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Full Length Research Paper

Potential role of *Nostoc muscorum* and *Nostoc rivulare* as biofertilizers for the enhancement of maize growth under different doses of n-fertilizer

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Strains of the genera *Nostoc* (7 species) and *Phormidium* (one species) were isolated from soil. Cyanobacterial isolates were tested for their ability to form associations with the roots of wheat seedlings grown in liquid culture as well as fixing of atomospheric N_2. The present study revealed that *Nostoc* colonization of wheat was tight association while *phormidium* colonization was loose association. In case of *Nostoc* association, its growth was in the form of aseriate packages on root surface. Moreover, our study reported that *Nostoc muscorum* isolate No. (12) had the ability to penetrate epidermal cells. In microcosms experiment, cyanobacterial isolates positively affected wheat growth as compared to the non-heterocystous isolate, *Phormidium*. *Nostoc rivulare* and *Nostoc muscorum* isolate No. (12) were more efficient in nitrogen fixing activity as compared to the rest of isolates. In case of 2,4-dichlorophenoxyacetic acid induced maize roots, nitrogenase activity of *Nostoc* significantly enhanced as compared to the untreated maize roots. *Nostoc muscorum* or *Nostoc rivulare* colonized externally at the junction of the para-nodules, and also abundance of *Nostoc* on the induced maize roots increased as compared to the untreated maize roots. Nitrogenase activity and abundance of *Nostoc muscorum* or *Nostoc rivulare* co-cultivated with maize roots was increased up to 1.7 times in the absence of combined nitrogen (nitrates) as compared to the nitrate treated plants. In pot experiments, biofertilization by *Nostoc muscorum* or *Nostoc rivulare* significantly increased shoot length and leaf area of maize either alone or in combination with N-fertilizer at 50 and 100 kg N/ha. Combination of biofertilization and N-fertilization, especially at 100 kg N/ha had more effect on the growth of maize compared to the biofertilization alone, as well as nitrogenase activity.

Key words: Cyanobacteria, maize, biofertilization, ammonium sulphate.

INTRODUCTION

The use of chemical fertilizer is considered inevitable for obtaining optimum yield of crops. But it has been observed that continuous use of chemical fertilizers may affect soil health and may lead to a negative impact on soil productivity. Bio-fertilization technique is used beside mineral fertilizers for the plant nutritional requirements in agriculture attempts for minimizing the use of mineral fertilizers due to their high cost as well as to avoid the environmental pollution problem (Bloom, 1998).

N_2 fixing cyanobacteria improve crop production by

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acting as natural fertilizers as they increase both carbon and nitrogen status of soils (Lange et al., 1994). Acea et al. (2003) had reported that the addition of Cyanobacteria to soils help in increasing of soil fertility. Several studies had reported that cyanobacteria can produce extracellular polymeric substances which help them to overcome conditions of water stress and also bind soil particles to be increased from structure and fertility of soil (Hill et al., 1994). Flaibani et al. (1989) had reported that exopolysaccarides producing cyanobacteria play a significant role in reclaiming and fertilization of desert soils.

El-Shahed and Abdel-Wahab (2006) showed that co-cultivation of *N. rivulare* and the wheat exhibited nitrogenase activities both in the presence or absence of nitrate but results also indicated that addition of nitrates significantly reduced nitrogenase activity. Several studies reported that colonization of cyanobacterial strains on the plant cultivars depends on cyanobacterial isolate, plant, and content of nitrogen and pesticides in liquid and soil cultures (Gantar et al., 1991a, b; Spiller et al., 1993; Patnaik et al., 1994).One strategy had been used the auxin (2,4-D) to induce the formation of tumor-like growth on roots called para-nodules. Previous studies reported that *Azospirillum, Herbazospirillum* and cyanobacterium *Nostoc* sp strain 2S9 B colonized paranodules of wheat roots and had ability to fix the nitrogen (Yu and Kennedy, 1995; El-Komy and Abdel-Wahab, 1998; Gantar and Elhai, 1999). El-Shahed and Abdel-Wahab (2006) showed nitrogenase activity of *N. rivulare* with 2,4-D (1 ppm) significantly enhanced as compared to those non-treated with 2,4-D.

It is well known that nitrogen fertilization plays a significant role in improving rice yield. Surendra et al. (1984) had reported that nitrogen addition in the soil increased its fertility, development of leaf area and productivity of plant crops. Fadl-Allah et al. (2010) had reported that biofertilization with 50% N-fertilization enhanced growth of wheat and also increased N-yield of wheat. Hussein and Radwan (2001) had found that increasing nitrogen application rates increased number of grains per spike, grain weight and wheat productivity. El-Kalla et al. (1988) had concluded that increasing nitrogen application rates up to 75 kg N/ha increased plant height, leaf area, spike weight and wheat productivity. Abd El-Rahman et al. (1992) had indicated that the application of 144 kg N/ha to the wheat plant enhanced spike length, 1000-grain weight, number of grains/spike and grain yield compared to the different weights of nitrogen fertilizer. Several research groups had studied effect combination between biological and chemical fertilization in the flooded rice system (Jeyaraman and Purushothaman, 1988) and reported that bio-fertilization was a better alternative for the extensive use of nitrogen fertilizers in rice production.

