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Screening of Cyanobacteria from Black Cotton Soil and Evaluate their Potential to Survive under Wet and Dry Condition for Biofertilizer Production

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Abstract: A Cyanobacterium is only oxygenic photosynthetic bacteria having nitrogen fixing ability. Therefore, it is used as biofertilizer in agriculture and grassland fields for improvement of soil fertility. Cyanobacterial strains were isolated from Black Cotton soil by serial dilution method and used to screen out best strain to survive in wet and dry conditions in laboratory. These strains were isolate from agriculture and grasslands soil on the basis of filamentous thalli and presence of heterocyst. Three isolates i.e. DR_1, DR_2 and NB_1 were selected out of six isolates. DR_1 and DR_2 was heterocystous filament while NB_1 was only filamentous strain. Maximum carbon, nitrogen and protein were increased by DR_1 and DR_2 than NB_1 but amount of nitrogen was increased by NB_1 was very low in both wet and dry conditions. Results showed that in 60-80% moisture, cyanobacterial strains were well grown and increased amount of carbon, nitrogen and protein. These BGA strains will be used for biofertilizer preparation to improve soil fertility of agriculture and grassland fields.
Keywords: Cyanobacteria, wetting, drying, carbon fixation, nitrogen fixation, biofertilizer

INTRODUCTION

Blue green algae are the most diverse photosynthetic prokaryotes found in terrestrial habitat and increased amount of carbon and nitrogen compounds in soil [1]. They are morphologically varied from unicellular to filamentous thalli where filamentous thalli contain heterocyst. Cyanobacteria liberate substantial quantities of extracellular nitrogenous compounds into the medium and also produced combined nitrogen in soluble form by nitrogen fixing BGA [2, 3]. Besides carbon and nitrogen, they also secrete growth promoting substances like vitamins, auxin and amino acids and improve soil fertility as a biofertilizer [4, 5].

Cyanobacterial strains formed mucilaginous colonies due to presence of sheath, made up of polysaccharide, and helps to dominant BGA in soil especially *Nostoc* spp. [6]. As a biofertilizer, comparative biomass production and nitrogen fixation has been examined in large number of cyanobacterial strains including members of Rivulariaceae such as *Gloeotrichia* and *Calothrix* and found that *Gloeotrichia* spp. exhibited higher dry weight while *Calothrix* higher nitrogen fixation [7, 8].

Nostoc commune had maximum nitrogen fixation rates at 22-126% moisture by weight in subtropical soil while in temperate grassland soil required 500% moisture for maximum nitrogen fixation by *Nostoc* [9, 10]. The nitrogen fixing capacity varies with moisture content and also between species [11]. *Scytonema* shows nitrogenase activity after 10 min of wetting in soil [12]. Dodds *et al.* [13] reported that soil sample have *Nostoc* spp dried for few days then wetted and found photosynthetic activity. Cyanobacteria are physiologically active only in wet condition. Therefore, their growth controlled by moisture [11, 14]. This moisture required for carbon fixation and photosynthetic products for nitrogen fixation. Benlap *et al.* [15] reported that soil crust cyanobacteria shows nitrogen fixation at values ranging from a water content of 6% dry weight to total water. *N. flagelliforme* containing soil sample was dried then exposed at different temperature with moisture shows photosynthetic activity [16].

Cyanobacteria are well adapted to the alternate wetting and drying cycle of climate zone where they live; their ability to tide over drought is due to production of extracellular polysaccharide which forms a protective sheath around the filament/colony [17]. It has been reported that addition of 0.02 g dried algal polysaccharide to 100 g soil increased the water stable aggregates of 0.1 mm diameter from 44 to 60 g. The inoculated algae multiply and bloom on the water film

around the soil particles which carry up to 40% or more moisture in the top one inch of the soil [18]. Blue green algae crust on the soil surface minimizes soil erosion, increase the soil aggregates size and optimize aeration, water movement, root development and fertilizer utilization [19]. Dubey [20] optimized drying - rewetting cycle in laboratory for cyanobacterial growth and found that their chlorophyll concentration was increased in drought and rewetting cycle at 60 - 80% moisture. The objective of this study will help in use of cyanobacterial strains for biofertilizer production to improve soil fertility of agriculture and grassland fields.

