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The Effects of Cyanobacterial (Blue Green Algae) Culture Filtrates on the Biomass and Biochemicals of *Withania somnifera* Dunal

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Abstract: The present investigation was aimed at studying the effect of culture filtrates of cyanobacteria (blue green algae) namely *Anabaena ambigua* Rao and *Oscillatoria foreaui* Fremi in different soil conditions on the growth of *Withania somnifera* Dunal in terms of biomass and biochemical characteristics along with active ingredients of the roots. Experiments conducted in field by replenishing the plant rhizosphere as foliar spray with increasing quantities of culture filtrates enhanced the growth of the plant more precisely in sandy loam soil when compared to red soil. The yield potentials of the roots also increased with the culture filtrate of *O. foreaui* along with the other parameters studied, attributing to the availability of the nutrients from the cyanobacterial culture filtrates.

Key words: Cyanobacterial culture filtrates, *Anabaena ambigua, Oscillatoria foreaui, Withania somnifera,* growth, biomass, active ingredients, red soil, sandy loam soil

INTRODUCTION

One of the important milestones of biological research in the last millennium is the understanding that soil microorganisms play a vital role in agriculture. The subsequent development of biofertilizer technology has coincided with the energy crisis in the 1970's and the compulsive need for contemplating alternate source of energies often than the chemical input. Nitrogen is an important constituent of amino acids, protein and protoplasts, directly influence the plant growth and development in terms of both morphological and biochemical characteristics. Since, nitrogen management is one of the major factors to attain higher productivity, particularly under limited water supply, where the use of higher dose of inorganic fertilizers is restricted, demands the integration of various sources of nitrogenous fertilizers in a more appropriate way because this not only reduces the use of inorganic fertilizers but also makes the environment eco-friendly.

A large number of fresh water and marine organisms excrete organic and inorganic substances in the medium in which they grow. It has been demonstrated that certain nitrogen fixing blue-green algae liberate appreciable amount of fixed nitrogen into the medium as polypeptide or free amino acids (Watanabe, 1951; Fogg, 1952) and were suggested as promising components of biofertilizer because of their ability to fix the atmospheric nitrogen both as free living organisms and components of symbiotic associations (Vessey *et al.*, 2005). Besides nitrogen fixation the cyanobacteria contain several extra cellular products like growth promoters, amino acids, vitamins, useful enzymes and nutrients like carbohydrates and nitrogen (Singh and Trehan, 1973; Malliga *et al.*, 2002) resulting in moderate but constant productivity in fields where nitrogen fertilizer is applied. Hence, the ideas of utilizing algal biofertilizers as an alternative or supplementary source of nitrogen have to be promoted for the potential biological system under low cost production technology. Since, cyanobacterial bio fertilizers contain (Ravishankar, 2000; Christopher *et al.*, 2007) the nutrients required by the plants and help to increase the quality of the soil with a natural microorganism in the environment and that foliar spray could be used in many different growing medium with excellent production resulting in its utilization as organic farming in gardening. Because one of the aspects of current interest in recent years is to use the available natural resources for the welfare of mankind and in this respect medicinal plants occupy a priority rank and since cyanobacteria are available naturally as a major biofertilizer, their exploitation is not completely understood and is demanding globally.

Study of the medicinal plants and using drugs obtained from natural products has been an important area of research during the last decade of the last century.

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Potential drug yielding plants and their compounds obtained are being studied for the pharmacological values. *Withania somnifera* Dunal of Solanaceae (also known as Indian Ginseng) is one such important tropical medicinal plant commonly used in ayurveda (Indian natural therapy) and other traditional system of medicines. Since more than 91% of pharmaceutical products are produced from the roots of this plant, there is an increasing demand for this herb in both national and international markets extending an enhancement in growth and desired bioactive ingredients. However, sustainable agricultural practices have been adopted for this plant all around India, implications of cyanobacterial input especially its extracellular products (that are available at a cheaper and economical value) have never been attempted so far. Although, cyanobacteria have been previously shown to enhance the growth of numerous plants of economic importance, a lacuna in the information of precise quality and quantity of growth promoting potential of commercial inoculants are to be standardized to formulate and facilitate studies on the comparison on potential benefits of cyanobacteria. Hence, a pioneering attempt has been made in this effort to fill the lacuna and to enumerate the effects of cyanobacterial culture filtrates for the effective growth of *Withania somnifera*.

