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Para-nodule Induction in Wheat, Maize and Rice with 2,4-D and its Infection with *Nostoc rivulare* Kützing

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Abstract: In water cultures, wheat, maize and rice seedlings treated with low concentrations (0.5, 1.0 and 3.0 ppm) of 2,4-D developed nodule-like structures (para-nodules) mainly along primary roots. *Nostoc rivulare* Kützing, colonized the para-nodules externally at the junction of the nodule and the root as well as at the top of the nodules. Efficient N_2 fixing activity was obtained in *N. rivulare* Kützing inoculated para-nodulated seedlings. Addition of 2,4-D (1 ppm) enhanced the rate of acetylene reduction of inoculated plants more than non-treated plants. Nitrogenase activity of plants co-cultivated with *N. rivulare* Kützing was increased up to eight times in the absence of combined nitrogen (nitrate) compared with nitrate-treated plants. Results of this study demonstrate that *N. rivulare* Kützing is a promising organism for achieving efficient association between N_2 fixing cyanobacteria and nonlegumes.

Key words: Para-nodules, 2,4-D, cyanobacteria, nostoc, cereals

INTRODUCTION

More than 15 years ago it was announced first by Tchan and Kennedy (1989) that wheat plants form nodule-like tumors (para-nodules) when inoculated with Rhizobium and treated with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Since then, the study on this phenomenon opened new and exciting aspects concerning the extension of biological nitrogen fixation in graminaceous plants. It has been demonstrated that para-nodules are potentially inhabited by introduced different diazotrophic bacteria which create an increased level of nitrogen fixation (Tchan and Zeman, 1995; Abdel-Wahab *et al*., 1995). Nitrogen fixing cyanobacteria, particularly Nostoc sp., form symbioses with different plant group: bryophytes such as Anthoceros (Meeks, 1990); the water-fern Azolla (Braun-Howland and Nierzwicki- Bauer, 1990); the gymnosperm Cycas (Lindblad and Bergman, 1990); and the angiosperm Gunnera (Bergman *et al*., 1992). Moreover, at present there is a great interest in establishing an intimate associations between gramineceous plants, such as wheat and the nitrogen-fixing cyanobacteria. Ulterastructure studies had revealed that the cyanbacterium Nostoc spp. had the ability to colonize and in some cases penetrate both root epidermis and cortex (Gantar *et al*., 1991a, b, 1993).

Cyanobacteria that enter into symbioses with plants fall within the class (Rippka *et al*., 1979) that differentiates specialized cells called heterocysts, which are sites of the enzyme nitrogenase. These cells devoid of photosystem II which is responsible for O_2 evolution and therefore they protect nitrogenase against inactivation by O_2. Because symbiotic cyanobacteria fix N_2 within heterocysts, they have no need for low O_2 tension to be maintained within para-nodules. The heterocyctous cyanobacterium *Nostoc rivulare* Kützing is characterized by its ability to form tight association with wheat roots and significantly improved its N_2-fixing capacity and consequently plant growth (El-Shahed, 2005). Investigations on the possibility of cyanobacteria to inhabit and form para-nodules with cereals are sporadic and scanty (Gantar, 2000a).

The present study aims at evaluation of the ability of *N. rivulare* to colonize and fix atmospheric N_2 within para-nodules induced by 2,4-D in wheat, maize and rice.

MATERIALS AND METHODS

Hydroponic growth conditions: The method described by Abdel-Wahab *et al*. (1995) was used for growing wheat, corn and rice in hydroponic solution. Plant seedlings were grown in sterile 15 L glass jars instead of test tubes (Fig. 1A). 1500 mL of sterile nitrogen-free hydroponic solution was added to each jar containing a sterile foam rubber plate previously perforated with a cork porer to support the plant seedlings. The hydroponic solution contained: KH_2PO_4, 40 μM; K_2SO_4, 80 μM; $CaCl_2$, 40 μM;

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$MgSO_4$. $7H_2O$, 60 μM; $MnSO_4$, 4 μM; and per liter, Fe-chelate, 10 mg; H_3BO_3, 120 μg; $ZnSO_4$. $7H_2O$, 46 μg; $NaMoO_4$. $2H_2O$, 10μg; $CuSO_4$. $5H_2O$, 15 μg; $COCl_2$. $6H_2O$, 4 μg. The pH was adjusted to 6.8 with K_2HPO_4, 0.1 M solution. This medium was used for testing the effect of combined nitrogen after the addition of KNO_3 at a rate of 0.81 g L^{-1}.

