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NITRATE LEVELS AND STAGES OF GROWTH IN HYPERNODULATING MUTANTS OF *LUPINUS ALBUS*. I. N_2 FIXATION POTENTIAL

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ABSTRACT

This work aimed to evaluate physiological parameters, nodulation response and N_2 fixation rate in mutants of *Lupinus albus* in comparison with the standard Multolupa cultivar. Two nitrate levels (0 and 5mM) and two evaluation periods (7 and 10 weeks) were used. Significant differences were observed among genotypes, in relation to fresh nodule weight, nitrate levels and growth stages. The overall average for nitrate level differed between them where 5mM severely inhibited the number of nodules, reaching a 49.5% reduction in relation to treatment without nitrate. There were no behaviour differences among genotypes, nor among evaluation periods. Although the level of nitrate did not influence the production of shoot dry matter in relation to the average among levels applied, the L-135 genotype, being an inefficient mutant, reached very low values. There were no significant differences in electron allocation coefficient (EAC) among nitrate levels, nor among genotypes studied. However, the evaluation periods revealed differences, where the EAC for the seventh week had a higher value than that for the tenth week, when a 5mM aplication was evaluated. The N_2 fixation rate (N_2 FIX) showed the existence of the nitrate interference in fixation, given that the application of 5mM severely reduced. However, there were no differences among the genotypes and it was noted that the fixation rate was much higher in those that received nitrate. The L-88 and L-62 genotypes were the ones that have shown best adaptability in this experiment, thus being able to be recommended for new studies with higher nitrate levels and different evaluation periods. The nitrate (5mM) interferes in the nitrogen fixation rate, given that all the genotypes were affected by the level applied.

Key words: Mutants, electron allocation coefficient, nitrogen fixation rate, *Bradyrhizobium* sp (*Lupinus*).

INTRODUCTION

Multiple effects of combined N on the legume nodulation function are conveniently divided into three classes: (a) The detrimental effect on the infection of legume roots by *Rhizobium*; (b) The negative effect of N on nitrogenase activity; c) The influence on nodule weight per plant (14). However, application of combined N to legumes may be required for maximization of yields and, therefore,

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economically justified. The mechanisms ruling the adverse effects on nodulation must be understood and controlled in such away that a manner of correcting the problem may be devised. Several forms of environmental stress and the supply of mineral N reduce nodulation and nitrogenase activity without altering the total N concentration in the plant tissue.

The inhibitory effects of nitrate could be accounted for by alterations in the partitioning of reducing sugars in soybean (16), alleviation of nitrate inhibition on nitrogenase activity by sucrose has been for intact pea plant. The reduction in carbohydrate transport to nodules could simply be a result of the loss of nitrogenase activity, i.e. the decline in photosynthates might lag behind the loss of activity in nitrate treated plants. According to Streeter (14), carbohydrate composition was similar in both high nitrate and zero nitrate treatments, suggesting that nitrogenase inhibition is linked to the availability of carbohydrates. Otherwise, it is well known that nitrate limits the infection process, the development of nodules, the subsequent expression of nitrogenase activity in bacteroids as well as hastening the breakdown of nodule tissue (6). The NO_3^- inhibited nodulation in nts (nitrate tolerant symbiosis) mutants. The difference is probably due to the different nitrate levels used, cultivation conditions, NO_3^- addition methods and growth period (4). The same authors showed that all mutants were sensitive to nodulation and N_2 fixation repression at high NO_3^- levels and suggest that the NO_3^- tolerant phenotype is the result of a failure in the self-regulation mechanism and not the mechanism responding to the NO_3^- in itself.

The influence of nitrate remains unclear, especially in the initial stages of nodulation, where there is no evidence as to whether the effects are due to the external presence of nitrate or to the events that occur following uptake and/or metabolism. Cho and Harper (3) provided evidence that the site of N-application primarily controls nodulation inhibition, probably through a decrease in the internal levels of root isoflavonoids. Also, the authors provided clear evidence that nitrate has an adverse effect on roots isoflavonoid compounds. A possible direct effect of nitrate on nodulation has been indicated by split-root studies with soybean, and by excised root experiments with common bean. In both kinds of research, Raggio *et al*. (12) demonstrated a positive nitrate and carbohydrates interaction on rhizobial root nodule formation. In another work, the incorporation of ammonium nitrate near the nodule zone (crown region) was more inhibitory to nodulation than was its placement below this zone (7).

This report compares the selected physiological parameters, nodulation response and N_2-fixation rate of the parent wild type *Lupinus albus* cv. Multolupa to two hypernodulating mutants, L-280 and L-88, one nitrate resistent mutant L-62, and one inefficient mutant L-135. Comparisons among cv. Multolupa and mutants at two growth stages are also reported.