As an effort in this respect, the present study aims at isolation and selection of the most efficient nitrogen fixing cyanobacterial isolates in order to be used as inoculants. Colonization of nitrogen fixing cyanobacteria to cereals roots and their effects on the plant growth were investigated through hydroponic solution (BG11 medium). Our study also aim to induce roots of maize by 2,4-D for formation of paranodules which are colonized with *N. revulare and N. muscorum* and its effect on nitrogenase activity. Field experiments were conducted to evaluate the effect of inoculation with *Nostoc* isolates selected as inoculants on the growth and yield of maize plant grown in sandy soil in absence or presence of different levels of nitrogen fertilizer in order to determine the extent to which the nitrogen-fixers could replace or decrease the application of nitrogen fertilizer.

MATERIALS AND METHODS

Cyanobacterium and growth condition

Soil samples collected from Minia Governorate, Egypt, is illustrated in Figure 1. The roots of selected plants were removed and gently shaken to remove free soil on surface. Five grams of free soil were placed in flask that contained 100 ml of sterile water after thoroughly shaking suitable dilutions were prepared. One ml of soil suspension was transferred to each sterile Petri- dish, which was then poured with melted but cooled nitrogen free BG11 medium (Rippka and Herdman, 1992) which is supplemented with agar for solidification (1%), at light intensity 3000 lux. Three plates were prepared for each sample; the plate's cultures were incubated at 24°C ±1 for 15 days according to the method described by Abdel-Hafez et al. (2000). Developing cultures were identified according to Desikachary (1959). Pure isolates were maintained on N_2 containing BG11 medium for further studies.

After 15 days, cyanobacterial cultures were harvested by centrifugation (8000 rpm for 10 min) and suspended in sterile H_2O (4g/ 250 ml), and used as inoculum of amount 10^6 heterocyst per seed of wheat plant (El-Shahed, 2005).

Nitrogenase activity of free living cyanobacterial isolates culture (*in vitro*)

Nitrogenase activity was evaluated in 15 days old culture using the acetylene reduction technique which was described by Hardy et al. (1973), using Gas Chromatograph, ATIUNICAM 610-GLC (UK) equipped with a glass column filled with activated alumina. A 10 ml aliquot of each culture was transferred to a flask with a 50 ml total volume, which was sealed with tight stopper. A 10% of free volume of air was replaced with pure acetylene using a gas tight syringe. Flasks were incubated for 2 h at 30°C. Results were expressed as nmole of C_2H_4 per µg Chlorophyll a.

Host plant

Seeds of host plant 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 2-3 days. Uncontaminated seedlings were transferred and grown in glass beakers or pots for experiments.

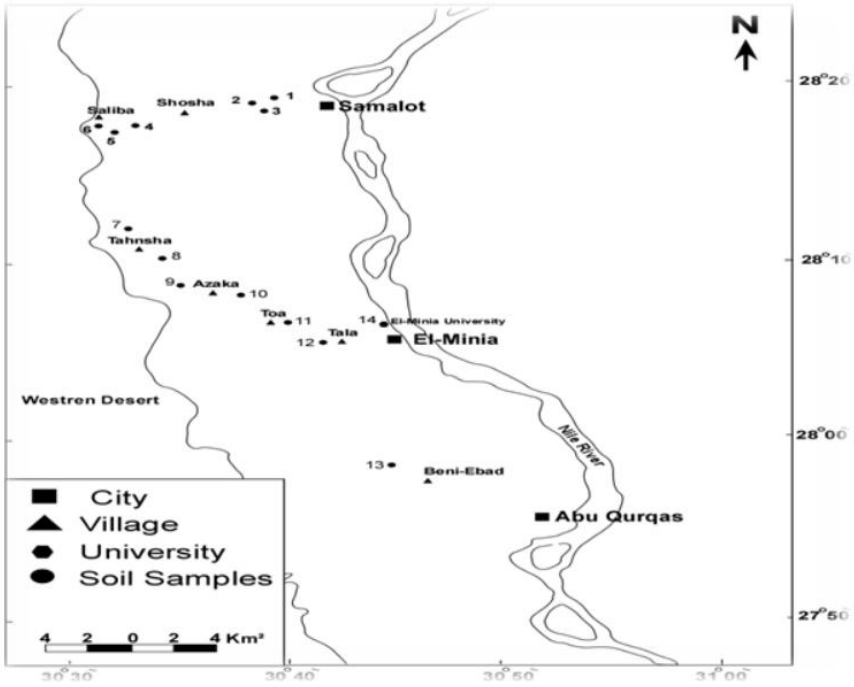

Figure 1. General location map shows localities of soil samples.

Hydroponic growth conditions

The method described by Gantar and Elhai (1999) was used for growing plant host in hydroponic solution (Rippka and Herdman, 1992). Pre-germinated host seeds were grown in sterile 250 ml glass beakers instead of tested tubes. 250 ml of sterile hydroponic solution was added to each beaker containing sterile foam rubber plate previously perforated with a cork borer to support the wheat seedling. 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..

Screening of N_2 fixing cyanobacteria associated with wheat roots in sterile soil (Microcosms experiment)

Four germinated wheat grains were transplanted in pots (500 ml volume) containing 500 g of sterilized sandy soil (2 sand: 1clay). Pots containing germinated wheat grains divided into nine groups with three replicates for each treatment as illustrated in Plate (2) followed with inoculation of different cyanobacteria (10^6 heterocysts per seedling). Seedlings of the first group represent the control. While those of the other groups were inoculated with tested cyanobacterial isolates. Pots were irrigated with distilled water according to field capacity. Pots incubated at continuous illumination, at room temperature. After 25 days from cultivation, growth parameters and nitrogenase activity were measured.