MATERIALS AND METHODS

The present study was carried out in Institute of Environment Management and Plant Sciences (IEMPS), Vikram University, Ujjain, Madhya Pradesh (India) in 2008. The experiments were conducted in a laboratory to show the effect of drying and wetting on growth of cyanobacterial isolates and estimated total carbon, nitrogen and protein from pot soil by standard protocols.

Isolation and identification of cyanobacteria

Six to eight soil samples were collected from each agricultural and grassland fields around Ujjain, M.P., India having Black Cotton soil. Composite sample was obtained via mixing all samples together. Ten gram soil was serially diluted via a standard dilution technique using Erlenmeyer conical flask having 95 ml 0.85% saline water [21] and sample was transferred to 90 ml saline water to get 10^{-2} dilution. One milliliter sample was transferred from 10^{-2} dilution to three test tubes having 10 ml BG-11 broth [22, 23]. The tubes were incubated at 30ºC in a continuous illuminated chamber for 3 weeks [20].

Microscopic observation was done by spreading cyanobacterial culture on glass slide and covered with glass cover slip. Low and high power objective lens of compound light microscope were used for observation. Unpurified cultures were again streaked on BG- 11 agar plate and incubated at 30ºC in a continuous light for 3 weeks then prepared microscopic slides and observed [24].

Pure forms of cyanobacteria were identified on the basis of morphological characteristics mentioned in Bergey's Manual of Determinative Bacteriology [25] and Bergey's Manual of Systematic Bacteriology, 2nd ed. Vol. 1 [26]. Identified cultures were preserved in continuous light and maintain as viable for further experiments.

Effect of wetting on growth of Cyanobacteria

Cyanobacterial isolates i.e. DR_1, DR_2 and NB_1 were transferred separately to BG-11 liquid medium tube and incubated at 30ºC for 3 weeks in continuous light then whole biomass was transferred to 100 ml broth in 250 ml capacity Erlenmeyer conical flask. The flasks were incubated at 30ºC for 3 weeks in continuous light and biomass was harvested by vacuum filtration. One gram fresh biomass of each experimental strain was separately mixed in 100 g of sterilized soil in plastic pot. The pots were wetted by adding distilled water to maintain 60 - 80% moisture for 1, 2, 3, 4, 16, 32 and 64 days, where un-inoculated pot was considered as control. The all pots were incubated in continuous light at 30ºC for 64 days then soil samples were collected from each pot after 30 and 60 days incubation time for estimation of total carbon, nitrogen and protein by standard protocols. The experiment was done in duplicates.

Effect of drying on growth of Cyanobacteria

One gram fresh biomass of each experimental strain i.e. DR_1, DR_2 and NB_1 were transferred to 100 g sterilized soil then 20, 40, 60, 80 and 100% moisture was maintained by adding distilled water while un-inoculated sterilized soil was used as control. All pots were incubated at 30ºC in continuous light for 50 days. Soil samples were collected from each pot after 10, 20 and 40th day incubation time to analyze total carbon, nitrogen and protein by standard protocols. The experiment was done in duplicates.

Estimation of total organic carbon by phenol sulfuric acid test

One gram soil was used from each pot for total organic carbon estimation by phenol sulfuric acid test [27]. Soil was thoroughly mixed with 12M 1.25 ml sulfuric acid and left for 16 hrs at 20ºC. Mixer was diluted up to 0.5M sulfuric acid by adding distilled water and heated at 100ºC for 5 hrs then cooled. Ten milligram digested soil was homogenized using Mortar and Pestle. Homogenized soil (0.1 ml) was taken in glass test tube and mixed with 1.9 ml distilled water. Add 0.05 ml 90% phenol reagent in diluted sample and vortex by cyclomixer. Thereafter, 5 ml sulfuric acid was added rapidly and incubated for 30 min at room temperature. Color density was recorded at 485 nm using Systronics spectrophotometer and compared with standard curve of glucose (1 mg/ml).