MATERIALS AND METHODS

The following *Lupinus albus* plant materials were used (Table 1), cultivar Multolupa (Wild-type, nod^+ fix^+) and mutants selected from H_2 families derived from Multolupa seed treated with ethyl methanesulfonate (0.04M), designed L-280, L-88, and treated with sodium azide (0.002M), designed L-62 and L-135. According to previous work done by C.I.D.A., Centro de Investigación y Desarrollo Agrario, Sevilla – Spain, the main characteristics of

Table 1. Characteristics of the *Lupinus albus* cv. Multolupa mutants.

Species	Selection year	Product utilized for mutagenesis process	Genotypes	Characteristics
Lupinus albus	1988	[1]EMS	280	High nodulation and Nif^+
Lupinus albus	1988	EMS	88	High nodulation and Nif^+
Lupinus albus	1989	[2]NNA_3	62	Resistant to NO_3^-
Lupinus albus	1989	NNA_3	135	Inefficient (f^-)
Lupinus albus cv. Multolupa	-	-	-	Control

[1]EMS - Ethyl methane sulfonate
[2]NNA_3 - Sodium azide

the cv. Multolupa mutants used in the study were: L-280 nod^+ fix^+; L-88 nod^+ fix^+; L-62 nod^-, resistant to NO_3^- and inefficient L-135 (nod^- fix^-).

Seeds of *Lupinus albus* cultivar Multolupa and selected mutants were scarified and surface sterilized by treating with ethanol (70%) for 20 min followed by 2 washes in sterile distilled water, before being planted in 2.5l Leonard jars using perlite as growth medium. After uniform germination of seedlings, three plants per jar remained for the experimental period. Innoculation was carried out twice, at planting and one week later, by placing 5 ml of a suspension containing 10^7-10^8 cells per ml of a strain mixture of *Bradyrhizobium* sp (*Lupinus*), namely L-750 and L-18C_2, with constitutive nitrate reductase, which belong to the C.I.D.A. collection.

The experiment was carried out during Autumn 1992, in a greenhouse at a temperature of 25°C during the day and 15°C at night. A N-free solution (4) was periodically supplied to the jars, and the nitrate level was built up by adding the appropriate volume of 5mM KNO_3. Additions of nutrient solution and KNO_3 were necessary for maintenance of the rooting medium as well as the combined N at the desired level.

The experiment was conducted according to a completely randomized block design, with four replications. Treatments were formed by a factorial arrangement with five genotypes, two growth stages (7 and 10 weeks) and two nitrate levels (0 and 5mM). F-tests for significance (0.05 level) were based upon ANOVA; where F-tests were significant, the LSD values were calculated to compare treatment means.

The plants were harvested at two growth stages, 7 and 10 weeks after emergence. At each harvest, the apparent nitrogenase activity (ANA) and total nitrogenase activity (TNA) were determined by measurements of H_2 evolution in air and ArO_2, respectively, of the whole root system incubated for 30 min., using a FI detector and a Poropak R column in a Shimadzu GC model RIA, as described in Chamber-Perez and Iruthaythas (1988). The N_2 fixation rate (NFR) was calculated = TNA-ANA/3 and also the electron allocation coefficient of nitrogenase (EAC)=(1-TNA/ANA) according to Hunt *et al*. (1987). Shoots and roots were dried (70°C for 72 hours) and weighed, and their total nitrogen measured with a Technicon 300B analyzer.

RESULTS AND DISCUSSION

In the fresh nodule weight (FNW) data shown in Table 2, significant differences among genotypes, nitrate levels and growth stages can be observed. The average nitrate level (5mM NO_3^-) significantly reduced the weight of the nodules, resulting in a value of 1.02g, compared to 1.74g in absence of nitrate. Comparing the behaviour of the genotypes at the 0mM level, L-88 was the one which produced the greatest nodule weight, similar to L-62, indicating that the inherent characteristics of these genotypes influenced the results, given that L-88 is a hypernodulating mutant and that L-62 is nitrate resistant. In spite of the lower values of FNW at 5mM in all genotypes, L-62 and L-88 showed the weights of 1.46 and 1.65g, respectively. Another factor that deserves attention is that the innefficient L-135

Table 2 -Effect of nitrate level and growth stage on number of nodules (NN) and fresh nodule weight (FNW) in five genotypes of *Lupinus albus*

