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Response of nodulating and non-nodulating *Pisum sativum* L. to nitrate

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Abstract

This study examined whether 'Risnod2' and 'Risnod27' non-nodulating mutants of pea (*Pisum sativum* L.) provided with increasing concentrations of nitrate could achieve a growth and nitrogen accumulation comparable to their parental N_2-fixing cv. Finale. In the cv. Finale, nodule number, nodule dry mass accumulation, total C_2H_2-reducing activity of nodulated roots (TAR) and estimated N_2 fixation were considerably inhibited at 5.0 and 10.0 mM root medium NO_3^- concentrations. In contrast a 0.63 mM level stimulated both the nodule dry mass and TAR. The cv. Finale N_2-fixing plants grown on 0 to 2.5 mM NO_3^- levels had higher shoot N concentrations than the Nod$^-$ mutants, but within the 5.0 to 10.0 mM levels the Nod$^-$ mutants approached or even overtopped the N concentration of the cv. Finale plants. Compared with a high positive correlation found in the Nod$^-$ mutants, shoot N concentration in the cv. Finale was negatively correlated with the root medium NO_3^- concentration. The pattern of nitrogen content in shoot dry mass was very similar to that seen in the shoot dry mass accumulation. The Nod$^-$ mutants grown on the 5.0 and/or 10.0 mM NO_3^- level had plant dry mass, shoot nitrogen concentration, shoot nitrogen content, and root/shoot dry mass ratio comparable with those of the nodulating cv. Finale grown on the same nitrate levels.

Key words: dry mass, mutants of pea, nitrogen content

Introduction

Mutants of legumes are considered a useful help in research on symbiotic dinitrogen fixation. Of several different classes of symbiotic genotypes (see *e.g.* Devine 1988, Vance *et al.* 1988 for reviews), so-called "non-nodulating" near-isogenic lines were selected in *Arachis hypogaea* (Gorbet and Burton 1979), *Cicer arietinum* (Davis *et al.* 1985), *Glycine max* (Williams and Lynch 1954, Burton *et al.* 1983, Carroll *et al.* 1986, Akao *et al.* 1991, George *et al.* 1993), *Melilotus alba annua* (Kneen and LaRue 1988), *Phaseolus vulgaris* (Davis *et al.* 1988), *Pisum sativum* (Jacobsen 1984,

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Kneen and LaRue 1984,1988, Engvild 1987, Duc and Messager 1989, Borisov *et al.* 1992) and *Vicia faba* (Duc and Picard 1986). Irrespective of the fact that some, mostly chlorosis-inducing *Rhizobium* (*Bradyrhizobium*) *japonicum* strains (Devine and Weber 1977) have a limited ability to overcome nonnodulation restriction in the *rj1rj1* homozygous soybean plants when applied at very high doses (La Favre and Eaglesham 1984), these plants do not nodulate in soil (Williams and Lynch 1954). The nodulation in *rj1*-possessing, non-nodulating soybean mutants was also induced by treatment with 2,4-dichlorophenoxyacetate (Akao *et al.* 1991), and restored to temperature-conditioned non-nodulating *sym 5* pea mutants treated with some ethylene inhibitors (Fearn and LaRue 1991).

It is reasonable that, in addition to study of the mechanisms of legume-*Rhizobium* symbioses, the non-nodulating legume mutants have been used as controls for measuring (both with ^{15}N-isotope dilution and/or with N difference method) the contribution of symbiotic N_2 fixation to the total nitrogen accumulation in the plants (*e.g.* Phillips *et al.* 1986, Rennie 1986, Vose and Victoria 1986). However, such non-nodulating isolines should comply with several requirements, among which they should have *e.g.* almost the same growing period as N_2-fixing plants and should have comparable nitrogen uptake and assimilation efficiencies (Rennie 1986, Witty 1983).

This study examined whether two non-nodulating mutants of pea (*Pisum sativum*) provided with increasing concentrations of nitrate could achieve a growth and nitrogen accumulation comparable to their parental N_2-fixing cultivar.