Anabaena ambigua Rao, (a heterocystous filamentous form) and *Oscillatoria foreaui* Fremi, (a non-heterocystous undifferentiated form) were chosen. Their culture filtrate response on the growth and biomass in terms of morphology and biochemical characteristics and active ingredients of *Withania somnifera* (Ashwagandha in Sanskrit, Amukura in Tamil) a herb extensively used as anti-inflammatory, anti-oxidant, anti-tumour, anti-stress, immuno-modulator and rejuvenator showing a positive influence on the endocrine, cardiopulmonary and central nervous system. The roots are recommended for hiccup, female disorders, cough, rheumatism and dropsy.

MATERIALS AND METHODS

Culture and culture conditions: Cultures of *Anabaena ambigua* Rao (A 100) and *Oscillatoria foreaui* Fremi (A1340) were obtained from the Culture Collection of Algae, Centre for Advanced Studies in Botany (CAS), University of Madras, Chennai. *A. ambigua* was maintained in nitrate free BG_{11} medium and *O. foreaui* was maintained in nitrate amended BG_{11} medium (Rippka *et al.*, 1979) at 27±1°C under fluorescent illumination of 40 μ Em^{-2} sec^{-1}. Cultures were incubated in growth chamber (12 /12 h light/dark cycle) fitted with Sangmo Weston Ltd., S650 313 F model automatic timer. Gentle shaking of the cultures was done manually every day to reduce the clumping of cells. Based on the growth studies (data not provided), cultures were harvested at the exponential phases (16th day for *A. ambigua* and 12th day for *O. foreaui*) by centrifugation at 11,424 x g for 20 min in a Beckman centrifuge. The culture filtrates obtained were further used as foliar sprays to analysis it performance on the growth and biochemicals of a potential herb.

Site characteristics: Experiment was carried out during the period 1999 to 2000 for two seasons at the experimental farm (Maduravoyal) of Botany Field Research Laboratory, Centre for Advanced Studies in Botany, University of Madras (13° 04' N latitude and 80° 14' E longitude) which is located at a distance of 13 km East of Chennai. The site contained sandy loam soil which, belonged to the Series-Hillsdale, Order-Alfisols, Type-Hapludalfs and Family of Coarse-loamy, mixed and active. The red soil collected from Poonamallee region belonged to the Series-Ruple, Order-Ultisols, Type-Rhodudults and Family of Fine, parasesquic and thermic (according to the US soil taxonomy). Initially, the physico-chemical properties of both soils were analyzed at the soil test laboratory, Kancheepuram, which showed pH of 6.1 and 6.5, Electrical conductivity of 0.26 and 0.35 (dS m^{-1}). The nutrients available were found to be Nitrogen (45 and 59 mg kg^{-1}), Phosphorus (10.4 and 13 mg kg^{-1}) and Potassium (149 and 165 mg kg^{-1}) in red and sandy loam soils, respectively.

Planting material and experimental design: The seeds of *Withania somnifera* were obtained from Indian Council of Agricultural Research (ICAR), Anand, Gujarat, India. The experiment was conducted with 0.1% mercuric chloride sterilized seeds in both soil types (red soil and sandy loam soil). Red soil was mixed with sand in the ratio of 2:1, double sterilized and transferred to pots of size 30×30×20 cm, while sandy loam soil of the site was directly transferred to same sized pots after double sterilization. About 10 kg of soil was packed in each bag and 60 mg of seeds was used for the experiment. The plants were harvested bimonthly up to six months. Besides, watering the plants every day, culture filtrates of both *A. ambigua* and *O. foreaui* at different quantities of 50, 100 and 200 mL was given individually and regularly as foliar spray on alternate days until its harvest. Triplicates were maintained for each set and were compared against the control (only water).

Growth parameters (biomass and biochemical characteristics): The effect of cyanobacterial culture filtrate on the growth and development of *W. somnifera* was measured in terms of shoot and root lengths, fresh and dry weights, fruit yield and size of leaf lamina, number of root branches and diameter of the roots per plant along with certain biochemical characteristics. The amount of total chlorophyll (Arnon, 1949), total nitrogen (Umbriet *et al.*, 1972), total carbohydrate (Clegg, 1956), total lipids (Folch and Stoare-Stanley, 1957) and total withanolides-extracted (Nittala *et al.*, 1981) and quantified (Ramaiah *et al.*, 1984).

Statistical analysis: All the data were subjected to One way Analysis of Variance (ANOVA) and the significant difference among the means were compared by Duncan's new Multiple Range Test (DMRT) at a threshold p-value of 0.05 to test the differences between the treatments using SPCC/PC + Student ware statistical software.