Host plants: Seeds of wheat (*Triticum aestivum* L. cv. Giza 167), corn (*Zea mays* L.) and rice (*Oryza sativa* L.) were first rinsed five times in distilled water prior to surface sterilization. After 2.5 min treatment with 0.1% $HgCl_2$, the seeds were rewashed as described above and germinated on sterile filter paper in sterile 9 cm plates for 2-3 days. Uncontaminated germinating seeds were transferred and grown in plant jars under continuous light (3000 lux) at room temperature (30±2°C). Laboratory experiments were conducted during the period from April to August 2005 in the Microbiological lab, Botany Department, Faculty of Science, Minia University, Egypt.

Organism: The cyanobacterium designated *Nostoc rivulare* was originally isolated by El-Shahed (2005) from a garden soil sample collected from Minia Governorate, Egypt. It was made axenic by antibiotic treatment (Vaara *et al*., 1979) and repeated isolation of individual hormogonia. The strain was maintained in Chu 10 medium (Bold and Wynne, 1978) at light intensity of 3000 lux and room temperature for 15 days. The organism was harvested by centrifugation and suspended in sterile distilled water and then used as inocula.

Induction and inoculation of para-nodules: When the roots of the plants were about 5 cm in length, aliquots of *N. rivulare* culture were added to the jars 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. Uninoculated jar containing 2, 4-D and inoculated jars without 2, 4-D were used as controls. After about 5 days para-nodules were well formed on the root systems and were ready for experimentation. Roots were examined and photographed using a Carl Zeiss (Jena Med2) phase contrast microscope, Jena, Germany after washing thoroughly in strong water stream from a washing bottle so as to remove loosely adhered filaments of *Nostoc*.

Nitrogenase assays: Nitrogenase activity was assayed by the acetylene reduction assay (ARA) using ATIUNICAM 610-GLC, Cambridge, UK, equipped with a glass column (5 ft. × 18 inch) filled with activated alumina. Each plant seedling had its remaining seed detached aseptically and its root 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 C_2H_2 and incubated at 30°C for 1 h under continuous illumination. ARA was performed after this incubation period.

Tetrazolium salt staining and microscopic examination: Para-nodulated seedlings were incubated overnight with a solution of 0.025% of triphenyltetrazolium chloride (TTC) to locate the site of strong reduction. Such sites were detached and examined by a KYOWA, Japan, dissecting stereomicroscope.

Experimental data were subjected to one way analysis of variance and the means were separated by the least significant difference (LSD).

RESULTS

Effect of different concentrations of 2,4-D or *Nostoc rivulare* inoculation on the growth and number of para-nodules on plant roots:

Results in Table 1 show that treatment of seedlings with 2,4-D influenced the growth of plant shoots and roots and induced formation of para-nodules. Root elongation and lateral root formation was strongly inhibited by different 2,4-D concentrations as compared with control plants. Application of *Nostoc rivulare* significantly enhanced shoot and root weights as well as plant height.

Results of our study also indicate that para-nodules were developed mainly on the main roots as swollen projections after 5 days of 2,4-D treatments (Fig. 1B,C and D). Para-nodules were not uniformly arranged along the main root. The greatest number was found at the tip of the root (Fig. 1B). Data presented in Fig. 2 also showed that high numbers of para-nodules (per plant) were obtained with 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 with *N. rivulare*: When nodulated seedlings were inoculated with *N. rivulare* and were incubated 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 Nostoc which found a possible better site for N_2 fixation (Fig. 1, C, D).

