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Neem Leaf Aqueous Extract Induced Growth, Pigments and Photosynthesis Responses of Cyanobacterium *Nostoc muscorum*

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Neem leaf aqueous extract (1, 2, 4 and 8%) induced effects on growth; photosynthetic pigments, and photosynthesis of cyanobacterium *Nostoc muscorum* were studied. The low concentrations (1 and 2%) of extract produced stimulating effects on growth, photosynthetic pigment contents, photosynthetic electron transport activity (PS II, PS I and whole chain), oxygen yield and ^{14}C-fixation. But 2% extract resulted a marginal decrease in ^{14}C- fixation. Moreover, extract at high concentration (4 and 8%) caused considerable reduction in above parameters. PSI activity was either little or not affected with high concentration of aqueous extract. However, a substantial lowering in PSII activity was observed. The partial recovery of PS II activity by artificial electron donors (DPC, NH_2OH, and $MnCl_2$) suggests that the PS II inhibition might have occurred as a result of interruption in electron flow at water oxidation side of PSII as well as reaction centre. Study also reveals that in addition to growth promoting concentrations, the high concentrations (4% and 8%) of extract also support the biomass production of cyanobacterium, when exposed for longer duration.

Key Words: *Azadirachta indica*, Bioactive ingredients, Biopesticide, Photosynthetic electron transport, ^{14}C- fixation

INTRODUCTION

Rice fields in tropical and subtropical countries are the best habitats for a large number of heterocystous and non-heterocystous cyanobacteria (blue-green algae), which fix atmospheric molecular nitrogen in aerobic and micro aerobic conditions by utilizing light energy. The importance of cyanobacteria in improving soil fertility for sustainable agriculture in submerged and irrigated rice cultivation is well recognized (Venkataraman 1981; Goyal 1993; Saikia & Bordoloi 1994; Tiwari et al. 2000). Since time immemorial, cyanobacteria are exploited for nutrient recycling and maintenance of organic matter in soil. In recent years, the greater exploitation of cyanobacteria as potential biofertilizer for rice cultivation has become very promising. Such inoculation with free living cyanobacteria (algalization technology) has been started in several countries such as the Phillippines, Japan, India, etc., to improve the productivity of paddy and other crops (Martinez et al. 1981, Watanabe et al. 1981, Singh and Bisoyi 1989, Kulasooriya 1998, Soliman 2000). Despite the congenial condition for the growth of cyanobacteria in paddy fields, the population of cyanobacteria is regulated by a variety of biotic and abiotic factors. Several invertebrate grazers viz. *Cypris*, *Cyclops*, *Daphnia*, *Mesocyclops*, mosquito larvae etc. feed on cyanobacteria (Roger & Kulasooriya 1980), and limit their growth in

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rice fields. Decline in the rate of biomass production of cyanobacteria can cause considerable reduction in the level of nitrogen and organic matters in soil, as a consequence of this the paddy productivity declines significantly (Venkataraman & Rajyalakshmi 1971, and Kaushik & Venkataraman 1983). In addition, abiotic factors such as synthetic agrochemicals: weedicides, herbicides, insecticides, and chemical fertilizers are used to enhance crop yield, however, indiscriminate application of synthetic agrochemicals had adverse effects on key metabolic activities of cyanobacteria (Anand and Subramanian 1997, Ravindran et al. 2000; Prasad et al. 2005). The chemical pesticides not only create serious threat to the non-target organisms such as crops and beneficial microorganisms, but also enter into the food chain and affect humans. Furthermore, as a result of biological magnification the level of toxic chemicals may reach several times above the acceptable limit.

In view of above facts, extensive efforts are being made to evolve cheap, biodegradable and ecofriendly pesticides from biological origin. In recent years, several bioactive ingredients such as azadirachtin, nimbin, nimbolide etc. have been isolated from neem plants (Schmutterer 1990; 1995). However, the use of such unfriendly pesticides in commercial formulation in rural areas is still very limited and scanty, probably due to high cost and non-availability. In recent years the aqueous extracts of some plants have been used to check the growth of pests and vectors of dreaded diseases (Prasad et al. 2002; Sharma et al. 2003), which are also the grazers of cyanobacteria in aquatic bodies.The effect of aqueous extract of neem cake and seed on the growth of some cyanobacteria has also been demonstrated (Mishra & Adhikary 1997; Watanabe et al. 1981), but detailed study relating to key metabolic activity of cyanobacteria in the presence of bioactive substances is lacking. Therefore, in this paper an effort has been made to study the neem (*Azadirachta indica*) leaf extract induced effects on growth, pigments, photosynthetic oxygen yield, photosynthetic electron transport activity, and ^{14}C-fixation rate in a diazotrophic filamentous cyanobacterium *Nostoc muscorum*.