Induction and colonization of para-nodules

Ganter and Elhai (1999) and El shahed and Abdel-Wahab (2006) described the used method. When the roots of maize seedlings were about 5 cm length, aliquots of *N. muscorum* and *N. revulare* culture were 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 concentration 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 continuous 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.

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.

Greenhouse pot experiments

Six germinating grains were transplanted into a pot containing 3 kg soil (2:1 sand and clay) respectively. This was followed by inoculation with *N. muscorum* isolate No. (12) or *N. rivulare* (10^6 heterocysts per seedling). After one week from transplantation of

Table 1. Isolation of cyanobacteria spp. from different localities in El Minia Governorate.

Index number	Source	Plant cultivar	Isolated cyanobacterial spp.
1	Free soil	*Trifoliumalexandrinum*	*Nostoc microscapicum*
2		*Allium sativum*	*Phormidium molle* (P.254)
4		*Zea mays*	*Nostoc punctiforme*
5		*Trifolium alexandrinum*	*Nostoc paseriniamum*
6		*Solanum lycopersicum*	*Nostoc muscorum* isolate No.(11) *Nostoc muscorum* isolate No.(12)
8		*Trifoliumalexandrinum*	*Nostoc muscorum* isolate No.(10)
14		*Triticum vulgare*	*Nostoc rivulare*

maize seedlings, Ammonium sulphate dose was added on three levels (100 kg N/ ha added on three levels, each stage 33.3 kg N/ ha). Pots were divided into 9 groups with three replicates for each treatment as illustrated in Plate (4a, b, c). At the beginning of the second week after sowing, plants were thinned down to four plants per pot. The experiment was performed in a wire proof greenhouse maintained at 30 ± 5°C under natural day light. Pots were irrigated with water as needed according to field capacity. After 45 days, Plants were harvested, growth parameters and nitrogenase activity were measured.

Nitrogenase assay *in situ*

Nitrogenase activity was assayed by acetylene reduction assay (ARA) using a Gas Chromatograph, ATIUNI CAM 610-GLC (UK) equipped with a glass column filled with activated alumina. The remaining seed and its detached root were aspectically washed in sterile nitrogen free mineral solution and were 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 injected at 30°C for 2 h. Results were expressed as nmole of C_2H_4 per gm of root (Turner and Gibson, 1980).

Determination of plant growth

The lengths of tested plants (cm) were measured and weighed for obtaining fresh weights (mg) and then dried in an oven at 105°C to constant mass for further analysis.

a) Determination of leaf area: Leaf area was determined according to Norman and Campbell (1994).
b) Determination of the photosynthetic pigments: Chlorophyll a, Chlorophyll b and carotenoids were determined using the spectrophotometeric method recommended by Metzner et al. (1965).
c) Determination of calcium and magnesium: The versene (disodium dihydrogen ethylenediamine tetraacetic acid) titration method (Schwarzenbeck and Biederman, 1948) was employed for the determination of Ca^{+2} and Mg^{+2} concentrations.
d) Determination of Sodium and Potassium: Sodium and potassium were determined in the samples photometrically by flame photometry according to the method of Golterman et al. (1978).

Physico-chemical properties of tested soil

Soil temperature was determined *in situ* using a Hg thermometer. pH value was immediately measured after transportation to the laboratory using pH meter. Electrical conductivity of collected soils was measured using conductivity meter. Soil texture was determined by mechanical analysis through soil texture triangular. Cl^-, Ca^{+2} and Mg^{+2} were determined by volumetric methods. Phosphate and ammonia were determined by spectrophotometric methods using a Perkin Elmer Spectrophotometer (Table 4).

a) Determination of chloride: Estimation of chloride in soil extract was performed according to the method described by Jackson (1960).
b) Determination of carbonate: The carbonate is determined in soil samples by the use of phenolphethalein as indicator and standard solution of the hydrochloric acid (0.05 N) as recommended by Jackson (1960).
c) Determination of orthophosphate: In general, all the glassware used in phosphorus determinations (bottles, pipettes, measuring cylinders, etc.) were washed with 10% H_2SO_4 to remove phosphates that may adhere to glassware and then rinsed with distilled water before use. The orthophosphate determination was performed according to the procedure reported in the American Public Health Association Publications (1995).
d) Determination of ammonia: Ammonia was determined colorimetrically by the method adapted by Naguib (1964)
e) Determination of nitrate: Nitrates were determined by sodium salicyate method (Deutsche Einheitsverfahren Zur Wasser-Abwasser Und Schlamm-untersuchung, 1960).