Materials and methods

Garden pea (*Pisum sativum* L.) cultivar Finale (wild-Nod^+Fix^+-phenotype) and its non-nodulating (Nod^-) 'Risnod2' and 'Risnod27' (Engvild 1987, Novák *et al.* 1993) mutants were obtained from Dr. K.C. Engvild, Risø National Laboratory, Roskilde, Denmark. The Nod^- mutants grown in non-symbiotic conditions were found to be indistinguishable from ('Risnod27') or even superior ('Risnod2') to their parental cv. Finale in leaf nitrate reductase activity (Novák *et al.* 1994).

Two-day-old seedlings inoculated with 3 cm^3 per seedling of inoculum of *Rhizobium leguminosarum* bv. *viceae* strain 248 were grown hydroponically under controlled environment (Škrdleta *et al.* 1994). The levels of nitrate used in the nutrient solutions were 0.0, 0.63, 1.25, 2.5, 5.0 and 10.0 mmol dm^{-3}. A constant ionic strength of the nutrient solutions was kept with Cl^- ions (Škrdleta *et al.* 1980). 5 h after the light was switched on, plants were sampled (n = 8) and their intact nodulated roots were immediately incubated individually at 23 °C for 13 min in 100-cm^3 serum bottles supplied with C_2H_2 (partial pressure of C_2H_2 *ca.* 10 kPa). Nitrogenase C_2H_2-C_2H_4-reducing activity (AR) was determined at 3 and 13 min of incubation and C_2H_4 concentration was measured in 0.5-cm^3 subsamples by gas chromatography. Nitrogen content in shoots was determined as described elsewhere (Škrdleta *et al.* 1994).

Results and discussion

N_2 fixation constituents: The nodule number per root in 34-d-old (early flowering stage) and 48-d-old (early pod filling stage) plants of cv. Finale was considerably

