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Full Length Research Paper

In vitro creation of artificial nitrogen fixing Cyanobacterium (*Nostoc muscorum*) association with wheat

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Using sonication technique and addition of 2,4 dichlorophenoxyacetic acid to wheat roots, increasing colonization of *Nostoc muscorum* on roots and enhancement of nitrogen fixation as novel associations between nitrogen fixing *Nostoc muscorum* and nonlegume wheat, had been occurred. Sonication of roots (10 and 20 s) enhanced abundance of *N. muscorum* within / on the root tissue.The *N. muscorum* penetrated the roots in the form of motile filaments (hormogonia) and once inside, they divided and transformed into aseriate packages. Sonication technique does not have significant effect on root growth, and there are no significant differences in shoot growth. Treatment of wheat roots with low concentrations of 2,4 dichlorophenoxyacetic acid (0.5, 1.0 and 3.0 ppm) developed nodule like structures (para-nodules) mainly along primary roots. *N. muscorum* colonized the paranodules externally at the junction of the paranodules. Addition of 2,4 dichlorophenoxyacetic acid significantly enhanced the rate of acetylene reduction of inoculated plants, and also abundance of *N. muscorum* on 2,4 dichlorophenoxyacetic acid induced wheat root compared to untreated wheat roots. Reduction of the growth of both root and shoot occured at a tested concentrations of 2,4 dichlorophenoxyacetic acid. Nitrogenase activity of *N. muscorum* co-cultivated with wheat roots was increased up to 1.9 times in the absence of combined nitrogen (nitrates) compared with nitrate treated plants, and also abundance of *N. muscorum* on roots increased in absence of combined nitrogen (nitrates). Results of this study revealed that *N. muscorum* is a promising organism for achieving efficient association between N_2 fixing cyanobacteria and nonlegumes by using sonication technique or 2,4 dichlorophenoxyacetic acid at low concentrations.

Key words: *Nostoc muscorum,* sonication, 2,4 dichlorophenoxyacetic acid (*2,4-D*), nonlegume.

INTRODUCTION

N_2 fixing cyanobacteria, particularly with the genus *Nostoc*, still enter into intimate relationships with some eukaryotic organisms that represent all plant divisions (Rai et al., 2000). Zeman et al. (1992) induced formation of para-nodules in the wheat and colonized it with *Azospirillum* that differentiates specialized cells called heterocysts, which protect nitrogenase against inactivation by oxygen.

Increasing the biological nitrogen fixation (BNF) is one of the main problems in the future. There are different possible solutions for extending BNF to economically important plant, as follows: (a) transformation the nitrogen-fixation genes (*nif* genes) into the plant genome, (b) increasing the efficiency of the existing symbiosis, (c) extending the host specificity of rhizobia, and (d) establishment of new artificial associations. At present,

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there is a great interest in establishing novel associations between higher plants particularly to cereals and a variety of nitrogen fixing cyanobacteria (e.g. *Nostoc* or *Anabaena* strains). The creation of novel symbioses with N_2 fixing microorganisms may provide an alternative method to the introduction of isolated *nif* genes.

Cereal grains and derivative products have an importance as they are consumed by millions of people and are considered the primary source of carbohydrates for humans and farm livestock. Among cereal products, bread is significant due to the fact that it provides more nutrients to the world population than any other single food source (Pomeranz, 1987).

One strategy has been to use the auxin 2,4 dichlorophenoxyacetic acid (2,4-D) to induce the formation of roots of tumor-like growth, called para-nodules have been created in wheat and shown to become infected with *Azospirillum* (Zeman et al., 1992; Chen et al., 1992; Yu and Kennedy, 1995), *Herbazospirillum* with wheat and corn (Abdel-Wahab et al., 1995), nitrogen fixing cyanobacterium *Nostoc* sp strain 2S9 B (Gantar and Elhai, 1999). El-Shahed and Abdel-Wahab (2006) showed that co-cultivation of *Nostoc rivulare* and the tested plant exhibited nitrogenase activities both in the presence or absence of 2,4-D (1 ppm) but results also indicated that addition of 2,4-D (1 ppm) significantly enhanced nitrogenase activity more than those non-treated with 2,4-D.

Additionally, Gantar (2000b) showed that mild sonication of the roots of wheat dramatically increased the number of cyanobacteria within the root tissue. Nitrate addition to N_2-fixing cyanobacteria cultures inhibits or reduces nitrogenase activity. Recently, El-Shahed and Abdel-Wahab (2006) showed that co-cultivation of *N. rivulare* and the tested plant exhibited nitrogenase activities both in the presence or absence of nitrate but results also indicated that addition of nitrates significantly reduced nitrogenase activity. This association depends on plant cultivars and cyanobacterial strains as well as nitrogen and pesticides application in liquid and soil cultures (Gantar et al., 1991a, 1991b; Spiller et al., 1993; Patnaik et al., 1994).

The present study aims at evaluation of the ability of *Nostoc muscorum* to colonize and fix atmospheric nitrogen within para-nodules induced by 2,4-D in wheat, increasing of openings on wheat roots by sonication to facilitate the colonization of the wheat endorhizosphere by *N. muscorum*.

MATERIALS AND METHODS

Hydroponic growth conditions

The method described by Gantar and Elhai (1999) was used for growing wheat in BG_{11} medium (Rippka and Herdman, 1992). Germinated wheat seeds were grown in sterile 250 ml glass beakers instead of tested tubes. 250 ml of sterile hydroponic solution (BG_{11}) was added to each beaker containing sterile foam rubber plate previously perforated with a cork porer to support the wheat seedling as illustrated in Plate (1a). This medium was used for testing the effect of combined nitrogen after the addition of $NaNO_3$ at a rate of 1.5 g/L.