Statistical analysis

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

RESULTS

Isolation of cyanobacteria spp. from different localities in El Minia governorate

Results in Table 1 showed that two genera represented with 8 of N_2-fixing cyanobacteria namely; *Nostoc rivulare, Nostoc muscorum* isolate No. (11), *Nostoc muscorum* Isolate No. (12), *Nostoc muscorum* Isolate No. (10), *Nostoc puncytforme*, *Nostoc paseriniamum*, *Nostoc*

Table 2. Nitrogenase activity of cyanobacterial isolates *in vitro.*

Cyanobacterial isolate	Nitrogenase activity (nmole C_2H_4 h-1 per µg of chl. a
Nostoc rivulare	16.2±0.2
N. microscapicum	10.9±0.3
N. passeriniamum	12.3±0.1
N. punctiforme	14±0.7
N. muscorum isolate No.(10)	13.6±0.1
N. muscorum isolate No. (12)	22.2±0.5
N. muscorum isolate No. (11)	16.8±0.4
Phormidium molle (p.254)	2.6±0.2

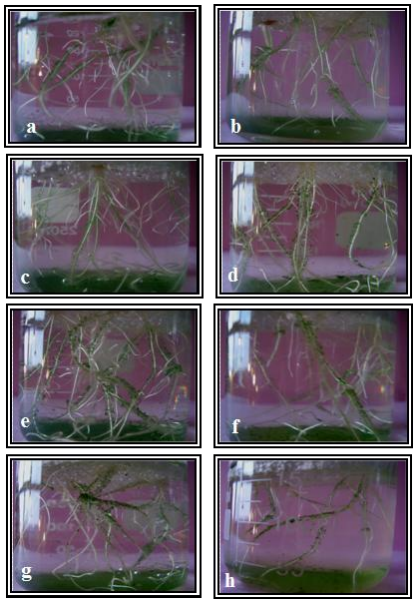

Plate 1. Colonization of wheat roots with different *Cyanobacterial* spp. a) *N. microscopicum*, b) *N. punctiforme*, c) *N. muscorum* isolate No. (11), d) *N. passeriniamum*, e) *N. muscorum* isolate No. (12), f) *N. muscorum* isolate No. (10), g) *N. rivulare*, h) *Phormidium molle* p.254.Scale bars are 2.25 cm.

microscapicum and *Phormidium molle* (P.254) were isolated from different types of cultivated soils which were collected from different localities in El Minia Governorate as shown in Figure 1 *Nostoc rivulare* was isolated from free clay soil cultivated with wheat (*Triticum vulgare*), Minia city. *N. microscapicum* was isolated from free clay soil cultivated with *Trifolium alexandrinum*, Samalot city. *N. puncyiforme* isolated from free loamy sandy soil cultivated with corn (*Zea mays*), Samalot city. *N. paseriniamum* was isolated from free loamy sandy soil cultivated with *Trifolium alexandrinum*, Samalot city. *N. muscorum* isolate No. (11) was isolated from free loamy sandy soil cultivated with *Solanum lycopersicum*, Samalot city. *N. muscorum* isolate No. (12) was isolated from free loamy sandy soil cultivated with *Solanum lycopersicum*, Samalot city. *N. muscorum* isolate No. (10) was isolated from free loamy sandy soil cultivated with *Triticum vulgaris*, Minia city. *Phormidium molle* (P.254) was isolated from free loamy sandy soil cultivated with onion (*Allium sativum*), Samalot city.

Nitrogenase activity of cyanobacterial isolates *in vitro*

All cyanobacterial isolates were tested for their ability to fix atmospheric nitrogen *in vitro*. Results in Table 2 showed that the eight isolates have the ability to fix nitrogen.

Colonization of wheat roots by different cyanobacterial isolates in hydroponic solution and their capacity on nitrogen fixing (*in situ*)

The isolated cyanobacteria were tested for their ability to colonize the roots of wheat plant in hydroponic solution. Results in Table 3 and Plate 1 showed that all cyanobacterial isolates have the ability to colonize the roots of wheat, and colonization of roots by *N. passeriniamum*, *N. muscorum* isolate No. (12), *Phormidium molle* was higher than other isolates. Results in Table 3 also showed that all cyanobacterial isolates have the ability to fix nitrogen *in situ*. The nitrogenase activity of *N. muscorum* isolate No. (12) and *N. rivulare* was the highest compared to other cyanobacterial isolates. Two different kinds of association between roots and cyanobacteria were observed, (a) loose attachement in case of *Phormidium*, (b) Tight association was observed in case of *Nostoc* isolates which showed tightly packed filaments of the *Nostoc* forming aseriate packages on a root surface. The first stage of colonization of wheat roots by these isolates was probably the migration of hormogonia, then hormogonia developed into long filamentous and the long filaments

Table 3. Colonization of wheat roots by different cyanobacterial isolates in hydroponic solution and their nitrogen fixation *in situ*.

Isolates	Chl. a content (abundance of isolates on root) (µg/g root)	Nitrogenase activity (nmole C_2H_4 $h^{-1}g^{-1}$ f.wt.)
N. rivulare	27.7±2	1.6±1.2
N. microscapicum	38.1±3	8.1±0.8
N. passeriniamum	53.4±1.5	8.8±1.2
N. punctiforme	25.6±1.2	15.5±2.1
N. muscorum isolate No. (10)	16±2.2	10.1±1.2
N. muscorum isolate No. (12)	69.5±1.9	24.4±2.1
N. muscorum isolate No. (11)	11.5±2	10.8±2
Phormidium molle (p.254)	56.4±2.4	4.3±1.7

Table 4. Physico-chemical properties of the used soil in the experiments.

pH	CO_3^{2-} (%)	P (ppm)	NH_4^+ (ppm)	NO_3^- (ppm)	K^+ (%)	Soil texture			Type of soil
						Sand %	Clay %	Silt %	
8	0.15	4.0	3.0	3.0	88	88	6.60	5.80	Sandy