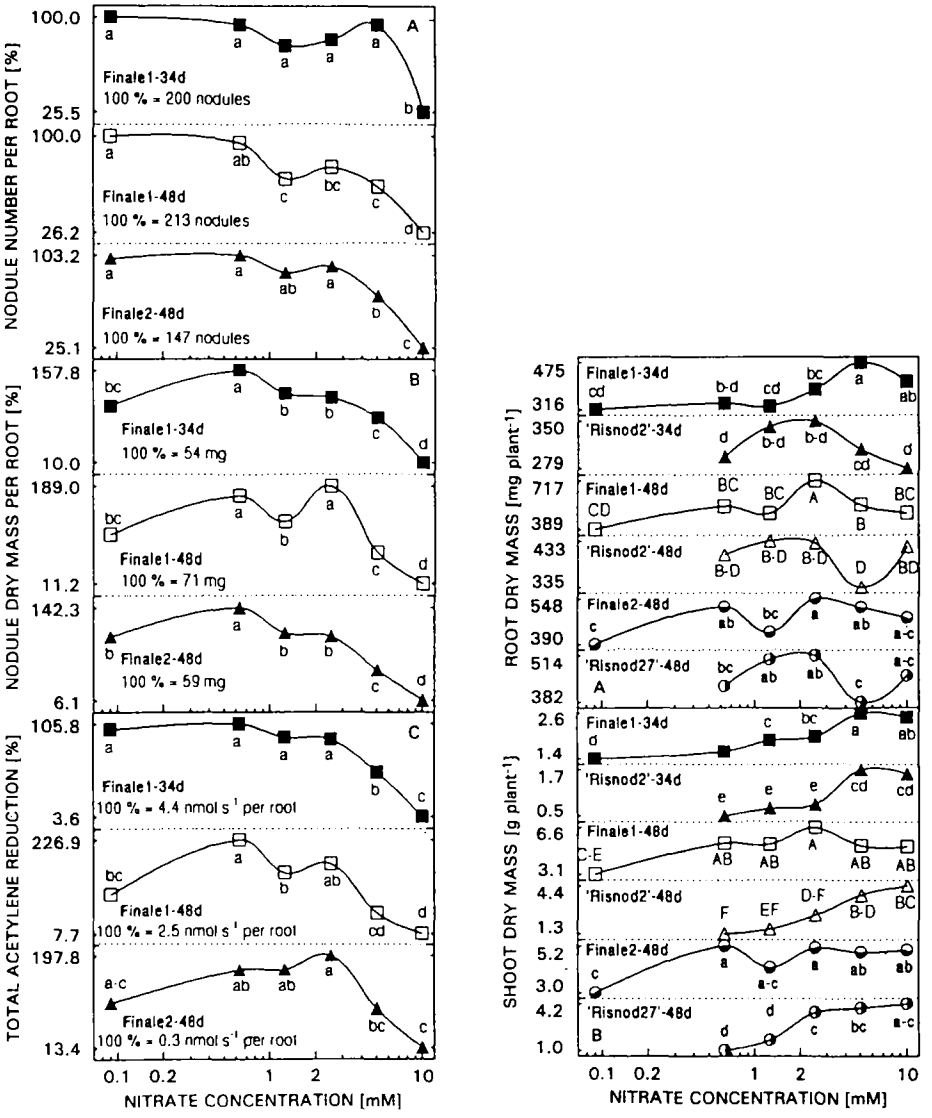

Fig. 1. Effect of increasing nitrate level on nodule number (*A*), nodule dry mass accumulation (*B*), and TAR activity (*C*) in the cv. Finale. Values marked with the same letter are not significantly different at $P \leq 0.05$ on the basis of Duncan's multiple range test.

Fig. 2. Effect of increasing nitrate level on root (in cv. Finale including root nodules - *A*) and shoot (including pods - *B*) dry mass accumulation in Nod$^-$ mutants 'Risnod2' and 'Risnod27', and their parental cv. Finale. Values marked with the same letter are not significantly different at $P \leq 0.05$ on the basis of Duncan's multiple range test.

inhibited at the 5.0 and 10.0 mM nitrate levels (Fig. 1A) and small transient decrease was also observed at the 1.25 mM level. In comparison with the N-free treatment the nodule dry mass accumulation (Fig. 1B) was more sensitive to nitrate showing a significant increase at 0.63 mM and a more pronounced decrease at the 5.0 and 10.0 mM levels. The above-mentioned effects of increasing nitrate levels on nodulation also generally held for total nitrogenase C_2H_2-reducing activity of nodulated roots (TAR; Fig. 1C). Compared with the N-free treatments the TAR activities were unequivocally depressed by the addition of 5.0 mM NO_3^- (by 11 to 48 %) and at the 10.0 mM level they were decreased by 87 to 96 % (Fig. 1C). In the Nod$^-$ mutants 'Risnod2' and 'Risnod27' no nodules were found, confirming the Nod$^-$ phenotype of these mutants if grown in soil and/or in hydroponic cultures (Engvild 1987, Novák *et al.* 1993). In our independent growth experiments (Fig. 1B,C) the low (0.63 mM) nitrate level undoubtedly stimulated both the nodule dry mass accumulation and TAR activities. This phenomenon has been repeatedly confirmed (for a review see *e.g.* Streeter 1988) and seems to be related to findings that some combined N level is required, at least for maximum yield of pulse legumes. However, irrespective of the fact that peas grown in the presence of nitrate always produced a higher dry mass, Waterer *et al.* (1993), using a continuous flow hydroponic culture, found the nitrate concentrations as low as 0.1, 0.2 and 0.5 mM to be inhibitory for nodulation and nitrogenase H_2-evolving activity.

Plant dry mass accumulation: Compared to the nodulated cv. Finale plants at 0.63 to 10.0 mM NO_3^- concentration, the Nod$^-$ mutants 'Risnod2' and 'Risnod27' had lower root dry mass (Fig. 2A) by 19 and 28 % (34-d- and 48-d-old 'Risnod2'), and by 8 % in the 48-d-old 'Risnod27'. In the 34-d-old 'Risnod2' plants grown at 0.63 to 10.0 mM NO_3^- shoot dry mass accumulation (Fig. 2B) was significantly lower than that in the

Table 1. Correlation coefficients for shoot dry mass, plant dry mass, shoot N concentration and shoot N content with nitrate concentration in root medium for Nod$^-$ mutants 'Risnod2' and 'Risnod27', and their parental cv. Finale.