Estimation of total protein by Lowry method

One gram soil was taken from each pot and separately mixed with 50 ml 0.5N sodium hydroxide then boiled for 20 min. The mixer cooled and extract was obtained by filtration using Whatman No 1 filter paper [28]. Reaction mixer including 0.2 ml soil extract and 2 ml Lowry reagent [29] was taken in glass test tubes and mix by cyclomixer. The test tubes were incubated at 37ºC for 20 min in water bath and added 0.2 ml Folin phenol reagent then mixed. Test tubes were again incubated at room temperature for 30 min and color density was recorded at 660 nm using Systronics spectrophotometer and compared with standard curve of Bovine serum albumin (BSA, 1 mg/ml).

Estimation of total nitrogen by Kjeldahl method

Soil sample was collected from each pot and dried in hot air oven at 50ºC for 24 - 48 hrs. Five gram soil was taken in an 800 ml capacity Kjeldahl flask [30, 31] and added 20 - 25 ml distilled water with 1 ml liquid paraffin and some glass beads. After that flask was connected with condenser and maintained cool tap water flow in condenser. In another conical flask, 4% 40 ml Boric acid solution was taken and 2 - 3 ml mixed indicator was added in 5:5 ratio (0.1% Methyl red and 0.15% Bromocresol green), showed pink color. This flask was connected with another outlet of condenser and dipped in the solution of conical flask.

Added 2.5% 20 ml sodium hydroxide in Kjeldahl flask and mouth was immediately closed with rubber stopper then heat by electronic heater. About 100 ml of distilled was collected in the conical flask having cooled solution of boric acid and indicators. The color of flask reagent was changed from pink to green then removed the conical flask and switched off the heater. A blank sample was also run simultaneously without taking soil sample. The solution of conical flask was titrated with standard 0.5N sulfuric acid till the pink color appeared and burette reading was noted then compared with control.

RESULTS AND DISCUSSION

Cyanobacteria are multiply in optimum moist condition and fix carbon and nitrogen, also produced other metabolites. In the present study, BG-11 media, 13 sterilized soil containing plastic pots and three cyanobacterial isolates were used and soil samples were taken from each pot to evaluate total carbon, nitrogen and protein in laboratory using standard protocols.

Isolation of Cyanobacteria

Table 1. Isolation of Cyanobacteria from soil.

S No.	Isolates Code	Isolates
1	NB_1	*Oscillatoria*
2	DR_1	*Fischerella*
3	DR_2	*Nostoc**
4	UC_1	*Synechocystis*
5	PH_1	*Nostoc*
6	AR_1	*Gloeocapsa*

Legends: PH- Agriculture and grassland fields of Pingleshver and Harsodhan, NB- Grassland area on Maxi Road (Navlakhi beed), UC- Grassland area of University Campus, DR- Agriculture fields of Dewas Road and AR- Agriculture fields of Agar Road.

The result showed that Cyanobacteria isolated from Black Cotton soil of agriculture and grassland fields were identified as *Nostoc*, *Synechocystis*, *Fischerella*, *Oscillatoria*, *Nostoc** and *Gloeocapsa*. All the cyanobacterial cultures were blue green in color but *Nostoc** was brown in color (Table 1). Out of these isolates, three cultures were heterocystous, one was non-heterocystous filament and two were unicellular. Hence, two heterocystous (*Fischerella* and *Nostoc**) and one non- heterocystous filament (*Oscillatoria*) culture was used to evaluate their potential to survive under wet and dry conditions for biofertilizer production.

Effect of wetting on growth of Cyanobacteria
Total carbon estimation

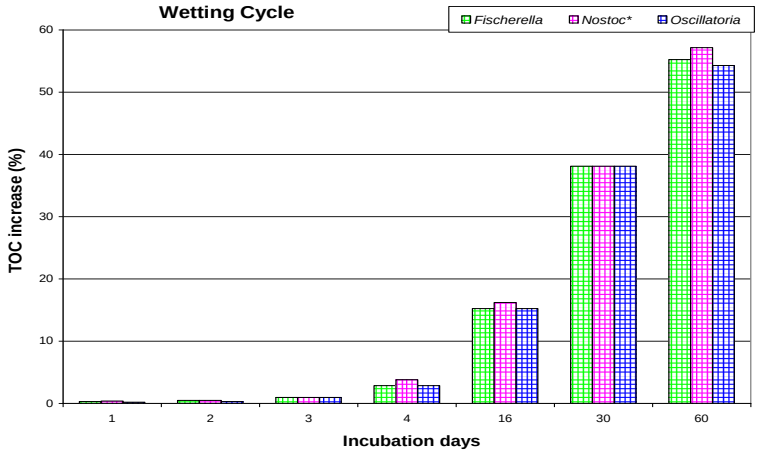

Fig. 1. Effect of wetting on addition of TOC in pot soil by cyanobacteria.