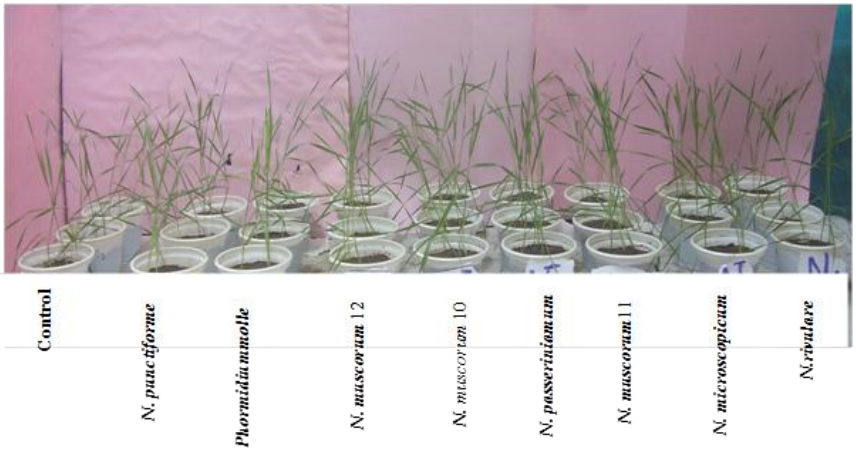

Plate 2. Screening of different cyanobacterial isolates to improve the growth of wheat grown in microcosms experiment. Bar = 8 cm.

developed of the aseriate stage which consisted of filaments tightly packed in a mucilaginous sheath. In addition to form a tight association, one strain (*N. muscorum* isolate No. (12)) also appeared to penetrate some root cells as cyanobacterial mass.

Microcosms experiment

Effect of different cyanobacterial isolates on wheat growth

Different cyanobacterial isolates were tested for their ability to affect the growth of wheat growing in sterilized loamy sand soil. Results in Table 5 and Plate (2) showed that most cyanobacterial isolates increased wheat root length. Data also showed that cyanobacterial inoculation increased lengths of shoot, while *Phormidium molle* and *N. muscorum* isolate No. (11) showed non-significant effect in length of shoot. The tested isolates except *Phormidium molle* and *N. passeriniamum* caused a significant increase in fresh weights of root and shoot of wheat plant. Cyanobacterial inoculation positively affected pigment content, *Phormidium molle* treatment showed that the highest effect. Most cyanobacterial isolates showed a significant increase in plant leaf area, but *N. punctiforme* was not affected significantly in plant leaf area. Data also showed cyanobacterial inoculation

Table 5. Effect of different cyanobacterial isolates on wheat growth and pigment contents.

Treatment	Root			Shoot			Leaf area (cm^2/ plant)	Pigments (µg /g plant)		
	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)		Chl. a	Chl. b	Carotein
Control	7.7	102	16.3	26.7	460	74.3	15.2	936.1	679.7	96
Nostoc rivulare	9.8	247	18.8	29.4	564	88.6	20.9	993.7	766.4	81.3
Nostoc microscapicum	9.6	264	20.0	28.8	561	87.1	20.0	970.5	679.8	120.0
Nostoc passeriniamum	10.4	215	16.0	28.8	663	90.0	20.5	929.2	902.6	8.2
Nostoc punctiforme	11.4	201	17.0	29.1	480	75.7	15.7	952.3	920.3	4.2
N. muscorum isolate No. (10)	10.2	275	17.0	30.7	667	90.0	21.4	915.4	903.5	24.9
N. muscorum isolate No. (12)	7.7	193	16.3	29.3	575	91.7	21.2	970.1	820.7	36.4
N. muscorum isolate No. (11)	9.9	186	17.0	28.6	607	90.0	20.02	950.4	887.8	2.7
Phormidium molle (p.254)	9.8	188	16.3	28.0	483	71.7	19.8	1012.6	1019.6	2.9.0
L.S.D (5 %)	3.8	70.0	3.5	1.3	90.0	4.0	4.18	21.0	87.0	24.0

Table 6. Nitrogen fixing efficiency (*in situ*) of different cyanobacterial isolates associated with wheat in microcosms experiment.

Cyanobacterial isolates	Nitrogenase activity (nmole C_2H_4 $h^{-1}g^{-1}$ f.wt.)
Control	0.00
Nostoc rivulare	17.80 ±1.2
N. microscapicum	6.00 ± 0.5
N. passeriniamum	4.20 ±0.42
N. punctiforme	0.38 ±0.05
N. muscorum isolate No. (10)	9.60 ± 0.85
N. muscorum isolate No. (12)	18.80 ±2
N. muscorum isolate No. (11)	2.00 ±0.35
Phormidium molle(p.254)	0.60 ±0.05

clearly increased plant shoot and root dry weight, while *Phormidium molle* did not exhibited such effects.

Nitrogen fixing efficiency of different cyanobacterial isolates associated with wheat in situ

Results in Table 6 showed that nitrogenase activity of different cyanobacterial isolates differed greatly and depended on the cyanobacterial isolate. All cyanobacterial isolates associated with wheat roots have ability to fix nitrogen in microcosms experiment. Acetylene reduction assay of *N. rivulare* and *N. muscorum* isolate No. (12) were the greatest among other isolates. According to these results, both *N. rivulare* and *N. muscorum* isolate No. (12) were selected for further studies.

Para-nodule induction in maize with 2,4-D and its infection with *N. muscorum* or *N. rivulare*

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

Results in Table 7 showed that treatment of seedlings of

Table 7. Effect of different 2,4-D levels and inoculation with *N. rivulare* and *N. muscorum* isolate No. (12) on growth of maize seedlings (15 days old).