Genotypes	Shoot dry mass	Plant dry mass	Shoot N concentration	Shoot N content
	experiment 1 34-d-old plants			
cv. Finale	0.6145**	0.6364**	-0.7586**	0.5599**
'Risnod2'	0.9502**	0.5929**	0.9555**	0.8871***
	48-d-old plants			
cv. Finale	0.1237	0.1206	-0.4509*	0.0387
'Risnod2'	0.6390**	0.6217**	0.7769**	0.8371***
	experiment 2 48-d-old plants			
cv. Finale	0.2629	0.2537	-0.4760*	0.1922
'Risnod27'	0.6699**	0.6534**	0.8083**	0.7877***

*,**,*** correlation coefficients significant at $P \leq 0.05$, 0.01 and 0.001 levels

cv. Finale. However, in the 48-d-old plants shoot dry mass accumulation considerably increased at 5.0 to 10.0 mM NO_3^- so it was not significantly different from that of the cv. Finale. In the 48-d-old 'Risnod27' mutant a nitrate concentration-dependent shoot dry mass accumulation was similar to the 'Risnod2' (Table 1). While in the Nod$^-$ mutants the shoot and plant dry mass accumulation was found to be highly correlated with the root medium nitrate concentration ($P \leq 0.001$) in both the 34- and 48-d-old plants in the cv. Finale this relationship was significant in the younger plants only. Moreover, compared with the cv. Finale both the Nod$^-$ mutants grown on the 5.0 and 10.0 mM NO_3^- levels showed an identical root/shoot dry mass ratio (Fig. 3).

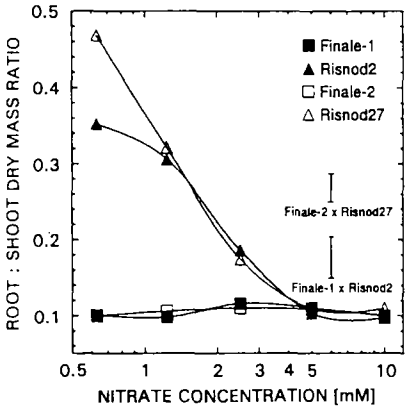

Fig. 3. Root:shoot dry mass ratio in 48-d-old peas as affected by increasing NO_3^- concetration in root medium. The Finale-1 × 'Risnod2' and Finale-2 × 'Risnod27' were independent growth experiments. Bars represent LSDs at $P \leq 0.05$.

Shoot nitrogen concentration and content: The cv. Finale N_2-fixing plants grown on 0.0 to 2.5 mM NO_3^- had higher shoot N concentrations than 'Risnod2' and 'Risnod27' mutants (Fig. 4A). However, at 5.0 to 10.0 mM NO_3^- the Nod$^-$ mutants approached or even overtopped the N concentration of the cv. Finale plants. Compared with a high positive correlation found in the Nod$^-$ mutants, shoot N concentration in the cv. Finale was negatively correlated with the root medium NO_3^- concentration (Table 1). The pattern of nitrogen content in shoot dry mass (Fig. 4B) was very similar to the shoot dry mass accumulation (Fig. 2B) and was positively correlated with the root medium NO_3^- concentration in the Nod$^-$ plants, but no correlation was found in the 48-d-old cv. Finale (Table 1).

Estimation of shoot nitrogen content derived from symbiotic dinitrogen fixation: In our experimental conditions the 5.0 and 10.0 mM NO_3^- levels in the root medium were necessary for the Nod$^-$ mutants to reach the shoot yield (Fig. 2B), shoot nitrogen content (Fig. 4B) and root/shoot dry mass ratio (Fig. 3) comparable to the control N_2-fixing cv. Finale. At the risk of underestimating an actual NO_3^- contribution to the shoot N content resulting from nitrogen-limited growth of the Nod$^-$ mutants at the low NO_3^- levels (Fig. 4B), their shoot nitrogen contents were used for estimating the contribution of N_2 fixation to total shoot N content in cv.