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		NN	FNW	NN	FNW	NN	FNW	NN	FNW	NN	FNW	NN	FNW	NN	FNW
N-level	0mM	116.8aA	1.5cdA	101.9aA	2.2abA	170.9aA	1.0dA	134.2aA	2.5aA	160.1aA	1.6bcA	135.6A	1.7A	10.6	0.7
	5mM	56.6aA	0.5bB	43.6aA	1.5aB	95.4aA	0.7bA	66.4aA	1.6aB	80.0aA	0.8bB	67.2B	1.0B		
	LSD[2]	3.4	0.2												
Growth stage	7 weeks	79.8bA	0.8cA	69.9bA	1.7aA	206.2aA	0.6cA	95.6abA	1.6abB	117.8abA	1.0bcA	109.4A	1.2B		
	10 weeks	88.3aA	1.2cA	69.6aA	1.9abA	71.9aB	1.0cA	99.1aA	2.5aA	115.5aA	1.4bcA	88.0A	1.6A		
	LSD[3]	3.4	0.2												
	Overall Mean	84.0a	1.0b	69.7a	1.8a	130.5a	0.8b	97.3a	2.1a	116.6a	1.2b				
	LSD[4]	7.5	0.5												

[1] LSD (0.05), between N levels and growth Stages, averaged across *Lupinus* genotypes
[2] LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] LSD (0.05), between two Growth Stages within a *Lupinus* genotype
[4] LSD (0.05), between *Lupinus* genotypes, averaged across N levels and Growth Stages

mutant did not differ in relation to the levels applied. These results are similar to those obtained by Carrol *et al*. (1), in which some Bragg soy mutants nodulated in the presence of a continuous supply of 5.5 mM NO_3^- in sand culture. These mutants, however, are intermediate or extremely supernodulating and have an altered self-regulating response. Similar behaviour was observed by Jacobsen and Feenstra (9), who studied a new mutant of *Pisum sativum* var Rondo with efficient nodulation in the presence of nitrate and noted that the use of 15mM KNO_3 strongly inhibited the cv. Rondo nodulation. These authors observed the behaviour of the mutant in relation to the cv. Rondo, and noted that without nitrate the mutant presented better nodulation. However, the concentration of nitrate was three times higher than that used in the present study. They also observed that the "high nodulation" mutant characteristic is monogenic and recessive. Nodulation behaviour in the nitrate medium differed from the *nod1 nod2* lineage, which showed great inhibition by 15mM nitrate, similar to the standard strain from which our mutant was derived. The mutant, however, was highly resistant to nitrate. The mutant gene was designated *nod3*. The analysis of growth stages showed that 10 weeks produced the highest values of FNW, perhaps due to the culture cycle, being this period the best time for the evaluation of this parameter. In 10 weeks, the L-88 genotype was the one which presented the highest value, which was different of the value at 7 weeks. The other genotypes showed no differences. On comparing the genotypes at the 7 week period separately, the nitrate resistant L-62 showed the highest value, not differing from L-88. However, at the tenth week, these genotypes showed higher values than the others, and L-88 presented a higher FNW than L-62.

Table 2 also shows that the nitrate average level differed among genotypes and 5mM strongly inhibited the number of nodules (NN), reaching a reduction of 49.5% in relation to treatment without nitrate addition. This Table also shows that there were no differences, in the behaviour of genotypes, and in the periods evaluated.

Table 3 indicate that the nitrate level did not influence the production of shoot dry matter (SDM). However, a difference among the genotypes was observed: at 0mM, the L-88 and L-62 genotypes presented SDM of 6.70 and 6.40g, respectively. Being an inefficient mutant, the L-135 genotype reached extremely low values of SDM. At 5mM level, genotypes showed similar behaviour, demonstrating that even in the absence of significant differences, the application of 5mM in some way affects the production of dry matter in *Lupinus*. These data disagree with Lee *et al* (10), who, in studying regulation in the nodules development in supernodulating mutants and soy standard, characterized the supernodulating mutants by lower root and shoot dry matter and high nodulation. In the nitrate tolerant mutants that showed lower root and shoot growth, with high number of nodules and dry nodule weight, they suggested that nitrate tolerant mutants are supernodulating due to the absence of a

Table 3 - Effect of nitrate level and growth stage on shoot dry weight (SDW) and root dry weight (RDM) in five genotypes of *Lupinus albus*, a control genotype (Multolupa); two hypernodulating (L-88; L-280); one nitrate resistant (L-62) and one inefficient genotype (L-135).

