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Effect of Inoculation and Nitrate on Nitrate Reductase Activity and Acetylene Reduction Activity in *Lotus* sp.-*Rhizobium loti* Symbiosis

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Abstract

The effects of nitrate and the *Rhizobium* strain used in the inoculation on acetylene reduction activity (ARA), nitrate reductase activity (NRA) and nitrate concentration in tissues of two *Lotus* sp. species inoculated with different *Rhizobium loti* strains were examined. ARA in the symbiosis *Lotus corniculatus-R. loti* T_1 strain was inhibited 90% by nitrate and 36% in the *L. tenuis-R. loti* Y_3 symbiosis. These results suggest that the nitrate inhibition is influenced by the legume-rhizobia combinations. NRA in leaves and nitrate concentration in stems were modified by inoculation. *L. tenuis* and *L. corniculatus* nodulated by *R. loti* T_1 strain had lower nitrate concentration in the stem than when the same plants were nodulated by *R. loti* Y_3 and U226 strains and the non-nodulated plants. Inoculation increased the NRA expression in leaf tissues of *Lotus* sp. nodulated by *R. loti* T_1 strain and the nitrate concentration was similar to that found in non-nodulated plants.

Keywords: *Lotus* sp., acetylene reduction activity, nitrate, nitrate reductase, *Rhizobium loti*, symbiosis

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1. Introduction

Members of the legume family may use nitrogen derived from symbiotic fixation and nitrate present in the soil. Nitrate inhibits the nitrogen fixation process, preventing nodule development and decreasing nitrogenase activity (Streeter, 1985).

Nitrate reductase (NR) catalyzes the reduction of nitrate to nitrite and is a good example of an enzyme inducible by its substrate (Campbell, 1988). Leguminous plants, like many other higher plants, can reduce nitrate in leaves, stems and roots. The stems of a wide range of legume species express high levels of nitrate reductase activity (NRA) (Andrews et al., 1984). Root nodules and bacteroids also contain an active NR (Kennedy et al., 1975; Becana et al., 1988; Stephens and Neyra, 1983).

The extent to which different plant organs participate in nitrate reduction and accumulation may vary with plant age, species, concentration of nitrate in the growth medium, strain of rhizobia that participate in the symbiosis, and environmental conditions to which the plant is exposed (Andrews, 1986; Ligero et al., 1987). Nodulation decreases nitrate absorption (Wych and Rains, 1978) and depresses NRA in leaves of soybean plants (Conejero et al., 1986). Ligero et al. (1987) found that nodulation affects NRA distribution and nitrate concentration in different tissues of pea cultivars. This modification in the NRA expression is due to interactions between the plant genotype and *Rhizobium* strain.

In preliminary studies, *Lotus corniculatus* plants inoculated with two strains of *R. loti* had equal acetylene reduction activity (ARA), but the plants inoculated with one of the strains showed higher NRA and total nitrogen content. This behavior could be attributed to the fact that the strain increased the NRA of the plant host affecting the plant nitrogen content (Monza et al., 1989).

Because nitrate affects ARA and nodulation influences nitrate metabolism we designed this study to obtain more information about the effect of nodulation by specific strains on the NRA and nitrate concentration in plant tissues of two *Lotus* species. We also examined the effect of nitrate on the ARA of six plant-bacteria combinations.

2. Materials and Methods

Plant material

Lotus corniculatus cv. La Estanzuela San Gabriel (Agrosan S. A., Montevideo, Uruguay) and *L. tenuis* cv. Chajá (obtained from J. Coll. INIA La Estanzuela,

Colonia, Uruguay) were grown in controlled conditions under 16/8 h light/dark cycle, 24/18°C day/night temperature and a photosynthetic photon flux density of 500 μmol m^{-2} s^{-1} (400 to 700 nm) supplied by a combination of fluorescent and incandescent lamps. Seeds were surface-sterilized, germinated and planted in sterile 0.5 l Leonard jar assemblies containing a mixture of river sand-vermiculite in a 3:1 ratio.

Plants were grown in sterile nutrient solution (Rigaud and Puppo, 1975) containing 1 mM KNO_3. The level of nutrient solution was kept approximately constant by changing it once a week and 48 hours before the assay. Jars containing 7 plants were inoculated with 10^8 rhizobia cells/plant. One set of each species remained uninoculated and was supplied with 1 mM KNO_3 while another set was inoculated but not supplied with KNO_3 so as to prove nitrate inhibition on ARA.