Finale. Depending on plant age and the Nod⁻ mutants used, application of 5.0 mM NO_3^- should decrease N_2 fixation by 19, 63 and 85 % (in comparison with the 34- and 40-d-old cv. Finale plants grown on zero nitrate level - Table 2). At 10.0 mM NO_3^-, nitrate inhibition of N_2 fixation in the 48-d-old plants was much more pronounced, leading to a nearly complete inhibition. In our experiments, nitrate

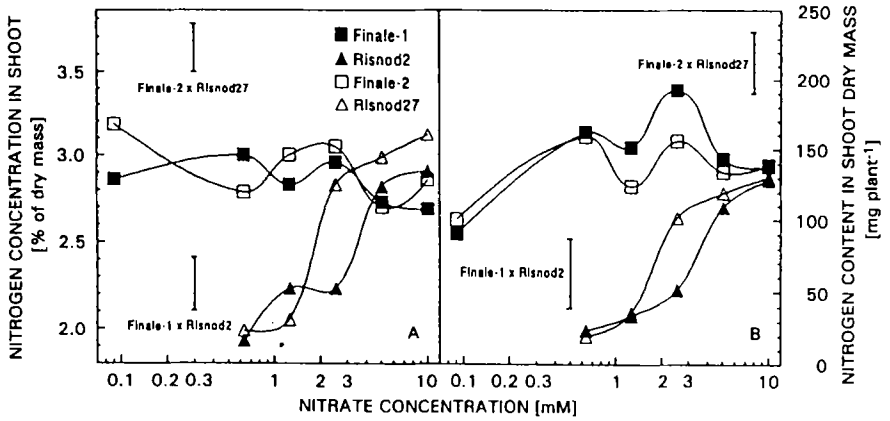

Fig. 4. Shoot nitrogen concentration (*A*) and shoot nitrogen content (*B*) in 48-d-old peas as affected by increasing NO_3^- concentration in root medium. The Finale-1 × 'Risnod2' and Finale-2 × 'Risnod27' were independent growth experiments. Bars represent LSD at $P \leq 0.05$.

Table 2. Nitrogen content in plant shoot derived from symbiotic dinitrogen fixation (BN_2F) in cv. Finale as affected by nitrate concentration (0, 0.63, 1.25, 2.5, 5.0 10.0 mM). BN_2F values were determined by subtracting the total N accumulation in the non-nodulating mutant 'Risnod2' (Experiment 1) or 'Risnod27' (Experiment 2) from that in the N_2-fixing cv. Finale. Percentage of nitrate-free treatment in parentheses.

Plant age [d]	BN_2F [mg N per shoot] 0.0	0.63	1.25	2.5	5.0	10.0
	experiment 1					
34	45.5 (100)	49.2 (108.1)	55.0 (120.9)	54.4 (119.6)	36.8 (80.9)	11.7 (25.7)
48	90.1 (100)	137.6 (152.7)	116.7 (129.5)	140.5 (156.0)	33.8 (37.5)	9.6 (10.7)
	experiment 2					
48	100.2 (100)	138.6 (138.4)	87.8 (87.7)	53.7 (53.6)	14.7 (14.7)	8.5 (8.5)

fertilization of the N_2-fixing cv. Finale resulted in a limited increase of shoot nitrogen content (Fig. 4B) indicating that NO_3^- assimilation mostly replaced N_2 fixation without any remarkable variation of the quantity of nitrogen accumulation by the plants. Sagan *et al.* (1993) found mineral N supply of about 50 g m^{-2}, allowing their Nod⁻ or Nod⁺Fix⁻ field-grown genotypes to reach the yield of the N_2-fixing parental genotype cv. Frisson and to inhibit dinitrogen fixation almost completely. Contrary to our observations they did not detect any significant genotype × N

treatment effect on the beginning of flowering. In our experiments, flowering was delayed compared with the cv. Finale, and the number of pods was somewhat lower in both the Nod$^-$ mutants used (not documented). Delayed flowering was also observed in common bean (Davis *et al.* 1988) and a lower seed yield in soybean (Talbott *et al.* 1985) and common bean non-nodulating mutants (Davis *et al.* 1988).

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