Host plant

Seeds of wheat (*Triticum aestivum* L.), were first rinsed five times in sterile distilled water prior to surface sterilization. After two minutes, seeds of selected plants were sterilized with a mixture of 1:1 solution of hydrogen peroxide (30%) and ethyl alcohol (70%), then they were rewashed with the distilled water and germinated on a sterile filter paper in sterile 9 cm Petri dishes for 2 to 3 days. Uncontaminated seedlings were transferred and grown in glass beakers.

Preparation of *N. muscorum* inoculum in liquid culture

N. muscorum is cultivated in BG_{11} medium for 20 days. Cyanobacterial cultures then harvested by centrifugation (8000 rpm for 10 min) and suspended in sterile H_2O (4g/250 ml), the inoculums size per seed is 10^6 heterocysts, and was counted by haemacytometer (El-Shahed, 2005).

Sonication of roots

In order to study the effect of sonication on cyanobacteria colonization of wheat root, the described method by Ganter (2000b) was used. Wheat grains were left to germinate on wet sterile filter paper in Petri dishes for 3 days in dark. Four germinated grains were transplanted into a beaker containing 250 ml of BG_{11} medium (hydroponic solution). Beakers were divided into six groups with three replicates for each treatment. When wheat roots were about 8 cm long, they were sonicated in an ultrasonic bath using sonicator (Cole Parmer, Chicagoi, Illinois 60648) by placing the plants directly into bath filled with BG_{11} medium. The roots were sonicated for 10 and 20 s, this was followed by inserting the seedlings into fresh BG11 medium and inoculation with *N. muscorum* at light intensity of 300 lux and room temperature for 20 days. When tested seedlings were 20 days old, seedlings harvested, growth parameters (represented in length of both root and shoot), nitrogenase activity and cyanobacterial abundance on root (Chl. a content) were determined.

Induction and colonization of para-nodules

The described method by Ganter and Elhai (1999) and El shahed and Abdel-Wahab (2006) was used. When the roots of wheat seedlings were about 5 cm length, aliquots of *N. muscorum* culture was added to the beakers containing 250 ml hydroponic solution to maintain a cell density of 10^6 heterocysts per seedling. Aliquots of sterile 2,4-D solution were also added to give a final concenteration of 0.5, 1.0 and 3.0 ppm and hence beakers were divided into five groups with three replicates. Non-inoculated and inoculated beakers without 2,4-D were used as controls at continous light intensity of 300 lux at room temperature for 15 days. After five days from treatment with 2,4-D, para-nodules were well formed on the root. Roots were examined and photographed using phase contrast microscope. When wheat seedlings were 15 days old, seedlings were harvested, growth parameters (represented in length of both root and shoot and weights of both root and shoot), nitrogenase activity and cyanobacterial abundance on root (Chl. a content) were determined.

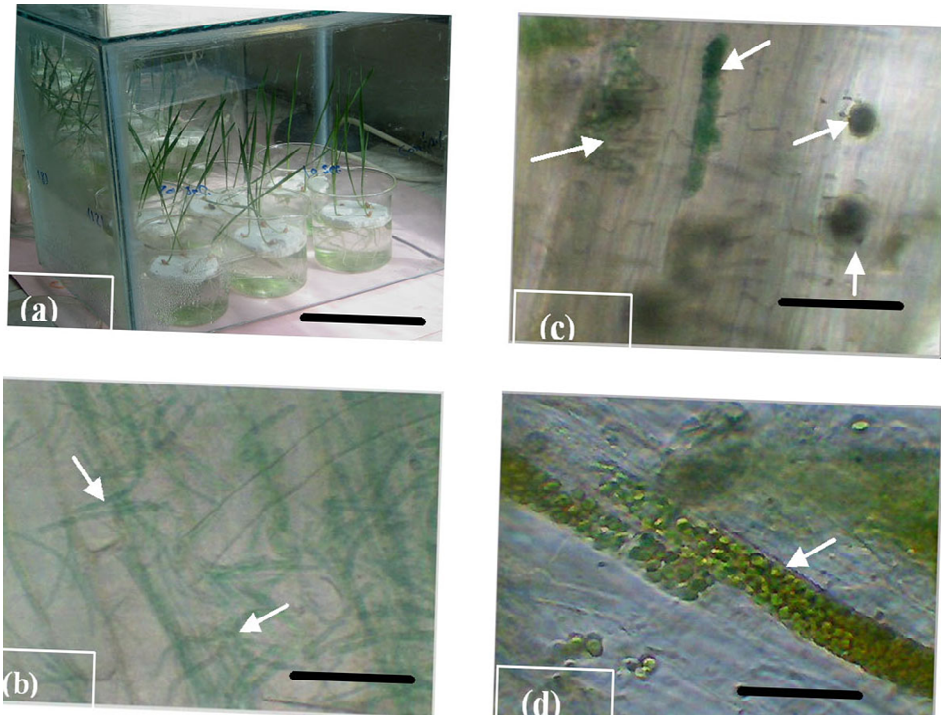

Plate 1. (a) Liquid culture for wheat growth and cyanobacterial inoculation. Bar = 7 cm. (b) Surface view of wheat root colonized by *N. muscorum* as filaments after 6 days from inoculation. Bar = 0.5 mm, (c) Surface view of a wheat root colonized by *N. muscorum* forming a tight association after 12 days from inoculation Bar =20 µm, (d) Intracellular association of *Nostoc muscorum* with epidermal cells of wheat roots. Arrows indicate cells containing associated cyanobacteria as cyanobacterial mass Bar = 50 µm.