Parameter	Growth of maize with *N. rivulare*						Growth of maize with *N. muscorum* isolate No. (12)					
	Root			Shoot			Root			Shoot		
	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)
Control	10.5	220	12.5	17.8	469.4	46.0	10.5	220	12.5	17.8	470.0	46.0
Inoculum	10.0	360	18.8	21.5	625	53.8	11.3	375.0	19.5	20.4	609.8	53.0
0.5 ppm (2,4D)	7.2	475	22.5	19	693.3	57.5	8.7	482.4	24.3	17.8	715.3	59.3
1.0 ppm (2,4D)	6.8	385	21.0	17.2	417.7	48.3	7.6	437.0	20.5	18.2	702.6	46.3
3.0 ppm (2,4 D)	5.8	330	18.8	14.5	360	35	6.5	367.6	17.5	16.5	390.5	37.5
L.S.D	2.22	12.0	4.0	4.9	230	5.7	2.24	49.0	6.5	2.84	75.0	6.7

maize with 2,4-D influenced the growth of both root and shoots. Applications of 2,4-D also induced formation of para-nodules as in Plate (3a). Root elongation, shoot length of both maize and lateral root formation were strongly inhibited by different concentration of 2,4-D compared with the control plants (2,4-D non-treated plants). Results in Table 7 also showed that inoculation of tested seedlings with *N. rivulare* or *N. muscorum* significantly increased weights of both root and shoot of maize seedlings as well as plant height compared with uninoculated seedlings.

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

Data presented in Table 8 showed that nitrogenase activity assayed by acetylene reduction assay of *N. rivulare* or *N. muscorum* associated with maize plants treated with 2,4-D was higher than those nontreated with 2,4-D. Generally, nitrogenase activity increase in the presence of 2,4-D.

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

Data presented in Table 8 showed that colonization of *N rivulare* or *N. muscorum* to maize 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 maize seedlings inoculated with N. rivulare or 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 3a). Data presented in Table 9 also showed that high numbers of para-nodules (per plant) were obtained at the range of 0.5 -1.0 ppm 2,4-D. The application of 2,4-D at a rate of 1.0 ppm did not repress plant development.

Colonization of para-nodules of maize with N. rivulare or N. muscorum

When nodulated seedlings were inoculated with *N. rivulare* or *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 N_2 fixation as indicated in (Plate 3b). Light microscopy examination revealed that *N. rivulare* or *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 3c).

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

Results in Table 10 showed that co-cultivation of maize seedlings with *N. rivulare* and *N. muscorum*

Table 8. Nitrogenase activity (*in situ*) and Abundance of *N. rivulare* and *N. muscorum* isolate No. (12) associated with maize at different 2,4-D concentrations (15 days old).

Treatment	Abundance (Chl. a content) (µg/g root)		Nitrogenase activity (nmole C_2H_4 $h^{-1}g^{-1}$ f.wt.)	
	N. rivulare	*N. muscorum*	*N. rivulare*	*N. muscorum*
inoculum	29.0±2	34.6±1.6	11.5±0.9	12.5±1
0.5 ppm (2,4-D)	35.0±3.5	81.7±2.1	12.1±1.2	17.5±2
1.0ppm (2,4-D)	91.3±1.3	143.1±1.1	37.1±1.5	31.0±1.7
3.0ppm (2,4-D)	59.4±1.5	105.0±1.9	25.3±2	28.5±1

Table 9. Effect of different 2,4-D concentrations on numbers of paranodules of maize seedlings inoculated with *N. rivulare* and *N. muscorum* isolate No. (12).

Treatment	Number of paranodules per plant inoculating with	
	N. muscorum	*N. rivulare*
inoculum	0	0
0.5 ppm (2,4-D)	40.7 ±2	41.7 ±1
1.0ppm (2,4-D)	51.3 ±3	51 ±2.3
3.0ppm (2,4-D)	39.3 ±0.5	37.5 ±1.5

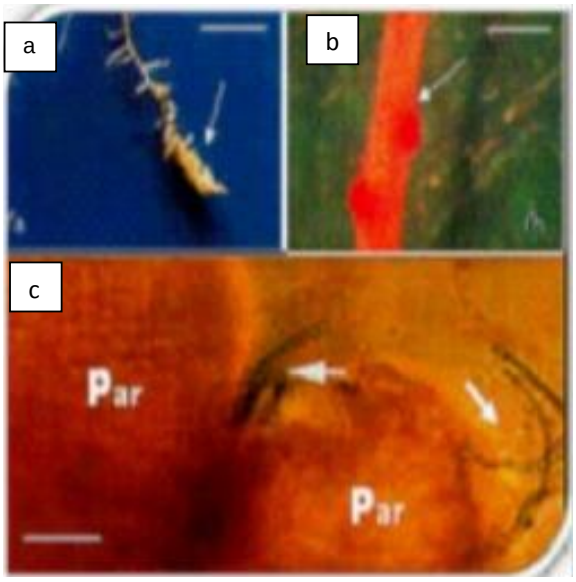

Plate 3. a) Colonization of paranoduled maize plants at the junction of the para-nodules (Para) with the root and at the top. Bar =50 µm. b) Para-nodules developed at the tip of maize main root. Bar = 1mm. c) Root stained with TTC showing reduction in para-nodules. Bar =2 mm.