Table 2. Effects of wetting cycle on addition of TOC (mg/g) by cyanobacteria in pot soil.

Incubation days	*Fischerella*	*Nostoc**	*Oscillatoria*
Control	10.5±0.02	-	-
1	10.53±0.05	10.54±0.07	10.52±0.08
2	10.55±0.08	10.55±0.05	10.53±0.02
3	10.6±0.04	10.6±0.05	10.6±0.04
4	10.8±0.09	10.9±0.06	10.8±0.05
16	12.1±0.10	12.2±0.07	12.1±0.06
30	14.5±0.05	14.5±0.03	14.5±0.04
60	16.3±0.09	16.5±0.12	16.2±0.10

The maximum amount of total organic carbon as 55.23, 57.14 and 54.28% was increased by *Fischerella*, *Nostoc** and *Oscillatoria* after 60 days incubation time as compared to control (Fig.- 1). Table 2 reveals that the concentration of organic carbon was increased observable after 16 days in all three isolates. 0.03, 0.04 and 0.02 mg/g and 5.8, 6 and 5.3 mg/g TOC was increased by *Fischerella*, *Nostoc** and *Oscillatoria* after 1 and 60 days incubation time respectively. Fig.- 1 also showed that the maximum amount of TOC was increased by *Nostoc** while minimum by *Oscillatoria* after 60 days incubation time. These all strains were photosynthetic hence, carbon di-oxide fixed by them but amount was differ due to growth, length of filament and survive in wet condition.

Total nitrogen estimation

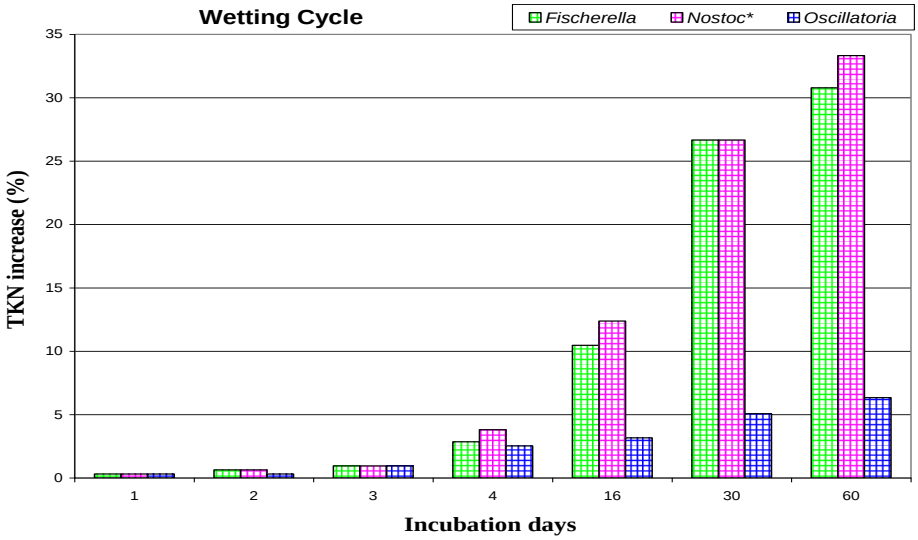

Fig. 2. Effect of wetting on addition of total nitrogen in pot soil by cyanobacteria.

Table 3. Effects of wetting cycle on addition of total nitrogen (kg/ha) by Cyanobacteria in pot soil.