Bacteria

R. loti T_1, Y_3 (Monza et al., 1992) and U226 (B816 strain obtained from the Department of Microbiology, University of New South Wales, Kensington, Australia) strains were used. The cultures were maintained asymbiotically on M79 slopes (Vincent, 1970) or as frozen suspensions containing 60% glycerin at –70°C.

Acetylene reduction assay

Nodule activity was estimated by the ARA (Hardy et al., 1973). Although ARA measured in closed vessels does not represent the true nitrogenase activity (Vessey, 1994; Minchin et al., 1994), it can be adequate in comparative experiments under controlled conditions (Cordovilla et al., 1994; Sicardi de Mallorca and Izaguirre Mayoral, 1993; Vessey, 1994).

Whole nodulated-roots were incubated in a hermetic flask wherein 10% (v/v) of air was substituted by acetylene gas. After 30 min of incubation (period wherein the ethylene production was linear) the ethylene produced was analyzed by a gas chromatograph provided with a flame ionization detector. ARA was expressed as μmol C_2H_4 formed g^{-1} nodule fresh weight h^{-1}.

Nitrate reductase assay

Determination of NRA was done following the *in vivo* assay described by Heuer and Plaut (1978) with slight modifications. Leaves, stems (5 mm width strips) and roots (10 mm length pieces) were harvested from 45 day old plants. Tissue samples weighing 0.05 g were placed into tubes containing 5 ml of an

incubation medium composed of 50 mM potassium phosphate buffer (pH 7.5), propanol 0.15 M, and Triton X-100 0.01%; with or without the addition of 100 mM KNO_3 (designated $+NO_3^-$ and $-NO_3^-$, respectively). Determination of NRA by this method gives two estimates which are considered to be indicators of the capacity of plant tissues to reduce nitrate (Andrews et al., 1984 and references therein). The tissues were vacuum-infiltrated twice at 50 kPa for 2 minutes each time. Mixtures were incubated in darkness at 30°C for 1 hour on a shaker (40 rpm) and then the tubes were boiled in a water bath for 10 min.

Nitrite was determined in 1 ml samples by the addition of 4 ml sulfanilamide-N-naphtylethylenediamine reagent (Nicholas and Nason, 1969). Absorbance was read at 540 nm and the NRA was expressed as µmol NO_2^- formed g^{-1} fresh weight h^{-1}.

Determination of nitrate

Nitrate present in plant tissue was extracted by adding 5 ml of boiling water to 0.1 g tissue samples and the tubes were boiled in a water bath for 10 min (Soares et al., 1985). Nitrate was analyzed by using the nitration salycilic acid method described by Cataldo et al. (1975). Samples of 0.2 ml were mixed with 0.8 ml of 5% (w/v) salycilic acid in concentrated H_2SO_4 and incubated for 20 min. This procedure was followed by the addition of 19 ml 2 M NaOH. Absorbance was read at 410 nm and the concentration was expressed as the µmol NO_3^- g^{-1} fresh weight.

Statistical design and analysis

The layout for the experiment was a randomized block design and the results were subjected to analysis of variance test. The experiment was repeated twice and NRA, ARA and nitrate concentration determinations were done in quadruplicate.

3. Results

Effect of nitrate on the ARA

No significant differences in the ARA were detected between *L. corniculatus* and *L. tenuis* nodulated by any of the three strains in the absence of nitrate (Table 1).

Table 1. ARA (expressed as μmol C_2H_4 g^{-1} FW nod. h^{-1}) in *Lotus* sp.nodulated by *R. loti* without and with 1 mM KNO_3 in the growth medium.

Host	Strain	Without NO_3^-	With NO_3^-	Inhibition (%)
L. corniculatus	T_1	20.2 ± 4.3	2.0 ± 0.8	90
	Y_3	24.2 ± 2.0	12.3 ± 2.6	49
	U226	20.5 ± 3.2	3.2 ± 1.0	84
L. tenuis	T_1	21.5 ± 3.0	6.5 ± 1.5	70
	Y_3	22.9 ± 1.5	14.6 ± 1.1	36
	U226	24.5 ± 2.5	9.6 ± 3.0	60

Values are mean of four replicates ± standard error of the mean.

The patterns of nitrate-mediated ARA varied among the different combinations of symbiotic partners. The addition of nitrate to growth media resulted in a higher ARA inhibition in the *R. loti* T_1 and U226 strains than in the *R. loti* Y_3 strain in plants of either species (Table 1). This inhibition varied between 49 and 90% for the *L. corniculatus* symbiosis and 36 and 70% for the *L. tenuis* symbiosis (Table 1).