MATERIALS AND METHODS

Organism and growth conditions

Diazotrophic filamentous cyanobacterium *Nostoc muscorum* ISU (ATCC 27893), obtained through kind courtesy of Prof. L. C. Rai, BHU, Varanasi, was grown axenically in culture room at 27± 2°C in modified Chu-10 medium (Gerloff et al. 1950) under photosynthetic photon flux density (PPFD) of 75 µmol photon $m^{-2}s^{-1}$, provided by white fluorescent tube with 14:10 hour light-dark period. Each experiment was performed with exponentially grown culture.

Preparation of neem leaf aqueous extract

Leaves of neem (*Azadirachta indica* A. Juss.) were collected from nearby areas of Allahabad City and they were dried under shade. The powder was prepared with the help of electric mixer grinder and the aqueous extract from the powder was obtained following the method of Prasad et al. (2002). For the preparation of aqueous extract, the powder of neem samples was mixed with sterilized Chu-10 aqueous medium in a ratio of 1:20 (w/v). The suspension was stirred for 10 h and thereafter left for the next 48 h at room temperature. At the end, the extract was filtered twice with eight-fold muslin cloth and filtrate was kept at low temperature. The crude neem extract was diluted serially to obtain the desired concentrations of 1%, 2%, 4%, and 8% (v/v) by mixing the stock extract in culture medium.

Measurement of growth and photosynthetic pigments

The cyanobacterial cultures were grown in absence and in presence of various concentrations (1%, 2%, 4%, and 8%) of neem leaf aqueous extract for 20 d under 75 µmol photon $m^{-2}s^{-1}$ at 27±2°C. At regular intervals a known volume of cyanobacterial suspension was withdrawn from extract treated and untreated cultures and dry mass was determined. Chlorophyll and carotenoids were extracted with 80% acetone and the amount of chlorophyll *a* and carotenoids were quantified by using the formulae of Arnon (1949) and Goodwin (1954), respectively. Phycocyanin was extracted in 2.5 mM phosphate buffer (pH 7.0) by repeated freezing and thawing, and the amount was estimated following the method of Blumwald & Tel-Or (1982).

Assay of photosynthetic oxygen yield, ^{14}C-fixation and photosynthetic electron transport activity

Photosynthetic oxygen yield in whole cells, exposed to various concentrations of extract for 4 days, was measured polarographically using Clark type oxygen analyzer (Digital Oxygen System, Model-10, Rank Brothers, UK) in presence of 360 µmol photon $m^{-2}s^{-1}$ photosynthetic active radiation (PAR) light. Photosynthetic rate is expressed as µmol O_2 evolved (mg chl a)$^{-1}h^{-1}$. To study ^{14}C-fixation ability of these cells, 5 mL culture was added with required amount of $NaH^{14}CO_3$ (0.5 µCi μmol^{-1}) and then exposed to PAR light of 360 µmol photon $m^{-2}s^{-1}$ at 27°C for 5 min. The rate of ^{14}C-fixation was determined according to the method of Prasad & Zeeshan (2004).

The reaction was terminated by addition of 0.5 mL 2N HCl and then the reaction mixture was flushed with air to remove the unincorporated $^{14}CO_2$ from solution. The rate of incorporation of ^{14}C was estimated by counting the radioactivity in acid stable compounds using liquid scintillation counter (LKB Wallace 1209, Rockbeta).