S. No.	Incubation days	*Fischerella*	*Nostoc**	*Oscillatoria*
1	Control	315±0.05	-	-
2	1	316±0.08	316±0.09	316±0.06
3	2	317±0.05	317±0.06	316±0.04
4	3	318±0.06	318±0.05	318±0.06
5	4	324±0.02	327±0.04	323±0.05
6	16	348±0.02	354±0.02	325±0.07
7	30	399±0.07	399±0.05	331±0.10
8	60	412±0.03	420±0.07	335±0.09

Initially only 1 kg/ha nitrogen was added by all three isolates in soil and amount was increased with incubation time as compared to control. Table 3 shows that 97, 105 and 20 kg/ha nitrogen was added by *Fischerella*, *Nostoc** and *Oscillatoria* after 60 days incubation time respectively. The maximum nitrogen

was increased by *Fischerella* and *Nostoc** (30.79 & 33.33%) as compared to *Oscillatoria* (6.34%) after 60 days incubation time. Both *Fischerella* and *Nostoc** have heterocyst but *Oscillatoria* has no heterocyst. Therefore, it was unable to fix atmospheric nitrogen under aerobic condition (Fig.- 2). The amount of nitrogen was maximum increased by *Nostoc** than *Fischerella* due to more nitrogen fixation in wet condition.

Total protein estimation

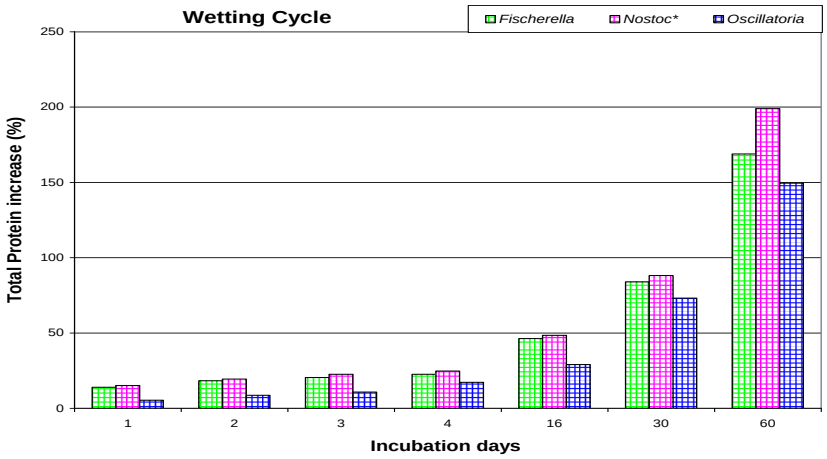

Fig. 3. Effect of wetting on addition of total protein in pot soil by cyanobacteria.

Table 4. Effects of wetting on addition of total protein (mg/g) by cyanobacteria in pot soil.

S. No.	Incubation days	*Fischerella*	*Nostoc**	*Oscillatoria*
1	Control	9.3±0.02	-	-
2	1	10.6±0.05	10.7±0.05	9.8±0.05
3	2	11.0±0.09	11.1±0.07	10.1±0.07
4	3	11.2±0.11	11.4±0.02	10.3±0.05
5	4	11.4±0.02	11.6±0.012	10.9±0.09
6	16	13.6±0.05	13.8±0.06	12.0±0.13
7	30	17.1±0.07	17.5±0.07	16.1±0.08
8	60	25.0±0.09	27.8±0.06	23.2±0.05

Table 4 reveals that initially 1.3, 1.4 and 0.5 mg/g protein was increased after 1 day incubation time by *Fischerella*, *Nostoc** and *Oscillatoria* respectively and amount was increased with incubation time. The maximum amount of protein was increased as 15.7, 18.5 and 13.9 mg/g after 60 days incubation time by *Fischerella*, *Nostoc** and *Oscillatoria* respectively. Further Fig.- 3 shows that 83.88, 88.17 & 73.14% and 168.14, 198.92 & 149.46% of protein was increased by *Fischerella*, *Nostoc** and *Oscillatoria* after 30 and 60 days incubation time respectively. The amount of protein was more increased by *Nostoc** than other two strains due to more growth and nitrogen fixing capacity in aerobic condition.

All three isolates increased more carbon, nitrogen and protein as compared to control due to photosynthetic and nitrogen fixing ability. In 60-80% moisture, growth of cyanobacteria flourished results as that concentration of carbon and nitrogen was increased. As compared to all three isolates, *Nostoc** and *Fischerella* showed maximum addition of those compounds then *Oscillatoria*. It can suggested that both *Nostoc** and *Fischerella* can be used for biofertilizer production to improve soil fertility in wet conditions.