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. rivulare* or *N. muscorum* was increased up to 1.7 times in the absence of nitrate compared with nitrate treated maize. Results in Table 10 also showed that colonization of *N. rivulare* or *N. muscorum* to maize increase in the absence of nitrate compared with the presence of nitrate.

Pot Experiment of Maize

Effect of N. rivulare or N. muscorum isolate No. (12) inoculation on the growth and nitrogen fixing of maize grown in pot experiment through the addition of different doses of combined nitrogen

Results in Tables 11 and 12 and Plate (4a, b, c) showed that plants fertilized with ammonium sulphate only showed improvement in measured parameters expressed as plant height, weights (dry and fresh) of both root and shoot and leaf area. Plants inoculated with *N. muscorum* isolate No. (12) and *N. rivulare* inhe presencet of combined nitrogen (half and full dose) showed a significant increase more than those of non-inoculated and fertilized plants (controls).

Results also indicated that the growth of maize inoculated with *N. muscorum* isolate No. (12) at half dose or full dose of combined nitrogen significantly increased compared with growth of maize inoculated with *N. rivulare* at half dose or full dose of combined nitrogen.

Table 10. Abundance and Abundanceof *N. rivulare* and *N. muscorum* isolate No. (12) (chlorophyll a content) colonized maize roots (15 days old) as affected by presence or absence of nitrate.

Treatment	Nitrogenase Activity (nmole C_2H_4 $h^{-1}g^{-1}$ f.wt.)		Abundance of isolate No. (12) (Chl. a content) (µg/g root)	
	N. rivulare	*N. muscorum*	*N. rivulare*	*N. muscorum*
With nitrates	35.5 ±1.9	38.9 ±2	15.6 ±2.5	85 ±4.3
Without nitrates	59.7 ±2.2	61.5 ±3.1	38.7 ±1.5	91.5 ±5.2

Table 11. Effect of *N. rivulare* and *N. muscorum* isolate No. (12) inoculation on the growth and nitrogen fixation of maize grown in pot experiment through different doses of combined nitrogen.

Treatment	Root			Shoot			Leaf area (cm^2/plant)	N.ase activity (nmole C_2H_4 h-1g-1f.wt.)
	Length (cm)	Fresh wt. (gm)	Dry wt. (gm)	Length (cm)	Fresh wt. (gm)	Dry wt. (gm)		
Control without combined N	13.3	0.35	0.17	27.40	0.75	0.28	62.74	0.0
Control with half dose N	16.3	0.97	0.16	36.67	2.50	0.61	91.41	0.0
Control with full dose N	15.4	1.08	0.17	42.50	4.80	1.25	177.29	0.0
N. rivulare without combined N	14.8	1.18	0.10	34.20	3.66	1.00	121.56	5.1 ±0.2
N. rivulare with half dose	20.0	1.43	0.19	40.10	3.70	1.89	258.42	13.7 ±0.5
N. rivulare with full dose	13.5	1.25	0.20	45.60	5.25	2.31	262.9	14.5 ±0.4
N. muscorum isolate No. (12) without combined N	15.0	1.18	0.15	40.60	6.50	1.50	238.6	5.0 ±0.1
N. muscorum isolate No. (12) with half dose	16.7	1.83	0.30	45.40	7.45	1.61	334.25	5.5 ±0.3
N. muscorum isolate No. (12) with full dose	14.0	1.75	0.28	50.70	10.30	5.00	357.42	16.6 ±0.1
L.S.D (5%)	2.57	0.26	0.07	8.16	0.97	0.27	75.1	

Table 12. Effect of *N. rivulare* and *N. muscorum* isolate no. (12) inoculation on the pigment content and mineral content of maize grown in pot experiment through different doses of combined nitrogen.

Treatment	Pigment (µg /g plant)			Mineral			
	Chl. a	Chl. b	Caroteins	Na^{+} µg/ g dry wt	K^{+} µg/ g dry wt	Ca^{+2} %	Mg^{+2} %
Control without combined N	45.4	96.5	7.7	7.76	24.65	1.08	1.0
Control with half dose N	42.1	159.0	11.7	11.02	4203	1.13	1.1
Control with full dose N	75.1	103.6	41.6	34.7	46.06	1.33	1.5
N. rivulare without combined N	75.1	116.1	1.2	3.68	32.73	1.60	1.1
N. rivulare with Half dose	74.9	148.5	8.6	6.53	40.81	1.60	1.2
N. rivulare with Full dose	80.4	145.3	3.2	11.84	44.44	1.60	1.8
N. muscorum isolate No.(12) *without combined N*	47.2	151.7	3.8	5.13	24.65	1.33	1.3
N. muscorum isolate No. (12) with Half dose	94.1	81.7	2.4	8.57	54.14	1.96	1.2
N. muscorum isolate No. (12) with Full dose	110.6	202.6	1.5.0	6.12	57.8	1.6	1.8
L.S.D (5 %)	9.3	4.2	0.7	0.2	0.5	0.05	0.07

Results in Table 12 showed that the addition of half or full dose of combined nitrogen caused an increase in pigment content and magnesium content in shoot. N-fertilization of maize caused a small increase in Ca^{+2} and Mg^{+2} content in shoot compared with the control plants. Results also indicated that inoculation of maize with *N. muscorum* isolate No. (12) and *N. rivulare* at addition of half or full dose of combined nitrogen improved from pigment content as well as potassium and sodium amount in shoot compared with maize treating alone with half dose or full dose of combined nitrogen. At the same time, it caused a small increase in calcium and magnesium content in shoot of maize.