Tetrazolium salt staining and microscopic examination

Para-nodules seedlings were incubated overnight with a solution of 0.025% triphenyltetrazolium chloride (TTC) to locate the site of strong reduction, such sites were detected and examined using a Kyowa, Japan, dissecting stereomicroscope.

Determination of cyanobacterial abundance on wheat root

The total cyanobacterial biomass in the root was determined as chlorophyll a content. The colonized roots were extracted in acetone 85% and chlorophyll a was estimated spectrophotometrically at 663 nm (Mackinney, 1941) and expressed as a root weight basis.

Nitrogenase assay of *N. muscorum* associated with roots of wheat *in situ*

Nitrogenase activity was assayed by acetylene reduction assay (ARA) using ATIUNI CAM 610-GLC (UK) equipped with a glass column filled with activated alumina. Each seedling had its remaining seed detached a septically and its root non washed in sterile nitrogen free mineral solution (Tchan and New, 1984) and was transferred to a 15 ml serum bottle containing 2 ml of that mineral solution. The serum bottles were stopped with sterile rubber stoppers and 10% of the gas reaction mixture was replaced with acetylene and inoculated at 30°C for 2 h. Results were expressed as nmole of C_2H_4 per g of root.

Statistical analysis

The triplicate sets of data for the various parameters evaluated were subjected to analysis of variance (ANOVA) in accordance with the experimental design (completely randomized design) using SPSS11 statistical packages to quantify and evaluate L.S.D. values were calculated at P level of 0.05% (Steel and Torrie, 1960).

RESULTS

Colonization of *N. muscorum* to wheat in hydroponic solution

N. muscorum isolated from clay soil collected from El Minia Governorate, *N. muscorum* was tested for their ability to colonize the roots of wheat plant in hydroponic solution. Results Plate (1b) showed that *N. muscorum* has the ability to colonize the roots of wheat. Tight association was observed in case of *N. muscorum*, our results in Plate (1c) showed that tightly packed filaments of the *N. muscorum* forming aseriate packages on a root surface. The first stage of colonization of wheat roots by *N. muscorum* was probably the migration of hormogonia, then hormogonia develobed into long filamentous as in Plate (1b), and the long filaments developed of the

Table 1. Effect of *N. muscorum* inoculation on length of wheat plant in hydroponic solution at different sonication times.

Treatment	Sonication time (s)	Height (cm)	
		Root	Shoot
Control	0	9.3 ± 0.6	25.0 ± 0.7
	10	9.5 ±1.0	25.0 ± 0.6
	20	10.0 ± 0.8	24.9 ± 0.3
N. muscorum	0	10.0 ± 0.9	26.0 ± 0.5
	10	11.5 ± 0.4	26.5 ± 0.8
	20	10.5 ± 0.2	26.8 ± 0.9

±mean standard deviation.

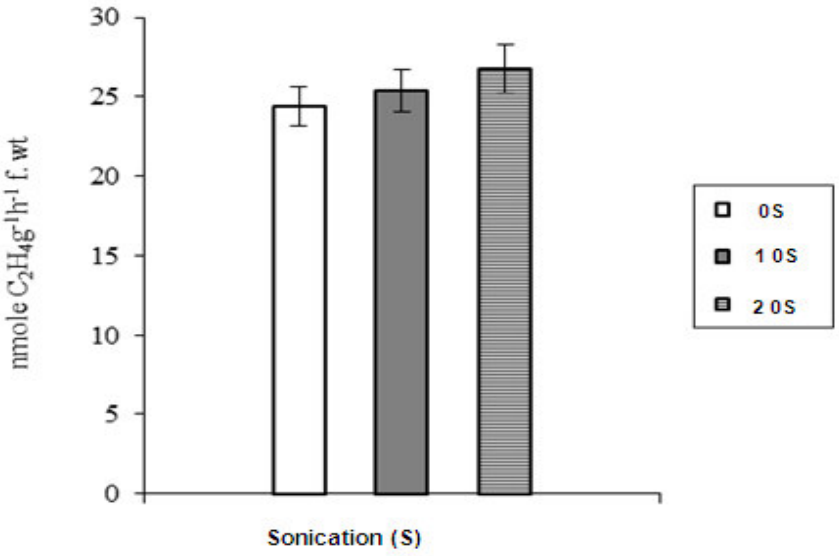

Figure 1. Effect of sonication on nitrogenase activity (*In situ*) of *N. muscorum* associated with wheat roots.

aseriate stage which consisted of filaments tightly packed in a mucilaginous sheath. Addition to form a tight association, *N. muscorum* also appeared to penetrate some root cells as cyanobacterial mass (Plate 1d).

Part 1: Sonication of wheat roots and its infection with *N. muscorum*

Effect of sonication on colonization and nitrogenase activity (in situ) of N. muscorum associated with wheat in hydroponic solution

Data presented in Table 1 showed that sonication treatments (10 and 20 s) in control plants did not significantly affect wheat growth. However, inoculation of sonicated wheat with *N. muscorum* improved the growth of wheat compared with control plants. Results in Figures 1 and 2 showed that nitrogen fixing capacity and cyanobacterial abundance were higher in case of plants sonicated and inoculated with *N. muscorum* than non-sonicated treatments. Nitrogenase activity and cyanobacterial abundance were increased by increasing the time of sonication up to zero.