Light microscopy examination revealed that *N. rivulare* colonized the para-nodules externally at both

Table 1: Effect of different 2,4- D levels or inoculation with *Nostoc rivulare* on growth, fresh weight and length, of wheat, maize and rice seedlings (15 days old)

	Wheat				Maize				Rice			
	Shoot		Root		Shoot		Root		Shoot		Root	
Treatments	Length (cm)	Weight (mg)	Length (cm)	Weight (mg)	Length (cm)	Weight (mg)	Length (cm)	Weight (mg)	Length (cm)	Weight (mg)	Length (cm)	Weight (mg)
0.0 ppm 2,4-D	16.2	280.0	15.0	260.0	16.3	300.0	25.0	460.0	10.5	50.0	10.8	80.0
0.5 ppm 2,4-D	11.7	240.0	9.9	224.0	7.6	110.0	7.6	120.0	2.8	30.0	2.5	50.0
1.0 ppm 2,4-D	7.6	110.0	7.7	120.0	9.7	120.0	8.9	160.0	2.7	40.0	2.8	35.0
3.0 ppm 2,4-D	9.7	120.0	8.9	160.0	8.4	140.0	10.0	90.0	1.5	25.0	2.1	20.0
Nostoc alone	18.4	320.0	18.1	290.0	20.1	380.6	26.6	490.1	12.3	66.3	11.9	93.5
LSD (5%)	0.91	6.7	1.1	8.1	0.81	8.8	1.2	7.9	0.9	5.6	0.5	9.5

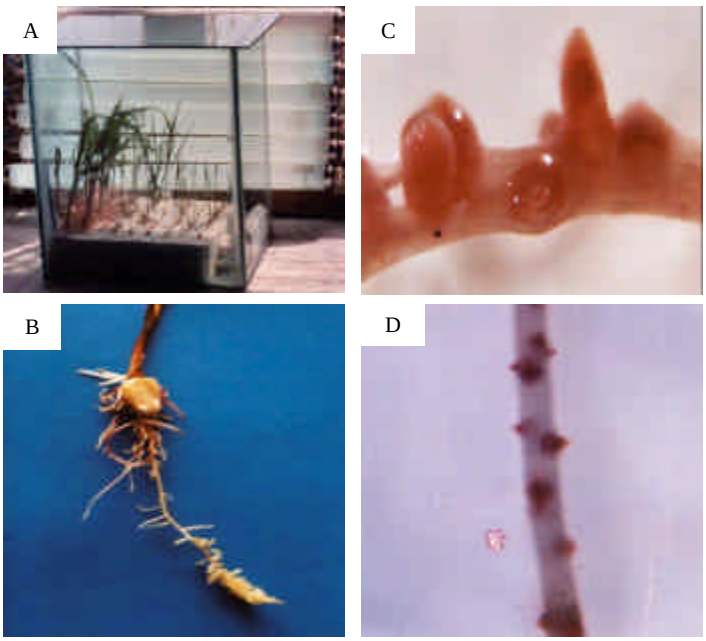

Fig. 1: A, liquid culture for induction of para-nodules in maize; B, para-nodules developed at the tip of maize main root; C and D, roots stained with TTC showing strong reduction in para-nodules. Scale bars are 5 cm, 1 mm, 2 cm and 2 mm in A, B, C and D, respectively

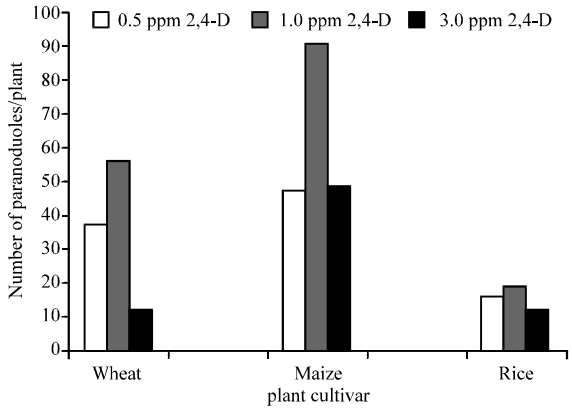

Fig. 2: Effect of different 2,4-D concentrations on numbers of para-nodules of wheat, maize and rice seedlings (15 days old)

the basal connection between the nodule and the root and at the top of the nodules as loosely arranged filaments (Fig. 3A). The surface epidermal layer of the plant root was also colonized with separate straight filaments of *N. rivulare* (Fig. 3B, D). *N. rivulare* filaments were also tightly packed as illustrated in Fig. 3B and C.