RESULTS AND DISCUSSION

Biomass

Shoot and root lengths: The shoot length of *W. somnifera* treated or amended with different quantities (also referred to quantities in the text) of both culture filtrates showed tremendous increase under both the soil conditions. With prolonged cyanobacterial culture filtrate inoculum the growth of the plant seemingly increased and was found to be higher especially in sandy loam soil by *O. foreaui*, which showed the maximum length of 155.15 and 137.54 cm in red soil at the highest quantity of 200 mL (Table 1). Although, *A. ambigua* was in-par with *O. foreaui*, increased the shoot lengths to 120.26 and 135.80 cm in red soil and sandy loam soil, respectively at the highest quantity of 200 mL (Table 1) was not as effective as that of *O. foreaui*. However, corresponding to the shoot length, the root length also increased by the culture filtrates in both soil types. Though the culture filtrates of both the organisms significantly increased the root length by two fold, maximum effect was observed by *O. foreaui* which, at 200 mL increased to 19, 30 and 54.8 cm especially in sandy loam soil during the 2nd, 4th and 6th months, respectively. Unlike that of *O. foreaui*, *A. ambigua*, increased to only 42 and 47 cm in red and sandy loam soils, respectively (Table 2).

Fresh and dry weights: The culture filtrate of cyanobacteria amended at different quantities proved to be very effective on the biomass in terms of fresh and dry weights in both the soil types. Significant increase in biomass of the plant was obtained in sandy loam soil than red soil with the higher volume of 200 mL culture filtrate of *O. foreaui* compared to *A. ambigua*. Biomass in terms of fresh weight increased to 31.2% by the culture filtrate of *O. foreaui* and to 28.9% by the culture filtrate of *A. ambigua* against the control in sandy loam soil (Table 3). Similarly, the dry weight was also increased to 73.8% by the culture filtrate of *O. foreaui* and to 71% by the culture filtrate of *A. ambigua* against the control in sandy loam soil compared to red soil (Table 3).

Fruit yield and leaf area index: Co-relating to the fresh and dry weights, the yield of the fruits measured at the final harvest was enhanced by the cyanobacterial culture filtrates in both soil types. Significant yield was achieved by the culture filtrate of *O. foreaui*, which showed 64.4 and 61.4% in sandy loam soil and red soil, respectively, while, less significant yield ($p<0.05$) was observed with the culture filtrate of *A. ambigua* yielding to just 43.9 and 44.7% against the control in sandy loam soil and red soil, respectively (Table 4). Likewise, leaf area

Table 1: Effect of cyanobacterial culture filtrates on the shoot length of *W. somnifera*

Soil types	Quantity of culture filtrates (mL)	*A. ambigua* 2nd month	4th month	6th month	*O. foreaui* 2nd month	4th month	6th month
Red soil	Control	17.9	42	60.70	18.0	42.0	57.20
	50	21.0	44	63.00	23.8	56.9	79.00
	100	23.0	63	101.00	35.0	80.0	102.00
	200	25.3	84	120.26	39.0	93.4	137.54
Sandy loam soil	Control	20.8	44	61.80	20.6	53.0	65.00
	50	24.0	46	72.00	36.8	74.0	92.60
	100	40.3	79	110.00	44.0	96.0	138.00
	200	45.0	105	135.80	59.6	122.0	155.15

Table 2: Effect of cyanobacterial culture filtrates on the root length of *W. somnifera*

Soil types	Quantity of culture filtrates (mL)	*A. ambigua* 2nd month	4th month	6th month	*O. foreaui* 2nd month	4th month	6th month
Red soil	Control	8.0	13.6	31.2	9.0	13.0	30.7
	50	11.0	17.0	37.0	13.0	17.8	33.0
	100	13.0	21.9	39.0	16.0	22.0	40.0
	200	16.4	23.8	42.0	18.0	25.0	50.0
Sandy loam soil	Control	10.0	14.6	30.0	11.8	16.0	31.0
	50	12.0	17.0	40.0	13.0	20.0	37.0
	100	14.0	22.0	43.2	16.6	23.2	48.0
	200	17.0	25.0	47.0	19.0	30.0	54.8

Table 3: Effect of cyanobacterial culture filtrates on the biomass of *W. somnifera*