Part 2: Para-nodule induction in wheat with 2,4-D and its infection with *N. muscorum*

Effect of different 2,4-D concentrations and inoculation with N. muscorum on growth of wheat seedlings (15 days old)

Results in Table 2 showed that treatment of seedlings of wheat with 2,4-D influenced the growth of both root and

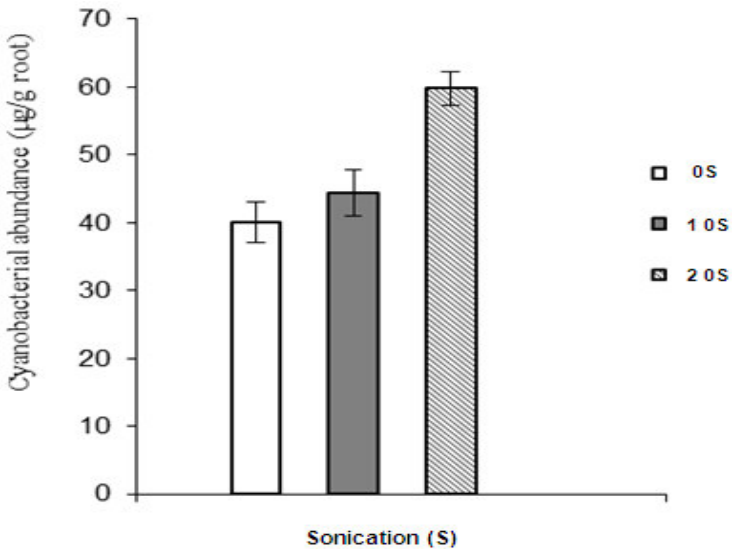

Figure 2. Effect of sonication on colonization of *N. muscorum* associated with wheat roots.

Table 2. Effect of different 2,4-D concentrations and inoculation with *N. muscorum* on growth of wheat seedlings (15 days old).

Treatments	Growth of wheat with *N. muscorum*					
	Root			Shoot		
	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)
control	9.3	41.1	4.0	20.5	131.7	21.6
Inoculum	7.6	95.7	4.0	24.2	140.0	22.3
0.5 ppm (2,4-D)	6.4	53.3	4.8	21.4	132.5	22.6
1.0 ppm (2,4-D)	6.2	51.4	4.8	19.4	115.7	26.3
3.0 ppm (2,4-D)	6.0	56.5	5.0	17.5	138.0	22.5
L.S.D (5%)	1.17	28.0	2.1	1.9	32.0	1.2

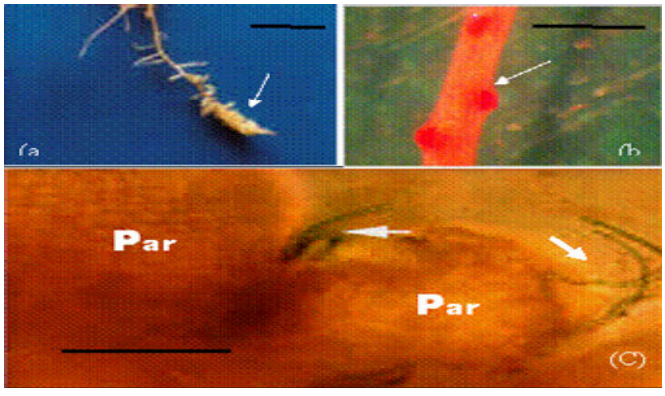

Plate 2. (a) Para-nodules developed at the tip of main root. Bar = 1 mm, (b) Root stained with TTC showing reduction in paranodules, Bar = 2 mm, (c) Colonization of paranoduled wheat plants at the junction of the para-nodules (Para) with the root and at the top, Bar =50 µm.

shoots. Applications of 2,4-D also induced formation of para-nodules as in Plate 2a. Root elongation, shoot length of both wheat and lateral root formation were strongly inhibited by different concentration of 2,4-D compared with control plants (2,4-D non-treated plants). Results in the table also showed that inoculation of tested seedlings with *N. muscorum* significantly increased weights of both root and shoot of wheat seedlings as well as plant height compared with uninoculated seedlings.

Nitrogenase activity (in situ) of and N. muscorum associated with wheat at different 2,4-D concentrations (15 days old)

Data presented in Figure 3 showed that nitrogenase activity assayed by acetylene reduction assay of *N. muscorum* associated with wheat plants treated with 2,4-

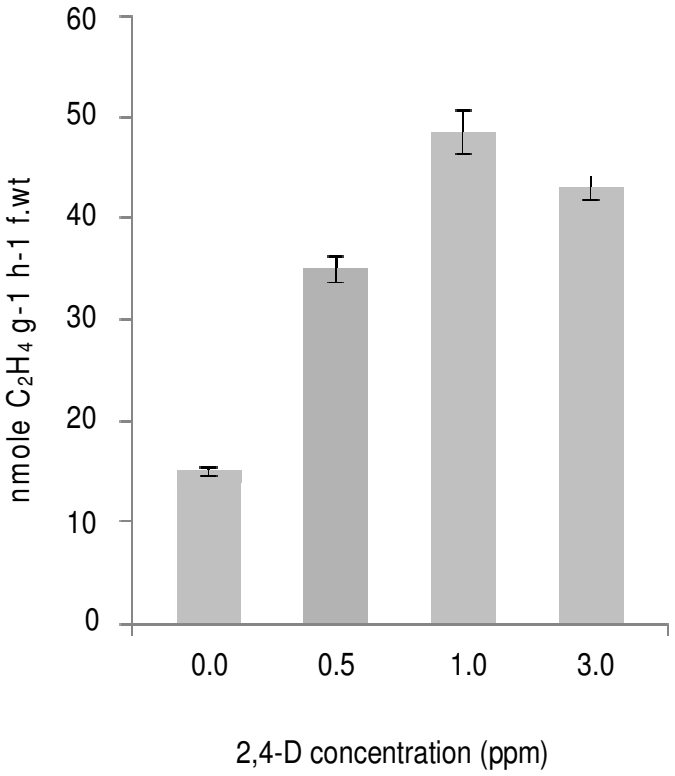

Figure 3. Nitrogenase activity (*in situ*) of *N. muscorum* associated with wheat at different concentrations of 2,4-D.