Nitrogen-fixing activity of *N. rivulare* associated with wheat, maize and rice as affected by the presence or absence of 2,4-D and nitrate: Data presented in Fig. 4 showed that co-cultivation of *N. rivulare* and the tested plants exhibited nitrogenase activities both in para-nodulated (treated with 2,4-D) and without 2,4-D application. Results also indicated that the addition of 2,4-D (1 ppm) significantly enhanced nitrogenase activity (especially in the absence of nitrate) more than

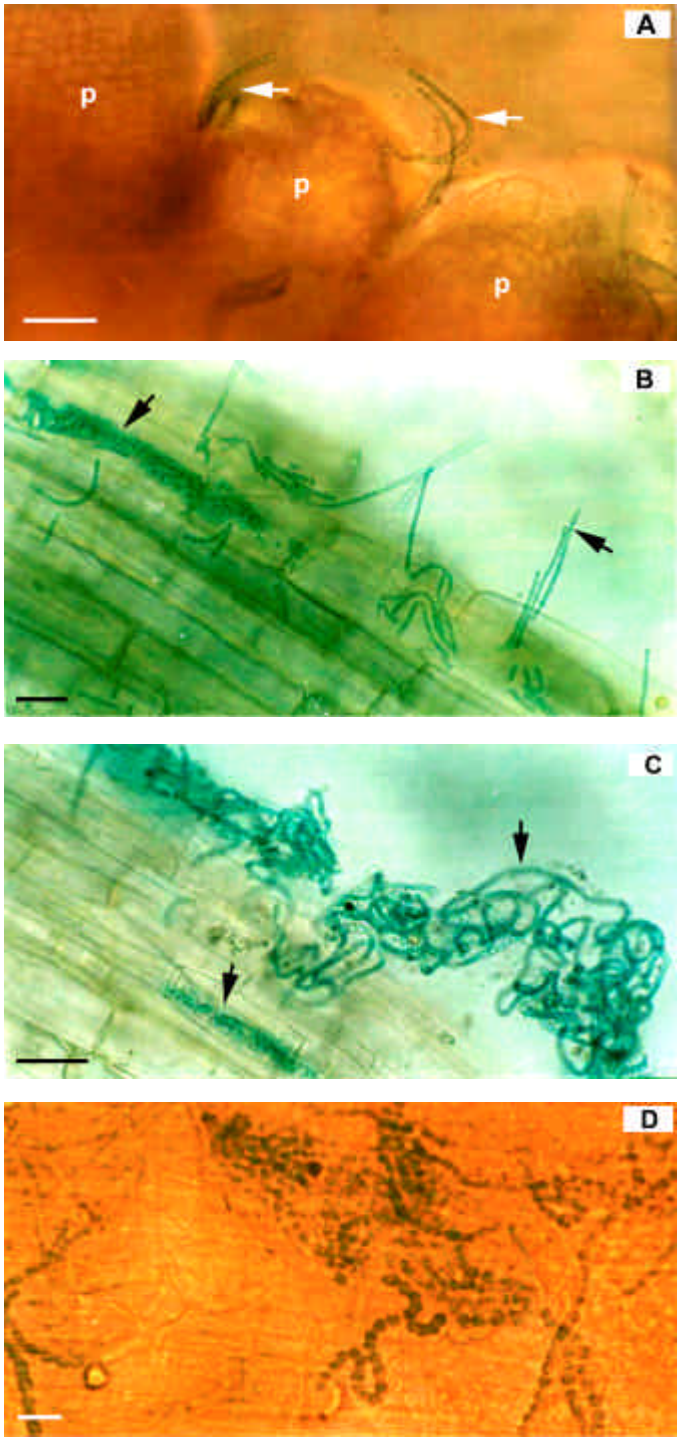

Fig. 3: A, colonization of para - nodulated wheat plants at the junction of the para-nodules with the root and at the top; B and C, straight and tightly packed filaments of *N. rivulare*; D, filaments adhered to root surface. photographed are root segments washed with a strong water stream . Arrows refer to appearance and position Nostoc filaments. p, para-nodules. Scale bars in A and C equal 50 μm; in B and D equal 20 μm