Photosynthetic electron transport activity was measured in spheroplasts, prepared in HEPES-NaOH buffer (pH 7.5) following the method of Spiller (1980). PSI activity was assayed in terms of O_2 consumption in the reaction mixture containing 3-(3, 4-dichloro diphenyl)-1, 1, dimethyl urea (DCMU, 10µM), methyl viologen (MV, 0.1 mM), 2, 6 dichlorophenol indophenol (DCPIP, 0.05 mM), sodium ascorbate (ASC, 0.5 mM), and sodium azide (NaN_3, 0.05 mM). PS II activity was determined polarographically in terms of O_2 evolution, using para-benzoquinone (p-BQ, 1mM) as electron acceptor. PSII mediated DCPIP photoreduction was also measured spectrophotometrically by recording the change in absorbance at 600nm in the presence of artificial electron donors: Diphenylcarbazide (DPC), NH_2OH and $MnCl_2$, which donate electrons at specific sites of water oxidizing side of PSII. Whole chain electron transport activity was assayed in term of O_2 consumption in presence of 0.05 mM NaN_3 and 0.1mM MV. In each experiment, required amount of aqueous extract of neem leaves was added to the reaction mixture 15 min prior to the estimation of activity.

RESULTS AND DISCUSSION

The application of various formulations especially azadirachtin based products to control the population of pests of crops is widely accepted. However, their impacts on crops and microflora are not adequately evaluated. In the present study the effects of various concentrations (1%, 2%, 4%, and 8%) of aqueous extract of neem leaves on growth, photosynthetic pigments, photosynthetic oxygen yield, ^{14}C-fixation and photosynthetic electron transport activity in a heterocystous cyanobacterium *N. muscorum* were investigated. Growth of cyanobacterium was found to be stimulated following treatment with low concentrations (1% and 2%) of extract (Fig. 1a). Contrary to this, high concentrations (4% and 8%) caused significant reduction. Our results at low concentration of extract are consistent with the earlier finding (Mishra & Adhikary 1997), where the aqueous extract of neem cake and seeds supported the growth of *Anabaena variabilis* UU147, which could be due to presence of amino acids, fats, K, Mg, Fe, and Zn in extract. Beside this, the appreciable decrease in growth of test organism at high concentrations (4% and 8%) might have occurred as a result of toxic action of active ingredients present in extract. Similarly, some organophosphorus based chemical pesticides at low doses enhanced the growth of cyanobacteria, whereas high doses caused severe damage (Prasad & Zeeshan 2004). Like growth, 1% of extract enhanced the synthesis of chlorophyll *a* and phycocyanin, while 4% and 8% declined the pigment contents appreciably (Figs. 1b & c). Interestingly, the toxic potential of extract declined considerably following longer exposure, and even with 2% extract the cyanobacterium after initial decrease exhibited stimulation in biomass and pigment contents particularly chlorophyll *a* and phycocyanin (Figs. 1b & c). The appreciable decrease in toxicity could be correlated with partial degradation of active ingredients by cyanobacterium as reported for endosulfan in *Plectonema boryanum* and *Mucor thermo-hyalospora* (Shetty et al. 2000; Prasad et al. 2005). The varied responses of carotenoids to high concentrations of extract could be explained on the basis of their protective role in stress condition (Fig. 1d).

Results pertaining to photosynthetic oxygen yield and ^{14}C-fixation in intact cells, and photosynthetic electron transport activity in spheroplasts of *N. muscorum*, treated with 1%, 2%, 4%, and 8% of extract are depicted in Table1. Low concentrations 1% and 2% of extract caused slight stimulation in whole cell photosynthetic O_2 evolution, but the activity decreased significantly with high concentrations 4% and 8% of extract as photosynthetic oxygen yield declined by 14% and 31%, respectively. Similar to whole cell oxygen evolution, the extract at low concentrations (1% and 2%) caused marginal stimulation in PS I, PS II and whole chain activities. However, high doses 4% and 8% of extract declined the PS II activity by 22% and 40%, and the corresponding decrease for whole chain was 24%, and 44%, respectively. In order to point out the action mechanism of active ingredients (extract) on PS II activity, DCPIP photoreduction in extract treated spheroplasts was studied in presence of artificial electron donors: DPC, NH_2OH and $MnCl_2$. Results presented in Table 2 reveal that artificial electron donors partially relieved the PS II inhibition, and hydroxylamine (NH_2OH) appeared to be more efficient indicating active ingredients induced inhibitory action on PS II reaction center together with other sites of water oxidation side. Compared to PS II, PS I showed little response suggesting its resistant nature as reported for other stresses (Almog et al. 1991; Prasad & Zeeshan 2004). Inhibition of PS II and whole chain electron transport activity in *N. muscorum* at high dose was in consonance with the earlier finding, where PS II reaction centre and oxygen evolving complex has been shown to be highly labile or art to change to stress (Prasad & Zeeshan 2004; Prasad et al. 2005). Besides this,