Irisarri *et al.* [1], Zhau *et al.* [16] , Goyal [18], Eldridge and Leys [19] and Dubey [20] has been isolate cyanobacteria from soil and found that filamentous heterocyst containing Cyanobacteria having polysaccharide sheath, helps to survived in rewetting cycle, and fixed atmospheric nitrogen and carbon. Therefore, cyanobacteria can be used as biofertilizer in agriculture and grassland fields.

Effect of Drying on growth of cyanobacteria
Total carbon estimation

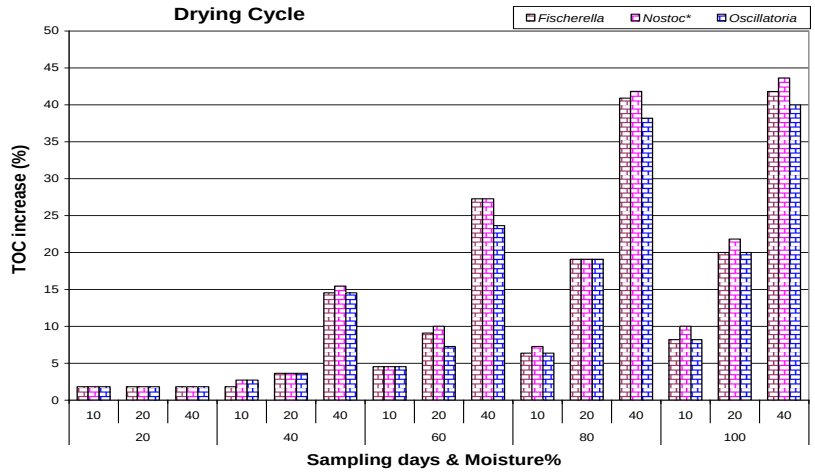

Fig. 4. Effect of drying on addition of TOC in pot soil by cyanobacteria.

Table 5. Effect of drying on addition of TOC (mg/g) by cyanobacteria in pot soil.

Moisture (%)	Sampling (Days)	*Fischerella*	*Nostoc**	*Oscillatoria*
0	Control	11.0±0.03	-	-
20	10	11.2±0.06	11.2±0.02	11.2±0.08
	20	11.2±0.08	11.2±0.05	11.2±0.06
	40	11.2±0.04	11.2±0.04	11.2±0.05
40	10	11.2±0.02	11.3±0.09	11.3±0.02
	20	11.4±0.06	11.4±0.08	11.4±0.04
	40	12.6±0.04	12.7±0.12	12.6±0.08
60	10	11.5±0.09	11.5±0.09	11.5±0.09
	20	12.0±0.08	12.1±0.05	11.8±0.10
	40	14.0±0.10	14.0±0.06	13.6±0.12
80	10	11.7±0.07	11.8±0.04	11.7±0.04
	20	13.1±0.06	13.1±0.12	13.1±0.05
	40	15.5±0.05	15.6±0.06	15.2±0.03
100	10	11.9±0.03	12.1±0.07	11.9±0.08
	20	13.2±0.04	13.4±0.05	13.2±0.02
	40	15.6±0.08	15.8±0.7	15.4±0.04

Fig.- 4 showed that only 3.63% carbon was increased by all three isolates in soil after 20 days incubation time with 40% moisture. In 60% moisture 1, 1.1 and 0.8 mg/g carbon was increased by *Fischerella*, *Nostoc** and *Oscillatoria* after 20 days incubation time respectively. After 40 days 3, 3 and 2.6 mg/g carbon was increased by *Fischerella*, *Nostoc** and *Oscillatoria* in 60% moisture respectively as compared to control (Table 5). Further Fig.- 4 shows that the amount of carbon was more increased in 100% moisture after 40 days incubation time as 41.8, 43.63 and 40% by *Fischerella*, *Nostoc** and *Oscillatoria* respectively. The amount of carbon was increased with moderate variation by all these isolates due to survival in 60 - 100% moisture and also availability of sufficient amount of moisture in pot soil.