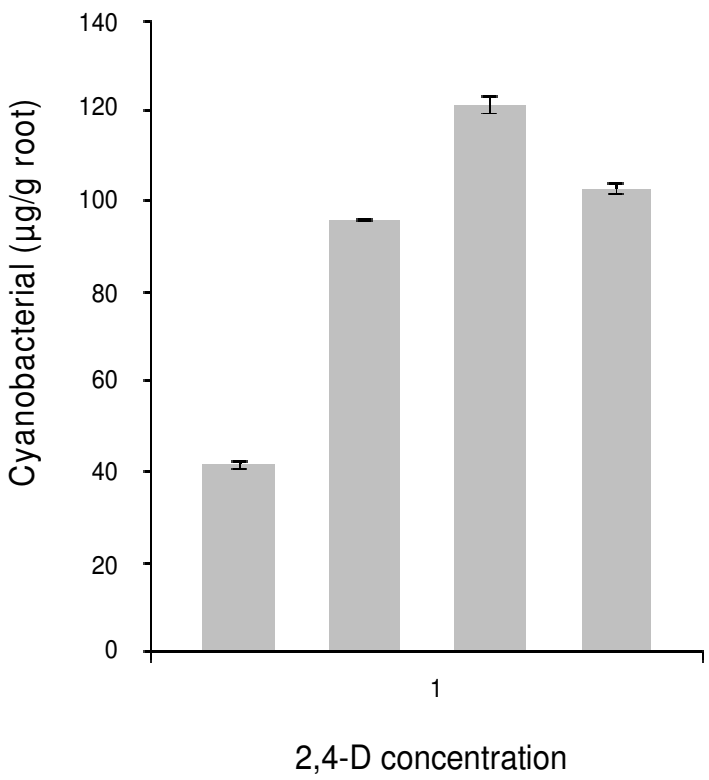

Figure 4. Abundance of *N. muscorum* colonized on wheat roots at different concentrations of 2,4-D.

D was higher than those nontreated with 2,4-D. Generally, nitrogenase activity increase in the presence of 2,4-D.

Abundance of N. muscorum (chlorophyll a content) colonized wheat roots at different concentrations of 2,4-D (15 days old)

Data presented in Figure 4 showed that colonization of *N. muscorum* to wheat roots enhanced by increasing 2,4-D concentration as compared with inoculated roots and not treated with 2,4-D.

Effect of different 2,4-D concentrations on numbers of para-nodules of wheat seedlings inoculated with N. muscorum

Results of our study indicate that para-nodules were developed mainly on the main roots as swollen projections after 5 days of 2,4-D treatments. The greatest number was found at the tip of the root (Plate 2a). Data presented in Figure 5 also showed that high numbers of para-nodules (per plant) were obtained at the range of 0.5 to 1.0 ppm 2,4-D. The application of 2,4-D at a rate of 1.0 ppm did not repress plant development.

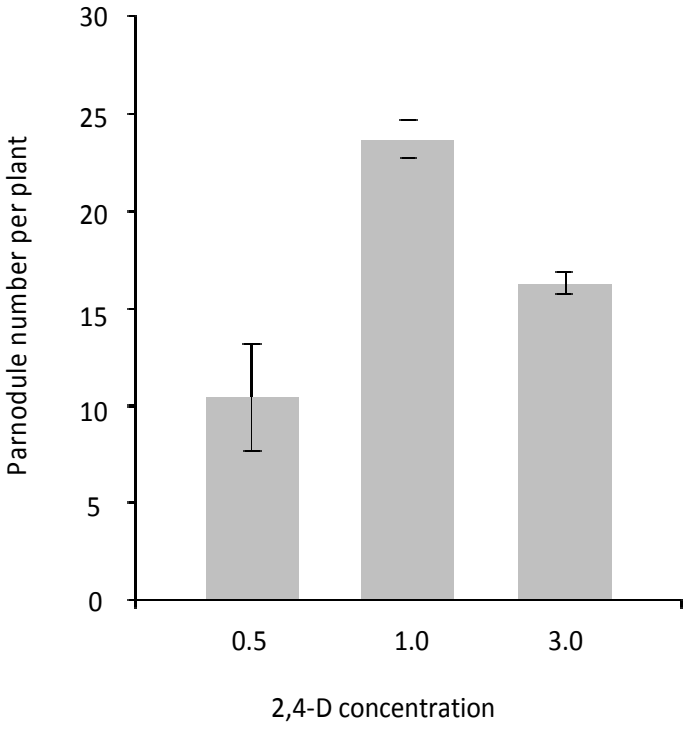

Figure 5. Effect of different concentrations of 2,4-D on numbers of paranodules of wheat seedlings inoculated with *N. muscorum*.