NRA in tissues of nodulated and non-nodulated plants

In *L. corniculatus* and *L. tenuis* nodulated plants, the analysis of the effect of each bacterial strain on NRA ($+NO_3^-$) indicated that leaf tissue NRA was significantly higher in plants nodulated by *R. loti* T_1 strain than in plants by the others strains or in non-nodulated plants (Table 2). The values of NRA ($+NO_3^-$) assay showed no differences between nodulated and non-nodulated plants (Table 2).

The stem NRA ($+NO_3^-$) of *L. corniculatus* was not modified by nodulation and was generally lower than in *L. tenuis*, considering both nodulated and non-nodulated plants (Table 2).

No host or strain dependent differences were found in root NRA, and NRA ($+NO_3^-$) behavior was similar to NRA ($+NO_3^-$) in both species.

Table 2. NRA (expressed as μmol NO_2^- g^{-1} FW h^{-1}) in leaf, stem and root of *Lotus* sp. inoculated by *R. loti*. Plants were grown with 1 mM KNO_3 during 45 days.

		Non-nodulated	Nodulated by strain T_1	Y_3	U226
In vivo $+NO_3^-$					
Leaf	*L. corniculatus*	0.84 c	1.30 a	0.84 c	0.65 c
	L. tenuis	0.73 c	1.05 b	0.82 c	0.64 c
Stem	*L. corniculatus*	1.07 bc	1.18 ab	0.98 c	0.95 c
	L. tenuis	1.29 a	1.22 ab	1.24 a	1.26 a
Root	*L. corniculatus*	0.35 a	0.38 a	0.40 a	0.47 a
	L. tenuis	0.42 a	0.40 a	0.32 a	0.33 a
In vivo $-NO_3^-$					
Leaf	*L. corniculatus*	0.69 abc	0.63 bcd	0.55 cd	0.54 cd
	L. tenuis	0.60 bcd	0.84 a	0.74 ab	0.48 d
Stem	*L. corniculatus*	0.87 abc	0.90 ab	0.97 a	0.82 abc
	L. tenuis	0.87 abc	0.78 bc	0.98 a	0.72 c
Root	*L. corniculatus*	0.38 a	0.42 a	0.28 a	0.44 a
	L. tenuis	0.32 a	0.30 a	0.35 a	0.30 a

Means in each plant organ were statistically compared by LSD. Numbers within each organ followed by the same letter(s) are not statistically different at $p<0.05$.

Nitrate concentration in tissues from nodulated and non-nodulated plants

Leaf and stem nitrate concentration of non-nodulated plants was higher in *L. corniculatus* than in *L. tenuis*. Root nitrate concentration was not significantly different in the two species (Table 3).

Leaf nitrate concentration of nodulated plants was higher in *L. corniculatus* than in *L. tenuis* and was not affected by the nodulating strain. Stem nitrate concentration in both species nodulated by *R. loti* T_1 strain was significantly lower than in plants nodulated by *R. loti* Y_3 and U226 strains and in non-nodulated plants (Table 3). Nitrate concentration in roots of nodulated plants was similar to that found in non-nodulated plants (Table 3).

Table 3. Nitrate concentration (expressed as μmol NO_3^- g^{-1} FW) in leaf, stem and root of *Lotus* sp. inoculated by *R. loti* and grown with 1 mM KNO_3.

		Non-nodulated	Nodulated by strain T_1	Y_3	U226
Leaf	*L. corniculatus*	17.8 a	12.4 ab	14.5 ab	11.7 abc
	L. tenuis	9.9 bcd	5.2 cd	5.5 cd	4.7 d
Stem	*L. corniculatus*	53.5 a	13.4 d	39.5 b	19.9 d
	L. tenuis	39.2 b	15.9 d	42.6 b	31.5 c
Root	*L. corniculatus*	12.6 a	13.5 a	11.5 a	12.7 a
	L. tenuis	9.2 a	14.1 a	13.2 a	11.9 a

Means in each plant organ were statistically compared by LSD. Numbers within each organ followed by the same letter(s) are not statistically different at $p<0.05$.

4. Discussion

Nitrate reduction and nitrogen fixation greatly contribute to the total nitrogen pool in legumes.

The inhibitory effect of nitrate on nitrogen fixation activity of legume root nodules has been under investigation for some time. While various hypotheses have been proposed to account for these phenomena, no single regulatory mechanism has been accepted (Streeter, 1985; Vessey et al., 1988).

It is well known that the degree of nitrate inhibition on the fixation activity varies among different legume-rhizobia combinations (Manhart and Wong, 1980). But, there are few reports about the effects of combined nitrogen on nitrogen fixation in different legume species nodulated by the same rhizobial isolate.