Total nitrogen estimation

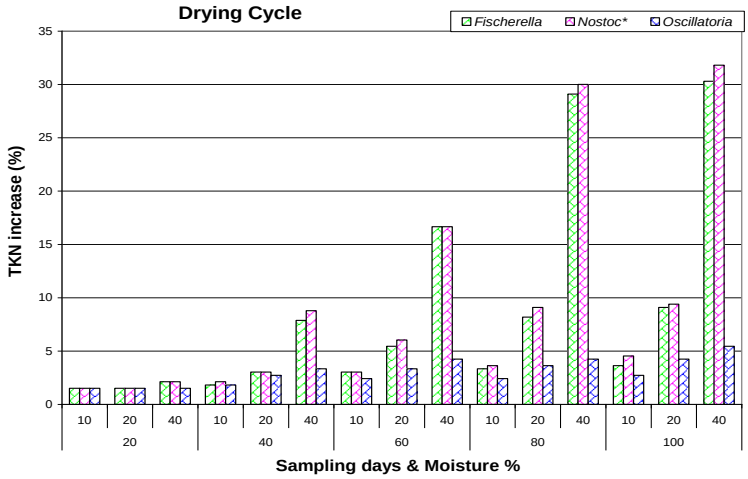

Fig. 5. Effect of drying on addition of total nitrogen in pot soil by cyanobacteria.

Table 6. Effect of drying on addition of total nitrogen (kg/ha) by cyanobacteria in pot soil.

Moisture (%)	Sampling (Days)	*Fischerella*)	*Nostoc**	*Oscillatoria*
0	Control	330±0.10	-	-
20	10	335±0.08	335±0.08	335±0.12
	20	335±0.03	335±0.02	335±0.05
	40	337±0.05	337±0.05	335±0.08
40	10	336±0.04	337±0.06	336±0.02
	20	340±0.07	340±0.07	339±0.06
	40	356±0.03	359±0.05	341±0.07
60	10	340±0.05	340±0.04	338±0.06
	20	348±0.06	351±0.09	341±0.03
	40	385±0.08	385±0.10	344±0.04
80	10	341±0.09	342±0.04	338±0.05
	20	357±0.10	360±0.05	342±0.08
	40	426±0.12	429±0.06	344±0.06
100	10	342±0.08	345±0.012	339±0.07
	20	360±0.06	361±0.02	344±0.08
	40	430±0.07	435±0.06	348±0.03

It was evident from Table 6 that 55, 55 and 14 kg/ha nitrogen was increased by *Fischerella*, *Nostoc** and *Oscillatoria* after 40 days incubation time in 60% moisture respectively in their respective control. In 80% moisture 69, 99 and 14 kg/ha nitrogen was increased by *Fischerella*, *Nostoc** and *Oscillatoria* after 40 days. Fig.- 5 reveals that the maximum amount of nitrogen was increased in pot soil as 30.30, 31.81 and 5.45% by *Fischerella*, *Nostoc** and *Oscillatoria* after 40 days incubation time with 100% moisture respectively as compared to control. The amount of nitrogen was more increased by both *Fischerella* and *Nostoc** as compared to *Oscillatoria* due to presence of heterocyst, helps in nitrogenase activity during aerobic condition.