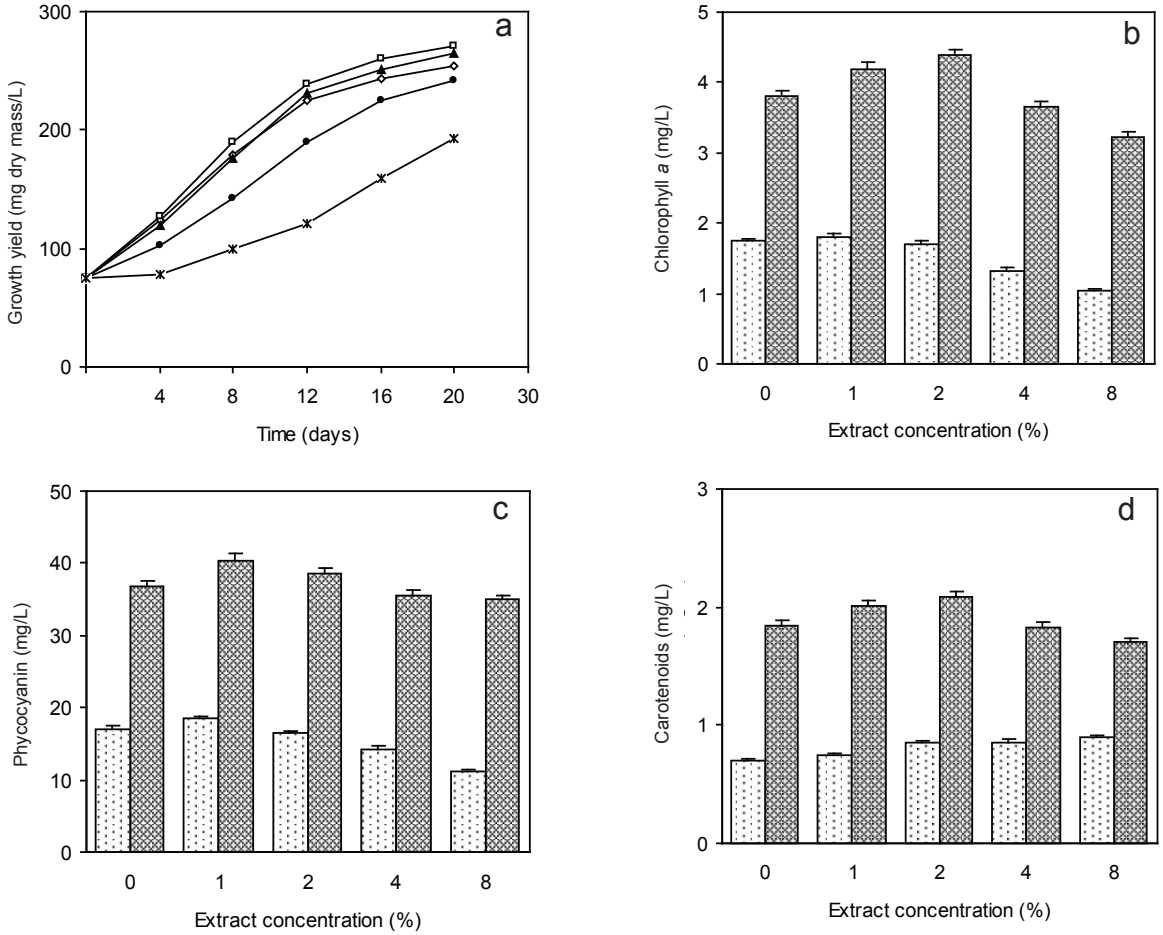

Figure 1. Effect of different concentrations of neem leaf aqueous extract on growth yields, chlorophyll *a*, carotenoid and phycocyanin contents of *N. muscorum*. Values are Mean ± SE with n=3. Photosynthetic pigments were estimated at 4th [▨] and 20th [▩] day of incubation. In fig.1a, the symbols ◇—◇, □—□, ▲—▲, ●—● and ×—× denote 0 (control), 1%, 2%, 4%, and 8% concentrations of aqueous extract, respectively