Nitrogenase activity (in situ) of maize roots grown in pot experiment

Results in Table 11 showed that *N. muscorum* isolate No. (12) and *N. rivulare* inoculation showed nitrogen fixing capacity on maize roots. The addition of N-fertilizer enhanced the nitrogen fixing activity using both *Nostoc* spp. than those of non-fertilized plants.

DISCUSSION

In our study, we described the association between cyanobacterial isolates and wheat plant in liquid culture in which there were two types of associations: a) Loose association as in case of *Phormidium*, b) Tight association as in case of *Nostoc*. These types of association with wheat had been previously reported by Spiller et al. (1993) and 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). Polysaccharides by which cyanobacteria strains were produced may play an important role in the attachement of cyanobacteria to roots as similar as previous study had been reported by Robins et al. (1986) for the attachment

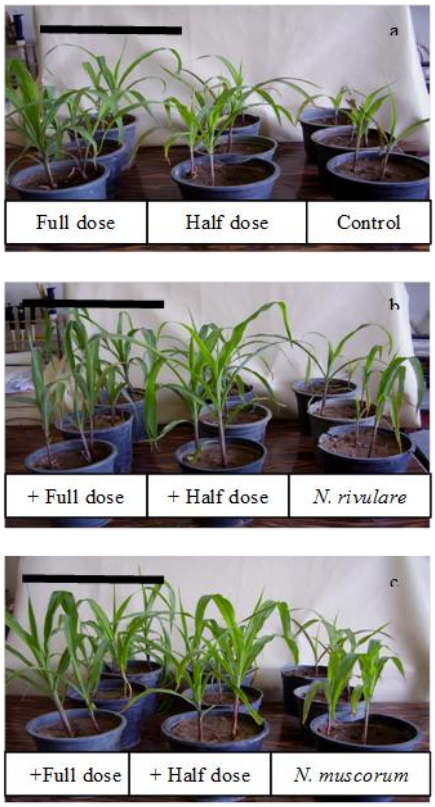

Plate 4. Pot experiment of maize; a) Effect of different doses of combined nitrogen on growth of maize plant. Bar = 13.8 cm. b) Effect of inoculation with *N. rivulare* on maize growth at different doses of combined nitrogen. Bar = 13.8 cm. c). Effect of inoculation with *N. muscorum* isolate No. (12) on maize growth at different doses of combined nitrogen. Bar = 3.8 cm

characterized by El-Shahed (2005) its ability to form tight association with wheat roots and fix the nitrogen.

In Microcosms experiment, results of the current study showed that inoculation of wheat plant with of cyanobacteria to plants cells. *N. rivulare* had been cyanobacterial isolates improved from wheat growth as well as positive effects of such association on plant growth which had been reported by Rai et al. (2000). The positive effects on wheat growth due to colonize cyanobacterial isolates the wheat roots, fix atmospheric N_2 and produce plant growth promoting compounds, as well as had been previously shown in *Nostoc*-wheat association (Gantar et al., 1995; Nanjappan et al., 2007; Sergeeva et al. 2002).

Preliminary studies showed that germinating maize 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 alcohol and hydrogen peroxide as well as had been previously shown by El-Shahed and Abdel-Wahab (2006). Thus, 2,4-D was applied up to the concentration of 3 ppm for the rest of the other plant in all our experiments. The present results showed that the auxin 2,4-D increased the colonization of roots of maize with *N. muscorum* or *N. rivulare* and hence, nitrogenase activity increased. These results are in accordance with previous results (Fadl-Allah et al., 2011; El-Shahed and Abdel-wahab, 2006; Nilsson et al., 2002; Gantar and Elhai, 1999). 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 N_2 fixation as well or b) 2,4-D had induced para-nodules on plant roots and these could had provided suitable sites for cyanobacterial colonization (Fadl-Allah et al., 2011; El-Shahed, 2005; Gantar and Elhai, 1999).

In the Pot experiment, results of the present study showed that inoculation of maize plants with *N. muscorum or N. rivulare* caused a significantly increase in growth of maize plants, represented in plant height, leaf area, weight of plant as well as legume weight of wheat, pigment and minerals content. These results are in accordance with previous results (Fadl-Allah et al., 2010; Nanjappan et al., 2007; Al-Noim and Hamad, 2004; Rai et al., 2000). Growth stimulation by *N. muscorum or N. rivulare* could be attributed to production of the auxins/ bioactive molecules (Nisha et al., 2007; Biondi et al., 2004; Aziz and Hashem, 2004, 2003; Sergeeva et al., 2002). The combination of biofertilization (*N. muscorum or N. rivulare*) and N-fertilization (half and full dose) significantly increased the growth of maize plants, compared to those of control non-inoculated and fertilized plants. These results are in accordance with previous observations (Fadl-Allah et al., 2010; Al-Noim et al., 2004; Nayak et al., 1986; Abou- Zeid et al., 1996; Hussein and Radwan, 2001; Bassal et al., 1996). The stimulatory effect of N-fertilizer addition on nitrogen fixing activity was due to stimulate root growth, as well as stimulated the growth of cyanobacterial population in soil cultivated with wheat, and thus colonization of cyanobacteria increased and also nitrogen fixing activity enhanced. Similar conclusions had been previously reported (Jha et al., 2001; Jha and Prasad; Singh et al., 1992; Wang, 1986).

Finally, our study shows that *N. muscorum or N. rivulare* is being 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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