Volumes of culture filtrate (mL)	*Anabaena ambigua*		*Oscillatoria foreaui*	
	Red soil	Sandy loam soil	Red soil	Sandy loam soil
Fresh weight (g plant⁻¹)				
Control	72.70±0.14	74.35±1.19	73.25±0.90	74.15±0.83
50	81.47±0.12	81.85±1.04	80.30±1.27	82.30±1.40
100	88.20±0.13	89.57±1.32	86.67±0.95	90.37±1.49
200	93.10±0.08*	95.75±0.89*	93.80±0.91*	97.42±1.02*
Dry weight (g plant⁻¹)				
Control	32.12±1.18	31.02±1.24	31.52±0.78	31.25±0.89
50	37.65±0.77	38.20±0.99	37.70±1.14	38.12±1.12
100	44.75±1.49	45.22±1.49	45.22±1.73	46.65±1.34
200	51.00±0.92*	53.07±0.88*	51.72±0.84*	54.32±1.13*

Values are mean±SE of four determinations, *: Indicates statistical significance at $p<0.05$ level in relation to control

Table 4: Effect of cyanobacterial culture filtrates on the vegetative growth of *W. somnifera*

Volumes of culture filtrate (mL)	*Anabaena ambigua*		*Oscillatoria foreaui*	
	Red soil	Sandy loam soil	Red soil	Sandy loam soil
Fruit yield (g plant⁻¹)				
Control	40.35±1.78	41.97±1.40	38.00±0.11	38.86±0.13
50	48.20±0.86	48.95±0.80	48.32±0.12	49.02±0.15
100	54.25±0.89	54.65±1.10	56.42±0.08	58.30±0.13
200	58.42±0.54*	60.40±0.80*	61.35±0.07*	63.92±0.05*
Leaf area (cm² plant⁻¹)				
Control	4.92±0.02	5.27±0.13	5.10±0.14	5.20±0.12
50	6.05±0.25	6.10±0.16	6.02±0.22	6.20±0.16
100	7.47±0.22	7.32±0.23	7.35±0.23	7.35±0.18
200	8.20±0.12*	8.55±0.13*	8.27±0.11*	8.52±0.16*

Values are mean±SE of four determinations, *: Indicates statistical significance at $p<0.05$ level in relation to control

Table 5: Effect of cyanobacterial culture filtrates on the root branches and diameter of *W. somnifera*

Soil types	Quantity of culture filtrates (mL)	*A. ambigua*			*O. foreaui*		
		2nd month	4th month	6th month	2nd month	4th month	6th month
Root branches (No.)							
Red soil	Control	4.00	6.0	7.00	4.0	6.0	7.00
	50	3.00	5.0	6.00	3.0	5.0	6.00
	100	3.00	4.0	5.00	3.0	4.0	5.00
	200	2.00	3.0	3.00	2.0	3.0	3.00
Sandy loam soil							
	Control	3.00	6.0	7.00	3.0	6.0	7.00
	50	3.00	5.0	5.00	3.0	5.0	5.00
	100	2.00	3.0	3.00	2.0	3.0	3.00
	200	2.00	2.0	2.00	2.0	2.0	2.00
Root diameter (cm branch⁻¹)							
Red soil	Control	2.50	3.7	4.10	2.4	3.7	4.30
	50	3.10	4.6	5.70	3.2	4.7	5.80
	100	4.00	5.3	6.10	4.0	5.5	6.30
	200	4.55	5.8	6.62	4.9	5.9	6.80
Sandy loam soil	Control	2.60	4.1	4.60	3.0	4.1	4.90
	50	3.30	4.5	5.80	3.9	5.3	6.20
	100	4.00	5.0	5.80	4.3	6.0	6.40
	200	4.10	5.3	6.20	5.3	6.8	7.20

index also significantly increased 63.8 and 62.2% with the highest volumes of 200 mL culture filtrate, with the culture filtrates of each *O. foreaui* and *A. ambigua*, respectively against the control (Table 4).

