Table – 5: Determination of Total N content of BGA Isolates (% ± SE)

BGA Isolates	Total N Content (%±SE)			
	Day 10	Day 20	Day 30	Day40
Cylindrospermum majus	0.60 ± 0.002	0.92 ± 0.001	1.38 ± 0.001	2.11±0.02
Nostoc muscorum	0.924 ± 0.001	0.964 ± 0.00	1.92 ± 0.00	2.62±0.00

The study of packed cell volume (PCV) also indicated that *Nostoc muscorum* was the efficient strains with highest amount of PCV (1.57ml pellet/ml) recorded at same very days, which was followed by *Cylindrospermum majus*. Table-6 shows the results of PCV.

Table –6: Determination of Packed Cell Volume (PCV) of BGA Isolates (ml pellet/10 ml of culture ± SE)

BGA Isolates	Packed Cell Volume (PCV) (ml pellet ml^{-1} ± SE)			
	Day 10	Day 20	Day 30	Day40
Cylindrospermum majus	0.212 ± 0.06	0.364 ± 0.02	0.62 ± 0.001	0.91±0.02
Nostoc muscorum	0.48 ± 0.02	0.62 ± 0.01	1.11 ± 0.01	1.57±0.06

There was no detectable amount of IAA like substances was determined in the uninoculated control medium. Among the three isolates, more quantity of IAA-like substances (4.0ppm)was detected in culture supernatant of *Nostoc muscorum* which was followed by *Cylindrospermum mujus* (2.6 ppm) **. Figure 2** describes the Indole-Acetic Acid (IAA) like substance production.

Figure-2: Content of IAA like substances in the culture supernatant of BGA isolates

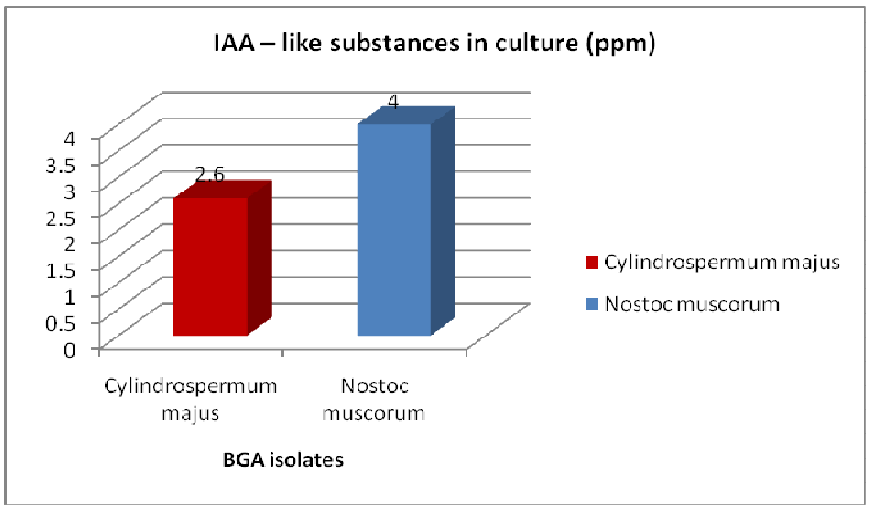


Table –7: Mono and multiple BGA isolates culture (*in vivo*) considering sterile garden soil as substrate

BGA Isolates	Substrate used (BG11 Media)	Biomass mg/100ml (Mean)	Total N content (%) (Mean)
Cylindrospermum majus	C_1	10.11	3.64
Nostoc muscorum	C_1	15.86	4.02
Cylindrospermum majus + Nostoc muscorum	C1	17.54	4.89
F-test		*	*
SE(d)		0.334	0.217
CD(1%)		1.051	1.008
CD(5%)		1.221	0.312
Substrate used (sterile soil)			
Cylindrospermum majus	T_1	13.33	5.08
Nostoc muscorum	T_1	22.16	8.92
Cylindrospermum majus + Nostoc muscorum	T1	25.08	9.11
F-test		*	*
SE(d)		0.703	0.453
CD(1%)		1.631	1.212
CD(5%)		1.080	1.121
Substrate used (non sterile soil)			
Cylindrospermum majus	T_2	16.73	8.4
Nostoc muscorum	T_2	26.07	11.16
Cylindrospermum majus + Nostoc muscorum	T_2	28.05	12.33
F-test		*	*
SE(d)		0.032	0.65
CD (1%)		0.102	1.82
CD (5%)		0.067	1.14

Thereafter, the growth study and mass multiplication of all the two strains were carried out in field condition considering sterile garden soil and non sterile garden soil as substrates. Table-1 shows the chemical characteristics of substrates). The mass multiplication of *N. muscorum* in earthen pot considering non sterile soil as a substrates found higher between the two strains **(**table-7) whereas the performance of *C. majus* in sterile soil and non sterile soil substrates was

found comparatively low than the *N. muscorum*. In comparison to control, the biomass and total N-content of two BGA isolates grown in pure form, recorded higher growth when non sterile garden soil taken as substrate . Similar effect in case of combine inoculation of the two strains on non sterile soil was also observed. Therefore, to develop multiple BGA inocula with soil and to improve N nutrition , the composite culture of two BGA isolate with non sterile soil substrate were identified as most suitable combination.