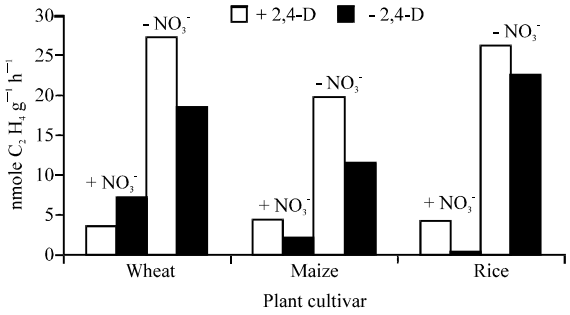

Fig. 4: Nitrogen-fixing activity by ARA (nmole $C_2H_4g^{-1}h^{-1}$) of *Nostoc rivulare* associated with wheat, maize and rice seedlings (15 days old) as affected by the presence or absence of 2,4-D and nitrate. 2,4-D dose = 1 ppm

those non-treated with 2,4-D. Nitrogenase activity by *N. rivulare* association was increased up to eight times in the absence of nitrate as compared with nitrate treated plants.

DISCUSSION

Preliminary studies showed that germinating wheat seedlings treated with high concentration of 2,4-D (5 ppm) alone (data not shown) exhibited fungal contamination compared with control plants in spite of sterilization of seeds with $HgCl_2$ (0.1%). This may be due to that at high 2,4-D concentration, the auxin-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 tested plants in all our experiments. Christiansen-Weniger (1997), indicated that the use of systemic fungicides such as Benomyl and Mancozeb in such experiments reduced the percentage of lost seedlings to 10% compared with 40-50% without fungicide, that author also indicated that the addition of fungicide did not affect root development.

The application of 2,4-D up to 1 ppm was also adopted for general use for para-nodule induction by several investigators (Tchan and Kennedy, 1989; Tchan and Zeman, 1995; Abdel Wahab *et al*., 1995). Moreover, this concentration of 2,4-D is 350 times lower than the dose currently applied as a herbicide (Glagoleva *et al*., 1997). It was also noted that better results were obtained by adding 2,4-D after the root system had reached a length of about 5 cm to allow enough space for para-nodule induction (Zeman *et al*., 1992).

Results of this study show that inoculation of plants with *N. rivulare* had stimulatory effect on plant growth. These results are in accordance with the earlier findings of Obreht *et al.* (1993), Rai *et al.* (2000) and Rai and Bergman (2002), who established that strains of *Nostoc* and Anabaena have been competent to colonize and positively affected plant growth with high contribution of N_2 fixing activity in both liquid and soil cultures.

Light microscopic studies revealed that *N. rivulare* successfully colonized para-nodules externally both at their basal connection with the root and on the epidermal root surfaces. Recently, Gantar and Elhai (1999), 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-nodule. Thus cyanobacteria were detected intercellularly, but never inside plant cells. Gantar (2000a) also indicated that *Nostoc* sp. strain 2S9B often appeared between the cells in a seriate state, in which filaments were tightly packed and contorted, in contrast with straight filaments that could be seen in free-living cultures.

Nitrogenase activity attributed to *N. rivulare* colonized roots was measured at ambient concentration of O_2, which indicates the role played by heterocysts that protect nitrogenase. Fay (1992) investigated the role played by heterocysts to protect nitrogenase and kept it active even at 20 Kpa of O_2.

The stimulatory effect of 2,4-D on nitrogenase activity specially in the absence of nitrates could be explained on the basis that either a) the auxin increases the amount 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 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 the cyanobacterium alone. Moreover, Gantar (2000 a, b) and Gantar and Elhai (1999), reported three times increase in the rate of acetylene reduction when wheat seedlings were treated with 2,4-D and co-cultivated with Nostoc sp. strain 2S9B, than untreated but colonized roots.

Stimulatory effect of the absence of combined N on nitrogenase activity which 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 N_2 fixing capacity (Flores and Herro, 1994). The inhibitory effect of high combined N levels on nitrogenase activity was reported earlier by Stewart *et al.* (1967).

Results of this study demonstrated that wheat, maize and rice para-nodules can be efficiently colonized by *N. rivulare* providing favourable conditions for N_2 fixation. Thus, it appears that *N. rivulare* is a promising organism for achieving efficient association between cyanobacteria and nonlegumes. 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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