Total protein estimation

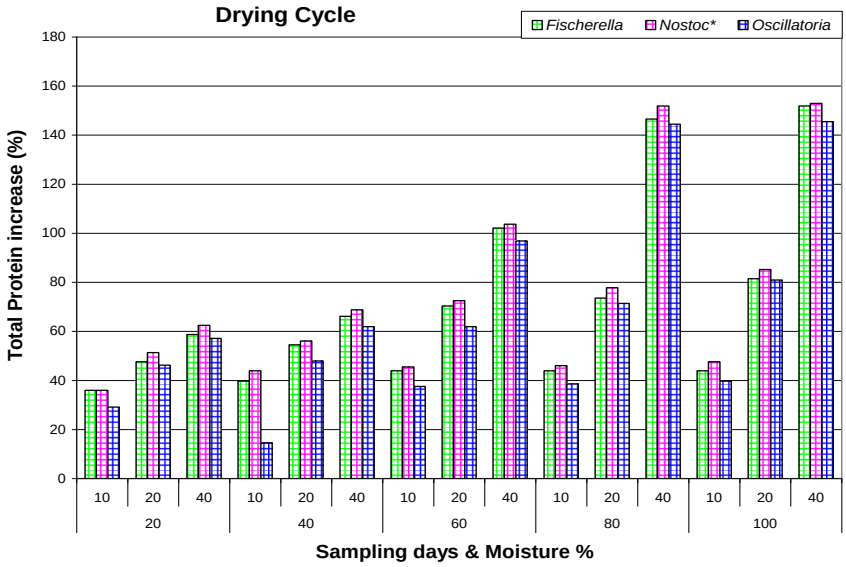

Fig. 6. Effect of drying on addition of total protein in pot soil by cyanobacteria.

Table 7. Effect of drying on addition of total protein (mg/g) by cyanobacteria in pot soil.

Moisture (%)	Sampling (Days)	*Fischerella*	*Nostoc**	*Oscillatoria*
0	Control	9.45±0.05	-	-
20	10	12.85±0.06	12.85±0.06	12.2±0.02
	20	13.95±0.02	14.3±0.08	13.82±0.03
	40	15.0±0.04	15.35±0.05	14.85±0.05
40	10	13.2±0.09	13.6±0.09	12.6±0.12
	20	14.6±0.04	14.75±0.04	13.98±0.05
	40	15.7±0.06	15.95±0.07	15.3±0.09
60	10	13.6±0.07	13.75±0.08	13.0±0.04
	20	16.1±0.09	16.3±0.05	15.3±0.02
	40	19.1±0.04	19.25±0.06	18.6±0.08
80	10	13.6±0.02	13.8±0.07	13.1±0.06
	20	16.4±0.04	16.8±0.05	16.2±0.07
	40	23.3±0.09	23.8±0.07	23.1±0.02
100	10	13.6±0.07	13.95±0.09	13.2±0.05
	20	17.15±0.10	17.5±0.02	17.1±0.03
	40	23.8±0.03	23.9±0.12	23.2±0.08

Results from Table 7 shows that the notable concentration of protein increased in soil was started from 60% moisture and continuously increased onwards 80 and 100% moisture. As BGA growth flourished the amount of protein was also increased in soil. Fig.- 6 shows that 14.35 (151.85%), 14.45 (152.95%) and 13.75 (145.5%) mg/g protein was increased in 100% moisture after 40 days incubation time by *Nostoc**, *Fischerella* and *Oscillatoria* respectively.

All three isolates increased at least same amount of carbon and protein in soil due to photosynthetic activity but amount of nitrogen was increased more by *Fischerella* and *Nostoc** as compared to *Oscillatoria*. In *Fischerella* and *Nostoc** a

specialized cell, known as heterocyst, was present for nitrogen fixation in aerobic condition while absent in *Oscillatoria*. Therefore, *Oscillatoria* was unable to fixed atmospheric nitrogen in aerobic condition.

Irisarri *et al.* [1], Belnap *et al.* [15], Zhau *et al.* [16] , Goyal [18], Eldridge and Leys [19] and Dubey [20] has been isolate cyanobacteria from soil and found that heterocystous cyanobacterial filament having polysaccharide sheath, helps to survived in drying - rewetting cycle, and fixed atmospheric nitrogen and carbon. Therefore, cyanobacteria can be used for biofertilizer production to improve agriculture and grassland fields soil.

CONCLUSION

To show effect of wetting and drying on growth of cyanobacteria, Black Cotton soil samples should be collected from agriculture and grassland fields. BG-11 medium should be prepared in distilled water and sterilized by steam then used for inoculation of soil sample for isolation. Total organic carbon, nitrogen and protein should be estimated from pot soil using standard protocols. The whole observation required control light and temperature, these organic and inorganic compounds increased fertility of soil due to photosynthesis and nitrogen fixing capacity. Therefore, cyanobacteria e.g. *Fischerella* and *Nostoc** can be used for biofertilizer production to improve agriculture and grassland soil fertility. They also provide nitrogen to plants and other organisms. The results also showed that both *Fischerella* and *Nostoc** survived well in both wet and dry condition with 60 - 100% moisture.

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