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW
N-level	0mM	3.8 bA	0.8bcA	6.4 aA	0.9abA	1.6 cA	0.4cA	6.7 aA	1.2 aA	3.9 bA	0.8abcA	4.5 A	0.8 A	1.8	0.4
	5mM	2.6 bA	0.6 cA	5.8 aA	1.1abA	2.2 bA	0.5 cA	6.6 aA	1.5 aA	3.0 bA	0.8bcA	4.0 A	0.9 A		
	LSD[2]	0.6	0.1												
Growth stage	7 weeks	2.2 bB	0.6 ca	5.6 aA	1.3[a]bA	1.4 bA	0.6 cA	5.7 aB	1.5 aA	3.2 bA	0.9 bcA	3.6 B	1.0 A	1.8	0.4
	10 weeks	4.1 bA	0.7 bA	6.6 aA	0.8bB	2.5 bA	0.4 bA	7.6 aA	1.3 aA	3.8 bA	0.8 bA	4.9 A	0.8 A		
	LSD[3]	0.6	0.1												
	Overall Mean	3.2 bc	0.7 cd	6.1 a	1.0 b	1.9 c	0.5d	6.7 a	1.4 a	3.5 b	0.8 bc				
	LSD[4]	1.3	0.3												

[1] LSD (0.05), between N levels and growth Stages, averaged across *Lupinus* genotypes
[2] LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] LSD (0.05), between two Growth Stages within a *Lupinus* genotype
[4] LSD (0.05), between *Lupinus* genotypes, averaged across N levels and Growth Stages

nodulation inhibitor instead of the presence of a nodule development activator. However, this inhibitor has not been clearly identified. For SDM, the evaluation of growth stages revealed differences, where in the second period, the value of dry matter in grams was greater than when evaluated in the first period. Among the genotypes, L-88 showed an overall average of 6.66g and L-62 6.09g, clearly showing that these genotypes were those that adapted best to the treatments used in the experiment. In Table 3 with regard to root dry matter (RDM), behaviour was very similar in relation to the level of nitrate applied, although among genotypes at 0mM, L-62, L-88 and L-280 did not differ among themselves, the hypernodulating genotypes having obtained the highest values of RDM. At the level of 5mM, L-88 also behaved equally towards the non-application of nitrate, followed by L-62. The analysis of growth stages showed higher SDM values in the first evaluation period than in the second. This would suggest that the reduction in SDM had been due to the limitation that the roots underwent as a consequence of the size (only 2.5l) of the vessel in which the plants were cultivated.

Table 4 indicates that the electron allocation coefficient (EAC) was a parameter that showed no significant difference among nitrate levels, nor among the genotypes studied. In compensation, the evaluation periods revealed differences, where the EAC in the seventh week showed a higher value than in the tenth week, when it was evaluated on application of 5mM. These results can be considered when Serrano and Chamber (13) report that the lack of homology in the DNA sequence *Hup* in *Bradyrhizobium* sp (*Lupinus*) can reflect different catalytic or physical properties among the enzymes. Some of these may represent a different period of *Hup* activity over the N_2 fixation cycle and persistence under stress conditions, such as the presence of a high nitrate level in the medium. According to Evans *et al*. (5), the average relative efficiency (RE) in the fixation of nitrogen (percentage of electron flow) through the nitrogenase which is allocated by N_2 to the 22 strains tested in symbiosis with *L. augustifolius* was 0.53, which is similar to the average value reported for other symbiotic pairs. With regard to the inability of some *Hup+ in Lupinus* rhizobium to induce hydrogenase activity in symbiosis with *Lupinus*, Murillo *et al*. (1989) interpreted this as an effect of the host observed in the phenotypical expression of hydrogenase. According to these authors, the interpretations were based on the idea that genes other than the hydrogenase structural genes are involved in the availability of H_2.

Table 4 also presents the N_2 fixation rate (N_2 FIX), which shows the existence of the interference of nitrate in fixation, since the application of 5mM greatly reduced the rate, there being no differences among the genotypes. It was also noted that the fixation rate in the genotypes was higher in those that received no nitrate. Specific nitrogenase activity,

Table 4 - Effect of nitrate level and growth stage on electron allocation coefficient (EAC) and nitrogen fixation rate (N_2FX) in five genotypes of *Lupinus albus*, a control genotype (Multolupa); two hypernodulating (L-88; L-280); one nitrate resistant (L-62) and one inefficient genotype (L-135).