Table 1. Effect of neem leaf aqueous extract on photosynthetic O_2 yield, ^{14}C-fixation and photosynthetic electron transport activity of the cyanobacterium *Nostoc muscorum*

Treatment [extract (%)]	Photosynthetic yield [μmol O_2 evolved (mg Chl *a*)$^{-1}h^{-1}$]	^{14}C-fixation [CPM (mg Chl *a*)$^{-1}h^{-1}$ x 10^2]	Photosynthetic electron transport activity [μmol O_2 evolved/consumed (mg Chl *a*)$^{-1}h^{-1}$]		
			PSI (DCPIP/ASC→MV)	PSII (H_2O→p-BQ)	Whole chain (H_2O→MV)
0	246±2	714±6	475±3	312±1	156±1
1	256+3	761±7	479±4ns	318±1	163±1
	(+4)	(+7)	(+1)	(+2)	(+4)
2	253±3	683±5	484±3 ns	321±1	166±1
	(+3)	(-4)	(+2)	(+3)	(+6)
4	212±3	586±4	466±2 *	243±3	118±3
	(-14)	(-18)	(-2)	(-22)	(-24)
8	170±3	443±3	465±3 *	187±3	87±2
	(-31)	(-38)	(-2)	(-40)	(-44)

All the values are means ± SE. Data in parenthesis denote % stimulation (+)/inhibition (-) over control. All treatments are significantly different ($P < 0.01$) and ($P^* < 0.05$) from control (student's 't' test). Photosynthetic O_2 yield and ^{14}C-fixation was studied after 4 days of incubation with neem leaf aqueous extract. ns= not significant.

Table 2. Effects of various electron donors on restoration of PS II activity in spheroplasts of *Nostoc muscorum* exposed to different doses of neem leaf aqueous extract

Artificial electron donors	PS II activity [μmol DCPIP photoreduced (mg Chl *a*)$^{-1}$h^{-1}]		
	Extract concentration		
	0	4%	8%
Without donor	62±0.5	50±0.5	36±0.4
		-19	-42
DPC	93±0.8	82±0.7	60±0.6
		-12	-35
NH_2OH	72±0.5	67±0.4	53±0.4
		-7	-26
$MnCl_2$	66±0.5	57±0.5	38±0.3
		-14	-42

All the values are means ± SE. Data in parenthesis denote % inhibition over control. All treatments are significantly different ($P < 0.01$) from control (student's 't' test).

inhibition on PS II may also result due to arrest of electron flow at reducing side as reported for binding of herbicides to PS II reaction centre D1 protein at native binding site for plastoquinone (Trebst 1991). Similar to photosynthetic oxygen yield and electron transport activity, ^{14}C-fixation rate also decreased at high doses of extract and the intensity of inhibition was comparatively more than that of photosynthetic oxygen evolution in intact cells (Table1). Even the 2% extract caused marginal decrease in ^{14}C-fixation rate. At high concentration the decrease in ^{14}C- fixation rate can be explained on the basis of possible reduction in ATP and NADPH pool, resulted due to extract induced inhibition of photosynthetic electron transport activity (Table 1). Similar explanation has also been suggested for herbicide 2, 4-D and endosulfan induced inhibition of CO_2 fixation in earlier findings (Moreland 1980, Prasad et al. 2005). The greater inhibition of ^{14}C-fixation rate might have also occurred as a result of direct interaction of active ingredient of extract with rubisco and other Calvin cycle enzymes.

CONCLUSIONS

Neem leaf extracts could be used at low concentration to stimulate the biomass production of cyanobacteria as this concentration enhanced the photosynthetic rate of test cyanobacterium on one side, while on the other side, it successfully limits the growth of common grazers of cyanobacteria (Singh 2002). Study also pointed out that even damaging concentrations of neem extract support the growth of cyanobacterium after prolonged exposure and exhibited its ecofriendly nature. Furthermore, easy preparation and safer application suggest the greater practical importance of aqueous extract of neem. Thus, with the current thrust on sustainable agriculture and organic farming, the use of neem products has a greater practical significance especially in biomass production of cyanobacterial biofertilizer and ecologically sound pest management in rice cultivation.

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