When the plants grew without nitrate ARA in nodules formed by the three bacterial strains in both plant species averaged 22.6 μmol C_2H_4 g^{-1} FW nod. h^{-1} and there were no significant differences among them (Table 1). However, ARA of the symbioses formed by *R. loti* Y_3 strain were less inhibited by nitrate than the symbioses formed by *R. loti* T_1 and U226 strains. These findings confirm that the *Rhizobium* strains in the symbiotic condition differ in their reactions to nitrate. Differences among strains in their response of ARA to nitrate addition were also obtained by other authors working with *P. sativum* (Nelson and Edie, 1988), *Phaseolus vulgaris* (Streeter, 1986) and *Glycine max* (Hardarson et al., 1984) nodulated plants.

Legumes differ in their response to nitrate (Lang et al., 1993). Manhart and Wong (1980) working with *Vigna unguiculata* and *Lupinus augustifolius* nodulated by the same *R.* sp 127E15 strain found differences among plant species in tolerance of nitrogen fixation to nitrate. Moreover, other authors (Chalifour and Nelson, 1987) have reported the same differences working with *Vicia faba* and *Pisum sativum*, both species nodulated by *R. leguminosarum* 175F9 and 17519 strains. But, it is important to point out that these studies were carried out with plants of different genera.

The data show that the ARA of *L. tenuis-R. loti* symbiosis was less inhibited by nitrate than the ARA of *L. corniculatus-R. loti* symbiosis. This result indicates that the tolerance to nitrate inhibition is also influenced by the plant species. However, these results do not allow us to determine which are the mechanisms used by the symbiotic partners to tolerate nitrate inhibition.

The effect of nodulation on nitrate utilization by the legume is well documented (Conejero et al., 1986; Hervas et al., 1991; Ligero et al., 1987 and Wych and Rains, 1978). In this work the results showed that nodulation influenced the NRA and nitrate concentration in leaves and stems of *Lotus* sp. grown with 1 mM KNO_3 (Tables 2 and 3).

Nodulation by *R. loti* T_1 strain increased the NRA of leaf tissues in *Lotus* sp. (Table 2); however, nitrate concentration was similar to that found in the leaves of non-nodulated plants (Table 3). The effect of the bacterial strain on NRA was also obtained in plant roots by Hervas et al. (1991) working with *Pisum sativum* nodulated by *R. leguminosarum* GRL19 and GRA19 strains. The discrepancies between the data obtained by Hervas et al. (1991) and the ones reported here might be related to intergeneric differences and the rhizobia strains used for inoculation. Nevertheless, the results presented here provide additional evidence of the effects of *Rhizobium* strains on the NRA expression in plant tissues.

Parsons et al. (1993) have suggested that the nitrogen solutes in the xylem and phloem stream may be a mechanism of regulation that could operate between the two symbiotic partners. In addition, Li and Gresshoff (1990) have reported allantoic acid which is synthesized in the nodule, as a possible signal molecule in soybean responsible for the increase NRA in leaves. A possible explanation for our results could be attributed to a nodule nitrogenous product that is transported to the leaves affecting NRA.

Leaf NRA was higher in nodulated plants but did not show any change either in the stem or root when compared with the non-nodulated plants. However, Ligero et al. (1987) found, in *Pisum sativum*, that stem NRA in plants nodulated by *R. leguminosarum* strains increased while it decreased in roots and leaves in reference to non-nodulated plants. These differences suggest that

NRA expression is influenced by both nodulating rhizobia strain and plant genotype.

Nodulation affected the nitrate concentration in stems. *L. tenuis* and *L. corniculatus* nodulated by *R. loti* T_1 strain had lower nitrate concentration in the stem than the other symbioses and the non-nodulated plants (Table 3). However, the same NRA was found in all stems (Table 2). Nitrate concentration of plants nodulated by *R. loti* T_1 strain would be enough to maintain the NRA expression in spite of the decrease in the stems. NRA values obtained in this work are equal to those reported in a preliminary study (Monza et al., 1989) that used 5 mM KNO_3 in the growth medium. This fact suggests that the nitrate concentration in the growth medium used in this work (1 mM KNO_3) is enough to obtain the maximum NRA values.

A correlation was found between nitrate concentration and NRA in roots ($r = 0.982$, $p = 0.001$); however, this correlation did not occur in stems and leaves. These results are different from those obtained by Hervas et al. (1991) who found a correlation between nitrate and NRA in stems and leaves, but not in roots, working with *Pisum sativum*. This could be related to the data shown by several authors in reference to the fact that nitrate content is not the only factor of the plant tissues regulated NRA (Shaner and Boyer, 1976; Srivastava, 1988).

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