Number of root branches and diameter: Corresponding to the growth, reduction in the number of root branches was influenced by the increasing quantities of the culture filtrates. The culture filtrate of both *A. ambigua* and *O. foreaui* proved to be very effective at higher volumes of 200 mL, where they reduced the number from 7 to 3 in red soil and from 7 to 2 in sandy loam soil during their final harvest at the 6th month (Table 5). The girth or diameter of the roots was also influenced by the cyanobacterial culture filtrates, which gradually increased with increasing volumes of 50, 100 and 200 mL,

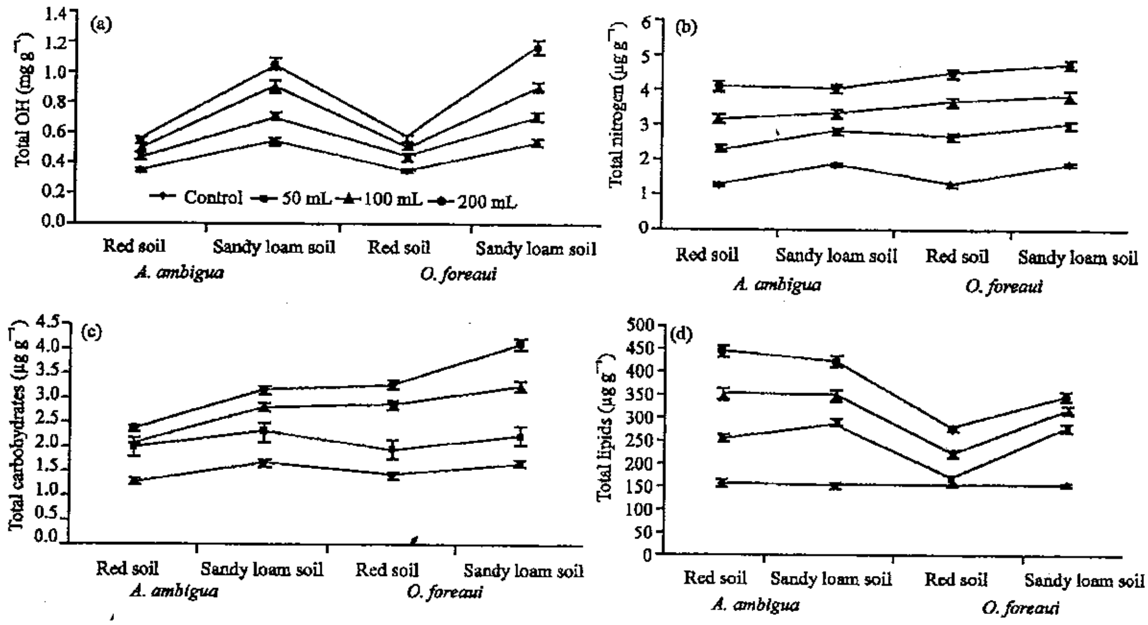

Fig. 1: Effects of cyanobacterial culture filtrates on the biochemical characteristics of *W. somnifera* (a) total chlorophyll content (b) total nitrogen content (c) total carbohydrates and (d) total lipids

respectively. However, the culture filtrate of *A. ambigua* increased the root diameter from 4.1 to 6.62 cm in red soil (Table 5). While, *O. foreaui* effectively promoted it from 4.9 to 7.20 cm in sandy loam soil (Table 5) signifying that the culture filtrates liberate an abundant quantity of many extra cellular organic compounds (Vaidya *et al.*, 1970) which have capacities to chelate with several micronutrient elements.

Biochemical characteristics: The biochemical characteristics analyzed revealed the effective response of the plant to the culture filtrates amended. Higher the volume, greater was the output by the plant. In general, total chlorophyll, total nitrogen and total carbohydrates analyzed showed manifold increase in plants treated with culture filtrates compared to the control plants. The plants grown in sandy loam soil recorded the highest chlorophyll content compared to red soil. Significant contents of 1.053 and 1.174 mg g^{-1} were recorded with the culture filtrates of *A. ambigua* and *O. foreaui*, respectively (Fig. 1a). The total nitrogen content varied with respect to the soil and organisms used. The highest content was observed in two combinations (i) *A. ambigua* with red soil which showed 4.102 µg g^{-1} and (ii) *O. foreaui* in combination with sandy loam with the content of 4.757 µg g^{-1}. Thus, it was increased by 42% in red soil and 68.2% in sandy loam soil with the culture filtrates of *A. ambigua* and *O. foreaui*, respectively (Fig. 1b). With respect to chlorophyll, the content of carbohydrate also increased

Table 6: Effect of cyanobacterial culture filtrates on the root withanolides of *W. somnifera*