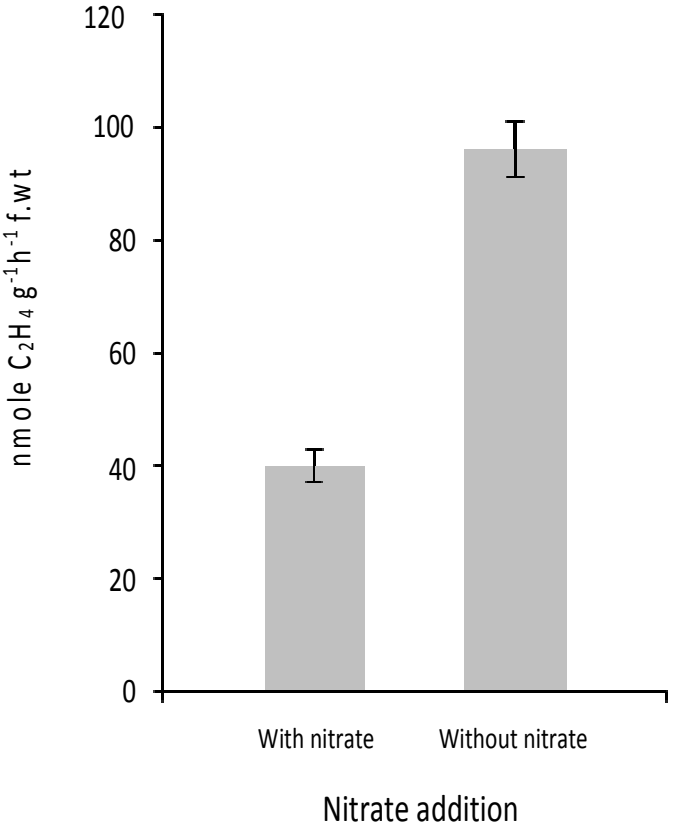

Figure 6. Nitrogenase activity of *N. muscorum* colonized wheat roots as affected by the presence or absence of nitrate.

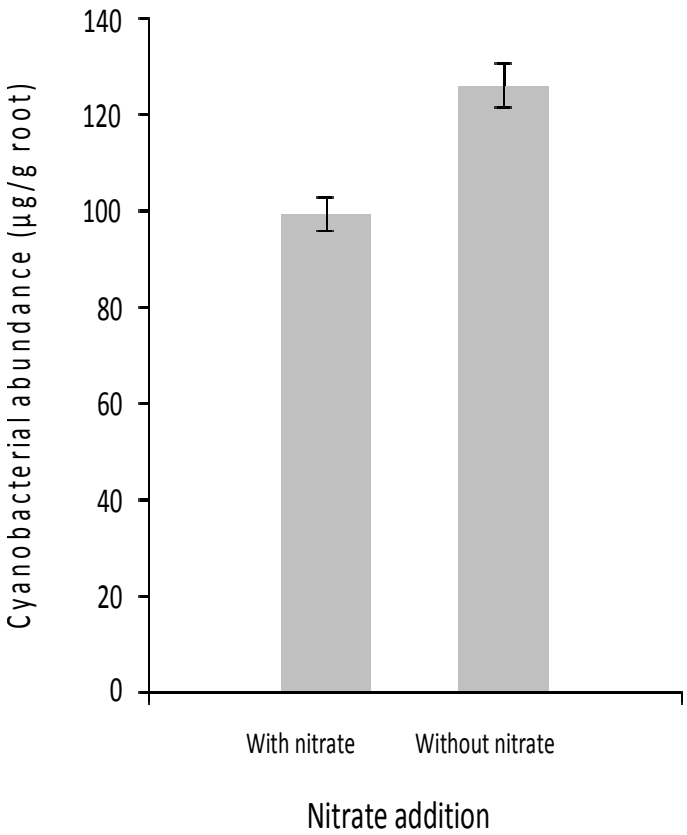

Figure 7. Abundance of *N. muscorum* colonized wheat roots as affected by the presence or absence of nitrate.

Colonization of para-nodules of wheat with N. muscorum

When nodulated seedlings were inoculated with *N. muscorum* and were inoculated overnight in a solution of 0.025% triphenyltetrazolium chloride (TTC), the whole para-nodule structure was stained red indicating that these structures had been colonized by *N. muscorum* which found a possible better site for N2 fixation as indicated in (Plate 2b).

Light microscopy examination revealed that *N. muscorum* colonized the para-nodules externally at both the basal connection between the nodule and the root and at the top of the nodules as loosely arranged filaments as indicated in (Plate 2c).

Nitrogenase activity of N. muscorum colonized wheat roots (treated 1 ppm 2,4-D) as affected by the presence or absence of nitrates

Results in Figure 6 showed that co-cultivation of wheat seedlings with *N. muscorum* exhibited nitrogenase activity in the presence or absence of nitrate. Results also indicated that absence of nitrate caused a large increase in nitrogenase activity with *N. muscorum*. Nitrogenase activity of wheat co-cultivated with *N. muscorum* was increased up to 1.9 times in absence of nitrate compared with nitrate treated wheat. Results in Figure 7 also showed that colonization of *N. muscorum* to wheat increase in absence of nitrate compared with in the presence of nitrate.

DISCUSSION

Bonoett and Silvester (1981) reported an association between the higher plant Gunnera and members of the genus *Nostoc*. Results of the current study showed that inoculation of wheat plant grown in liquid culture with *N. muscorum* improved wheat growth, and was agreed with previous studies (Obreht et al., 1993; Rai et al., 2000; Whitton and potts, 2000). In addition to its ability to fix and supply nitrogen to plants, cyanobacteria have been shown to produce compounds that stimulate the growth of plants. Najappan et al. (2007) evaluated the potential of plant growth promoting cyanobacteria as biofertilizer for wheat. *N. rivulare* is characterized by its ability to form tight association with wheat roots and significantly improve wheat growth (El-Shahed, 2005; El-Shahed and

Abdel-Wahab, 2006). In a previous study by Sergeeva et al. (2002), it was proven that cyanobacteria have the ability to produce phytohormone IAA. We described the association between cyanobacterial isolate and wheat grown in liquid culture in which there is tight association. This type of association previously reported for wheat (Gantar et al., 1991a, b; Spiller et al., 1993; Gantar, 2000a).