The performance of the BGA isolates throughout the year (2008-2009) and the seasonal variation of different parameters were also studied. The growth and biomass of aforementioned strains varied in different seasons. *Nostoc muscorum* (NM_0) performed significantly better in terms of biomass and total N-content compared to that of other BGA strain in summer season. It was also observed that the growth was gradually decreased after the summer season and again increased gradually during winter. During a whole year study, a maximum biomass and total N-content by *Nostoc muscorum* and *Cylindrospermum majus* (Table-8) was recorded respectively. Analysis of above results suggest that the summer season is the most favourable season for the highest growth of each BGA strain.

So the above findings depicts the potentiality of the BGA isolates as efficient biofertilizer strain in the North-Eastern region which can be exploited commercially, Further research will help to improve the biofertilizer technology which is more suitable over chemical fertilizers.

Table –8: Seasonal variation (2008-09) of BGA Isolates

Month/ Season	Cm_1			Nm_o		
	Biomass (mg/100 ml)±SE	**Chl-a (mg/ml)±SE**	**Total N Content (%)± SE**	**Biomass (mg/100 ml)±SE**	**Chl-a (mg/ml)±SE**	**Total N Content (%)± SE**
Summer						
March	10.16 ± 0.16	0.710 ± 0.001	4.26 ± 0.00	14.03 ± 0.26	0.910 ± 0.00	5.46 ± 0.00
April	12.01 ± 0.12	0.842 ± 0.004	5.16 ± 0.001	12.74 ± 0.15	0.814 ± 0.001	5.12 ± 0.001
May	12.16 ± 0.10	0.871 ± 0.002	5.24 ± 0.0004	7.68 ± 0.068	0.633 ± 0.002	3.78 ± 0.00
June	8.87 ± 0.13	0.603 ± 0.001	3.01 ± 0.003	6.05 ± 0.004	0.541 ± 0.002	3.14 ± 0.002
Monsoon						
July	5.54 ± 0.09	0.416 ± 0.00	2.16 ± 0.001	6.03 ± 0.004	0.536 ± 0.00	3.10 ± 0.001
August	4.06 ± 0.098	0.041 ± 0.001	1.72 ± 0.00	6.74 ± 0.003	0.552 ± 0.001	3.41 ± 0.00
September	4.71 ± 0.003	0.463 ± 0.002	1.91 ± 0.001	8.06 ± 0.002	0.674 ± 0.00	4.02 ± 0.00
October	6.02 ± 0.002	0.514 ± 0.001	2.94 ± 0.002	8.32 ± 0.002	0.692 ± 0.004	3.41 ± 0.001
Winter						
November	7.72 ± 0.016	0.504 ± 0.001	3.42 ± 0.002	10.42 ± 0.051	0.782 ± 0.00	4.81 ± 0.001
December	9.09 ± 0.0043	0.511 ± 0.00	3.14 ± 0.00	11.03 ± 0.026	0.842 ± 0.001	5.16 ± 0.002
January	9.41 ± 0.012	0.524 ± 0.004	4.04 ± 0.00	13.24 ± 0.23	0.882 ± 0.00	5.72 ± 0.004
February	9.93 ± 0.014	0.601 ± 0.00	4.11 ± 0.00	16.01 ± 0.087	16.01 ± 0.087	6.16 ± 0.003

CONCLUSION

The modern day intensive crop cultivation requires the use of nitrogen fertilizers. However, fertilizers are in short supply and expensive in developing countries. Therefore, it is important to explore the possibility of supplementing nitrogen fertilizers with biofertilizers of microbial origin. Microbial processes are fast and consume relatively less energy than industrial processes. [43].

Many cyanobacteria are also capable of using atmospheric dinitrogen (N2) as the source of nitrogen. Many studies have been reported on the use of dried cyanobacteria to inoculate soils as

a means of aiding fertility, and the effect of adding cyanobacteria to soil on rice yield was first studied in the 1950s in Japan. The term 'algalization' is now applied to the use of a defined mixture of cyanobacterial species to inoculate soil, and research on algalization is going on in all major rice producing countries [44].

The outcome of the above experiment proved that biomass obtained from cultivation of *N.muscorum* in non sterile soil substrates was the best way of cultivation of nitrogen fixing cyanobacteria with low cost . The technique can be exploited for the commercial production of biofertilizers which is much efficient and environment friendly procedure also. The biotechnological importance and advantage of using potential BGA strain such as *Nostoc muscorum and Cylindrospermum majus,* for biofertilizer production extent to meet the biofertilizer demand in India. The experiment conducted also aims to study the behavior and productivity of the aforementioned BGA strains through out the year, the result depicts that the summer season is much favorable for both the strain, which recorded highest biomass and N-content and out of the two, *N. muscorum* showed best result.

Developing countries like India, cyanobacteria based biofertilizer technology is under much attention. As this technology can be promising for enriching the soil fertility and improving crop yields. However, the technology needs to be improved further for better exploitation under sustainable agriculture systems. A detailed understanding of cyanobacterial growth behavior and kinetics under whole annual cycle in agriculture systems has to be studied [44]. So a quest for innoculum improvement and search for an efficient BGA strain having good abiotic resistance capability is in great demand.

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