Volumes of culture filtrate (mL)	*Anabaena ambigua* Red soil	*Anabaena ambigua* Sandy loam soil	*Oscillatoria foreaui* Red soil	*Oscillatoria foreaui* Sandy loam soil
Control	0.173	0.22	0.180	0.230
50	0.220	0.25	0.235	0.270
100	0.270	0.29	0.315	0.320
200	0.290	0.31	0.340	0.355

in sandy loam soil by both the organisms. It showed 3.147 and 4.097 µg g^{-1} with the culture filtrates of *A. ambigua* and *O. foreaui*, respectively (Fig. 1c). Contemporarily, the total lipid content was increased manifold by the culture filtrate of *A. ambigua*, which, showed 444.50 µg g^{-1} in red soil and 423.62 µg g^{-1} in sandy loam soil, whereas, the culture filtrate of *O. foreaui* showed 279.62 and 347.75 µg g^{-1} in red and sandy loam soil, respectively (Fig. 1d), which was in accordance with the results of (Kerby *et al.*, 1987; Katyal and Carter, 1989), according to whom, cyanobacteria apart from fixing nitrogen, plays a major role in enriching the soil fertility as well chelating the micronutrients present in the soil to be available to the plants as such by either forming a consortium or liberating abundant quantity of many extra cellular organic compounds from its culture as leachate (Selvarani, 1983; Kumar and Mohan, 1997; Ravishankar, 2000).

Total withanolides of root: In relation to the enhanced growth of the plants in terms of both biomass and biochemical characteristics, the total withanolide content of the roots estimated increased with the culture filtrates of cyanobacteria at highest volumes of 200 mL. The active principle (withanolides) increased to about 15 and 13%, respectively against the control in both red soil and sandy loam soils when compared. Although, both the organisms significantly increased the quantity of withanolide compared to control, maximum quantification was possible with the plants treated with the culture filtrate of *O. foreaui* (0.35 μg g^{-1} DW) showing the minimum of 4% increase against the plants treated with the culture filtrate of *A. ambigu* (0.31 μg g^{-1} DW) (Table 6).

All parameters studied have proved the potentials of cyanobacteria, which, besides increasing nitrogen fertility, also benefited the plant by enriching and increasing their yields by its growth-promoting substances such as gibberellins (Metting, 1988), whose effect has been proved in paddy (Gupta and Shukla, 1972), mangroves (Toledo *et al*., 1995) and some vegetables like tomato and Cucumber (Ordoz and Pulz, 1996) especially by *Arthonema africanum* (Stirk *et al*., 1999). The nutrient requirements for the crops are not one and the same for all cultivars due to ecology and several other management practices (Reddy and Mohammed, 2000) and since nutrient requirement has become one of the most sparkling priority areas of research, requires the utilization of bio resources; especially cyanobacteria, which have the capacity to fix several forms of nitrogen.

Very few attempts made in the past to study the plant growth promoter nature of extra cellular or water-soluble products of *Calothrix* sp., *Anabaena* sp. and *Stratonostoc* sp., have proved the rhizogenous and stimulatory effect on the plant organs of wheat and mulberry (Koptiyeva and Tantsiurenka, 1971) and against certain chemical fertilizers (Zeenat and Sharma, 1990), there appears no record as far as the medicinal plants are concerned, except for *Solanum nigrum* (Lakshmi *et al*., 2005). Since, more direct evidence for hormonal effects of culture filtrates primarily with pre-soaking of rice seeds, decreased the losses from sulphate reducing processes attributing to the enhancement of germination and a faster seedling growth (Christopher *et al*., 2007), the present report also evidences similar effect and suggest the favorable role of cyanobacterial culture filtrates on the tested herb. A significant increase in sandy loam soil compared to red soil, could also be due to the fact that more porosity was provided on soil surface creating a way for the roots to penetrate deep further, thereby increasing the root length as already suggested by Bisoyi and Singh (1986) and Vessey *et al*. (2005).

CONCLUSION

Many of our most important drugs are obtained from natural sources particularly plants and that almost all drugs used in medicine today have been developed by modifying compounds originally obtained from plants. Therefore, herbs are important in not only the past and present but in the future. Most of the medicinal plants are generally wildly distributed and their effective utilization, proper cultivation with enhanced biomass and potentials becomes mandatory. Thus, the experiments formulated to optimize the level of nutrient supply and potentials of cyanobacterial culture filtrates on the yield potentials and economy of *Withania somnifera* produced certain interesting and significant observations. However, the useful interaction of microbial and plant systems are bound to exist in natural ecosystems and the efforts of scientists lies in the identification of such systems that can be beneficial to humankind. The present study is probably one such attempt to make use of two systems analyzed by the scientists in the last century-the cyanobacterial biofertilizers and the medicinal plants agrotechniques, which requires further detailed investigation in future to be commercialized.

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