Tight association may be related to characteristic developmental life cycle of *Nostoc* heterocyctous filaments, hormogonia and an aseriate stage that consists of heterocystous filaments packaged tightly together (Lazaroff, 1973). Other factors must be important, these could involve recognition of specific molecules including lectins (Mellor et al., 1981; Ladha and Watanabe, 1984; McCowen et al., 1987) as well as root exudates. Polysaccharides of plant and cyanobacterial origin may play an important role in the attachement of cyanobacteria to roots, as has been previously shown for the attachment of cyanobacteria to plants cells and inert surfaces (Robins et al., 1986).

To facilitate the colonization of cyanobacteria root system of higher plants, sonication technique increases number of openings to the root surface and hence root colonization increases. Our study indicated that mechanical damage of the wheat roots (roots sonication at 10 and 20 s) increased colonization of *N. muscorum* on wheat roots. Our study is in agreement with Gantar (2000b) reported that roots sonication provided a geater number of openings on the roots surface than other treatments, eventually leading to a more abundant presence of cyanobacteria. Bacteria are found together with cyanobacteria and it is possible that penetration is facilitated by the production of hydrolytic enzymes of bacterial origin (Ozawa and Yamaguchi, 1980). Indeed, a method has been employed to introduce rhizobia into roots of rice seedlings, without need for plant tissue culture, relies on treatment of the plants with hydrolytic enzymes and polyethylene glycol (Al-Mallah et al., 1989). Our results also showed that N_2 fixing activity of both *Nostoc* sp. increased with increasing of sonication time, as the result of increasing of colonization on wheat roots with soinication. Mild sonication of roots should be considered as a method for creating artificial plant cyanobacteria association (Gantar, 2000b).

Preliminary studies showed that germinating wheat seedling treated with high concentration of 2,4-D (5 ppm) alone exhibited fungal contamination compared with control plants in spite of sterilization of seeds with mixture of ethyl alochol and hydrogen perioxide (El-Shahed and Abdel-Wahab, 2006). This may be due to that at high 2,4-D concentration. 2,4-D treated seedlings showed symptoms of disorder and their root development was severely affected and thus became susceptible to fungal contamination. Thus, 2,4-D was applied up to the concentration of 3 ppm for the rest of the other plant in all our experiments.

Additionally, the present results showed that the auxin 2,4-D increases the colonization of roots of wheat with *N. muscorum*, and also increased nitrogenase activity. These results are in accordance with the earlier findings of Gantar and Elhai (1999), El-Shahed and Abdel-wahab (2006) and Nilsson et al. (2002) who established that the auxin 2,4-D increased colonization of cyanobacterial on root as well as nitrogenase activity. The stimulatory effect of 2,4-D on colonization of cyanobacteria on root and nitrogenase activity specially in the absence of nitrates could be explained on the basis that either a) the auxin increases amounts of cyanobacteria bound to the root surfaces and thus the extent of N2 fixation as well or b) 2,4-D had induced para-nodules on plant roots and these could have provided suitable sites for cyanobacterial colonization. A previous study (El-Shahed, 2005), showed that co-application of *N. rivulare* and 2,4-D (0.2 ppm) significantly enhanced nitrogenase activity recording two fold increase as compared with the application of cyanobacteria alone. Moreover, Gantar and Elhai (1999); recently El-Shahed and Abdel-Wahab (2006) reported clearly increase in nitrogen fixing activity when wheat seedlings were treated with 2,4-D and co-cultivated with *N. muscorum*, than untreated but colonized roots.

Using light microscopic technique, our data revealed that *N. muscorum* successfully colonized para-nodules externally both at their basal connection with the root and on the epidermal root surfaces. Recently El-Shahed and Abdel-Wahab (2006) and Gantar and Elhai (1999) who established that the cyanobacteria penetrated wheat para-nodules by migrating in between loosely arranged cells that covered their surfaces or by penetrating the spaces at the junction of root and para-nodules.

Stimulatory effect of the absence of nitrate on nitrogenase activity that is frequently attributed to members of the Nostocales could be related to the differentiation of more heterocysts that protect nitrogenase from inactivation by oxygen and thus increase their N2 fixing capacity (Flores and Herro, 1994). Results of the present study demonstrated that wheat para-nodules can be efficiently colonized by *N. muscorum* providing favourable conditions for N2 fixation. Thus, it appears that *N. muscorum* is a promising organism for achieving efficient association between cyanobacteria and non-legume plants. However, further studies on these relationships will promote the practical application of para-nodules for improving the nitrogen nutrition of cereals.

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REFERENCES

Abdel-Wahab AM, El-Komy HM, Sholkamy MA (1995). Para-nodule induction in wheat with *2,4-D* and its infection with *Herbaspirillum* sp. in 10th International Colloquim for the optimization of plant nutrition. April 8-13, Cairo-Egypt.

Al-Mallah MK, Davey MR, Cocking EC (1989). Formation of nodular structures on rice seedlings by rhizobia. J. Exp. Bot., 40: 473-478.

Bonoett HT, Silvester WB (1981). Specificity in the *Gunnera-Nostoc* endosymbiosis. New Phytol., 89: 121-128.

Chen TW, Scherer S, Bosger P (1992). Nitrogen fixation of *Azorhizobium* in artificially induced root para-nodules in wheat. Science in China, (series B), 35: 1463-1470.

El-Shahed AM, Abdel-Wahab MA (2006). Para-nodule induction in wheat, maize and rice with *2,4-D* and its infection with *Nostoc rivulare* Kutzing. Pakistan J. Biol. Sci., 9: 1693-1699.