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX
N-level	0mM	0.9 aA	1.0 aA	0.7 aB	0.9 aA	0.9 aA	0.8 aA	0.8 aAB	1.2 aA	0.8 aAB	1.2 aA	0.8 AB	1.0 A	0.2	1.0
	5mM	0.8 aAB	0.4 bA	0.7 aB	0.4 bA	0.9 aA	0.3 bA	0.8aAB	0.6 bcA	0.8 aAB	0.3 cA	0.8 AB	0.4 A		
	LSD[2]	0.1	0.3												
Growth stage	7 weeks	0.9 aA	0.8 aA	0.8 aA	0.7 abA	0.9 aA	0.7 aA	0.9 aA	0.8 bA	0.8 aA	0.9abA	0.8 A	0.8 A	0.2	1.0
	10 weeks	0.8 aA	0.7 aA	0.7 aA	0.6 bA	0.8 aA	0.5 abA	0.8 aA	1.0 abA	0.8 aA	0.6 bcA	0.8 A	0.7 A		
	LSD[3]	0.1	0.3												
	Overall Mean	0.8 a	0.7 a	0.7 a	0.7 ab	0.9 a	0.6 ab	0.8 a	0.9 ab	0.8 a	0.8 b				
	LSD[4]	0.1	0.7												

[1] LSD (0.05), between N levels and growth Stages, averaged across *Lupinus* genotypes
[2] LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] LSD (0.05), between two Growth Stages within a *Lupinus* genotype
[4] LSD (0.05), between *Lupinus* genotypes, averaged across N levels and Growth Stages

in nodules of supernodulating plants, is reduced and there is little information available, that is an increase in nodulation results in an increase in fixation (4). According to Jacobsen and Feenstra (9), the reduction of acetylene per plant in a medium without nitrate was greater in the mutant than in the cv. Rondo, this also occurring with the genotypes used in the present work. According to Jacobsen and Feenstra (9), the effect of nitrate on the reduction of acetylene was related to competition for energy and/ or carbohydrates. However, studies have shown that the balance between nitrogen fixation and the reduction of nitrate in the mutant is different from the standard strain. Neither did the evaluation period interfere in the N_2 fixation rate, possibly owing to the periods in which they were analysed not having been the best for the genotypes studied.

From these results we can observe that L-88 and L-62 genotypes were those that adapted best in this experiment, thus being recomendable for new studies with higher nitrate levels and different evaluation periods. Generally speaking it has become clear that the nitrate (5mM) interferes in the N_2 fixation rate, given that all the genotypes were affected by the level applied.

RESUMO

Influência dos diferentes níveis de nitrato e estágio de crescimento em mutantes hipernodulantes de *Lupinus albus* I. potencial de fixação de N_2

Este trabalho teve como objetivo avaliar parâmetros fisiológicos, resposta da nodulação e a taxa de fixação de N_2 em mutantes de *Lupinus albus* comparando com a cultivar padrão Multolupa. Foram utilizados dois níveis de nitrato (0 e 5mM) e dois diferentes períodos de avaliação (7 e 10 semanas). No peso fresco dos nódulos, diferenças significativas entre genótipos em relação aos níveis de nitrato e estágios de crescimento foram observadas. Nível de nitrato de 5mM inibiu fortemente a produção de nódulos, chegando a ter uma redução de 49,5% em relação ao tratamento onde não se adicionou o nitrato. Em relação aos genótipos, os desempenhos não diferiram entre si, o mesmo acontecendo com os períodos avaliados. O nível de nitrato não influenciou na produção de matéria seca da parte aérea em relação à média entre os níveis aplicados. Entretanto, entre os genótipos existiu diferença, onde 0mM, os genótipos L-88 e L-62 apresentaram 6,7 e 6,4g, respectivamente. O genótipo L-135, por ser um mutante ineficiente alcançou valores extremamente baixos de matéria seca da parte aérea. Para o coeficiente de alocação de elétrons (EAC), não houve diferença significativa entre os níveis de nitrato, nem entre os genótipos estudados. Em compensação os períodos avaliados tiveram diferenças: na sétima semana a EAC apresentou valor superior à EAC na décima semana, quando foi avaliada na aplicação de 5mM. A taxa de fixação de N_2 (N_2 FIX) mostrou que existe interferência do nitrato na fixação, uma vez que, a aplicação de 5mM reduziu muito a fixação, apesar de que entre os genótipos não houve diferença entre si. Com estes resultados observamos que os genótipos L-88 e L-62 foram os que melhor se adaptaram podendo desta forma ser recomendados para novos estudos com maiores níveis de nitrato aplicados e diferentes períodos de avaliação. De uma forma geral ficou nítido que o nitrato (5mM) interfere na taxa de fixação de N_2, uma vez que, todos os genótipos foram afetados pelo nível aplicado.

Palavras-chave: Mutantes, coeficiente de alocação de elétrons, taxa de fixação de N_2, *Bradyrhizobium* sp (*Lupinus*).

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