El-Shahed AM (2005). Interaction of *2,4-D* with a nitrogen fixing *Nostoc rivulare* Kutzing associated with wheat plants. Bull. Fac. Sci. Assiut Univ., 15: 123-132.

Flores E, Herro A (1994). Assimilatory nitrogen metabolism and its regulation, In: The molecular biology of cyanobacteria. Bryant DA (ed.). Kluwer Academic Publishers, Dardrech, The Netherlands, pp. 487-517.

Gantar M (2000a). Co-cultivation of N2-fixing cyanobacterium *Nostoc* sp. strain 2S9B and wheat callus. Symbiosis, 29: 1-18.

Gantar M (2000b). Mechanical damage of root provides enhanced colonization of wheat endorhizosphere by N2-fixng cyanobacterium *Nostoc* sp. strain 2S9B. Biol. Fertil. Soils, 32: 250-255.

Gantar M, Elhai J (1999). Colonization of Wheat Para nodules by the N2- fixing Cyanobacterium *Nostoc* sp. strain 2S9B. New Phytol., 141: 373-379.

Gantar M, Kerby NW, Obreht Z, Rowell P (1991a). Colonization of wheat (*Triticum Vulgare* L.) I. A. Survey of soil cyanobacterial isolates forming association with roots. New Phytol., 118: 447-483.

Gantar M, Kerby NW, Rowell P (1991b). Colonization of wheat (*Triticum vulgare*) by N2-fixing cyanobacteria: II. An ultrastructural study. New Phytol., 118: 485-492.

Ladha JK, Watanabe I (1984). Antigenic similarities among *Anabaena azolla* separated from different species of *Azolla*. Biochem. Biophys. Res. Commun., 109: 675-682.

Lazaroff N (1973). Photo-morphogenesis and Nostocacean development. In: The biology of blue green algae, Carr NG, Whitton BA (eds.). Oxford UK Blackwell Scientific Publications, pp. 273-319.

Mackinney G (1941). Absorption of light by chlorophyll solutions. J. Biol. Chem., 140: 315-322.

McCowen SM, Macarthur L, Gates JE (1987). *Azolla* fern lectins that specifically recognize endosymbiotic cyanobacteria. Curr. Microbial., 14: 329-333.

Mellor RB, Gadd GM, Rowell P, Stewart WDP (1981). A phyto-hemagglutinin from *Anabaena-Azolla* symbiosis. Biochem. Biophys. Res. Commun., 99: 348-353.

Nanjappan K, Radha P, Lata Nain, Brahama DK (2007). Evaluation the potential of plant growth promoting cyanobacterial as inoculants for wheat. Indian Agricultural Research institute (IARI) European J. Soil Biol., 43: 23-30.

Nilsson M, Bhattacharya J, Rai NA, Bergman B (2002). Colonization of roots of rice (*Oryza sativa*) by symbiotic *Nostoc* strains. New Phytol., 156: 517-525.

Obreht Z, N. Kerby W, Gantar M, Rowell P (1993). Effect of root-associated N2-fixing cyanobacteria on the growth and nitrogen content of wheat (*Triticum vulgare*) seedlings. Biol. Fert. Soils., 15: 68-72.

Ozawa T, Yamaguchi M (1980). Increase in cellulase activity in cultured soybean cells caused by *Rhizobium japonicum*. Plant Cell Physiol., 21: 331-337.

Patnaik GK, Kanungo PK, Rao V (1994). Interaction of *2,4-D* with nitrogen fixing bacterial populations and nitrogen fixation associated with rice. Microbiol. Res., 149: 291-295.

Pomeranz Y (1987). Modern Cereal Science and Technology. VCH New York VCH, p. 258.

Rai AN, Soderback E, Bergman B (2000). Tinsley Review: cyanobacterium–plant symbioses. New Phytol., 147: 449–481.

Rippka R, Herdman M (1992). Pasteur Culture Collection of Cyanobacterial Strains in Axenic Culture. Catalogue of strains. Institut Pasteur, Paris, France, 1: 103.

Robins RJ, Hall DO, Shi DJ, Turner RJ, Rhodes MJC (1986). Mucilage acts to adhere cyanobacteria and cultured plant cells to biological and inert surfaces. FEMS Microbiol. Lett., 34: 155-160.

Sergeeva E, Liaimer A, Bergman B (2002). Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. Planta, 38 (2): 215-229.

Spiller H, Stallings JW, Woods T, Gunasekaran M (1993). Requirement for direct association of ammonia-excreting Anabaena uariabilis mutant (SA-1) with roots for maximal growth and yield of wheat. Appl. Microbiol. Biotechnol., 41: 557-566.

Steel RG, Torrie JH (1960). Principles and procedures of statistics McGraw-Hall Book Co. New York.

Tchan YT, New PB (1984). Azotobacteriaceae, In: Bergy`s manual of Systematic Bacteriolgy, Williams and Wikins (eds.), Baltimore, pp. 220-230.

Whitton BA, Potts M (2000). The ecology of cyanobacteria, In: Their diversity in time and space. Dordrecht, Kluwer L (Eds.) I A survey of soil cyanobacterial isolates forming associations with roots. New Phytol., 118: 477-483.

Yu D, Kennedy IR (1995). Nitrogenase activity (C2H2 reduction) of *Azorhizobium* in *2,4-D*-induced root structures of wheat. Soil Biol. Biochem., 27: 459-462.

Zeman AMM, Tchan YT, Elmerich C, Kennedy IR (1992). Nitrogenase activity in wheat seedlings bearing para-nodules induced by 2,4dichlorophenoxyacetic acid (2,4-D) and inoculated with *Azospirillum*. Research in Microbiology Institut Pasteur Elsevier, 143: 847-855.