The Effects of Nitrate and Asparagine on Nitrogen Fixation (The Acetylene Reduction Activity) in Inoculated Faba Bean

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Abstract

The response of nitrogen fixation (as assessed by the acetylene reduction assay) in *Vicia faba* cv. Fiord, inoculated with three different strains of *Rhizobium leguminosarum* bv *viciae* (NA 533, SU 391 and CC 305) to two sources of combined nitrogen, nitrate and the amino-acid asparagine, applied exogenously, were investigated in a pot experiment. Inoculated seedlings were established under sterile conditions in sterilised coarse river sand and transferred to pots covered with sterilised polypropylene lids in a laminar flow cabinet. These were later transferred to a growth room. Thirty-five days after sowing (DAS), the plant roots were flushed daily with nutrient solutions containing 0, 2.5 and 10 m*M* nitrate or asparagine using a 'pour through' system. Dry matter, nodule weight, N accumulation and acetylene reduction (ARA) were monitored for a further 8 days. Inoculation with strain NA 533 gave highest dry matter yield, nodulation and ARA compared to inoculations with the other two strains, indicating that the symbiosis with strain NA 533 was highly effective. Both nitrate and asparagine were apparently taken up by the plants and contributed to total N, but plant growth was not significantly affected by nitrate or asparagine in all symbioses during the experimental period and there were no significant interactions between treatments. While inoculation with strain NA 533 appeared to supply all the N needed for growth, plants inoculated by strains SU 391 and CC 305 took up more N from both nitrate and asparagine, the uptake being highest in plants inoculated by strain SU 391, indicating this symbiosis to be less effective. The contribution to total plant N was significantly greater with asparagine than with nitrate. The higher contribution of N to plants from the 'less effective' symbioses did not lead to rapid increases in dry weight since differences in plant performance had already been established by the time treatments were imposed. Nodule dry weight and ARA were significantly depressed by nitrate and asparagine in all symbioses and in proportion to the concentrations applied. Also, the decline in ARA induced by asparagine was always greater than that with nitrate. This experiment suggests that plant size and the efficiency of the symbiosis may indirectly contribute to making a particular symbiosis less sensitive to combined N.

Key words: Acetylene reduction activity, asparagine, faba bean, nitrate, nitrogen fixation, nodules

Introduction

Nitrate is the most common form of N found in agricultural soils and the most potent inhibitor of N_2 fixation (Hageman, 1979). It is usually supplied at sowing so that the responses to NO_3^- usually attributed to effects on nodule functioning represent an interaction between many processes. Strains of *Rhizobium* are also known to vary in their capacity to nodulate the same host in the presence or absence of combined N (Pate & Dart 1961; Hoglund, 1973; Munns, 1977; Harper & Gibson 1984). However, little appears to be known about the differential responses to mineral N by mature symbioses established by different strains of *Rhizobium*, i.e. the direct effects of NO_3^- on N_2 fixation. The basis for these effects is also not well known.

One of the hypotheses advanced to explain combined N effects on N_2 fixation is the diversion of assimilates from nodule functioning to enzymes associated with the reduction of combined N when it is supplied to an active symbiosis (Houwaard, 1980). The responses of different symbioses to different sources of N which may have different energy requirements for their reduction could, therefore, lead to the selection of nitrogen sources which may allow symbioses to operate at their maximum potential for N_2 fixation at moderate levels of applied N. This has important implications for agriculture particularly in developing countries where mixed cropping involving cereals and legumes is usually practised. In such cases the cereal component may require N for increased yields but the addition of N may re- duce the potential N_2 fixation of the legume component thereby decreasing the benefits that can be derived from the exploitation of atmospheric N_2.

Streeter (1986) compared the effect of high levels of nitrate (12 m*M*) on two established symbioses of *Phaseolus vulgaris* which were raised on 1 m*M* nitrate from sowing, and found small but significant differences in acetylene reduction (AR) and in sugar consumption of the nodules. He suggested that nodules with the greatest rate of carbon utilisation and ammonium formation were the most sensitive to high levels of nitrate. In contrast, McNeil (1982) found no differences in the sensitivity of four soybean symbioses to 10 m*M* nitrate. The aim of this experiment was to investigate the responses of established symbioses of faba bean to two contrasting sources of nitrogen, nitrate and asparagine, an oxidised versus a reduced form of nitrogen.

Materials and methods

Plant culture

Seeds of *Vicia faba* cv. Fiord were surface sterilised by immersion in 45% ethanol for 1 min, rinsed with sterile water and again immersed in 0.2% $HgCl_2$ for 5 min. The seeds were washed free of $HgCl_2$ with several changes of sterile water and sown in 2l capacity sterilised containers of washed, steam-sterilised river sand moistened to field capacity with sterile water in a laminar flow cabinet. Seeds were inoculated with one of three strains of *Rhizobium leguminosarum* bv. *viciae*. Infection and nodulation were achieved by applying 150 ml of a yeast/mannitol broth of the appropriate strain evenly over the surface of the sand after sowing. The containers were completely covered with Glad Wrap to prevent any contamination and placed under lights (approximately 700μ mol quanta^{-2}s^{-1}) in the growth room.

After 5 days, the sand was moistened again with sterile water in the laminar flow cabinet and after a further 7 days, germinated seedlings were transplanted into pots again in the laminar flow cabinet. The tops of pots were covered with sterilised polypropylene lids with five holes, four for plants and one as a watering port. Seedling were led through these holes, inoculated again with 20 ml of the appropriate strain of *Rhizobium* and covered at their bases with sterile cotton wool to reduce contamination. The watering port was also plugged. In an earlier trial, uninoculated faba bean plants prepared in this way were examined for nodulation after 30 days growth and found to have no nodules, showing that the method provided an adequately sterile root environment. Plants were raised in the growth room at 20 °C (±1 °C) with a 12-h photoperiod.High pressure sodium 'lucolux' lamps (GTE Sylvania) provided photon irradiance of approximately 700μ mol quanta m^{-2}sec^{-1}.

Experimental design and treatments

The experiment was a factorial design replicated three times. Treatments consisted of three strains of *Rhizobium leguminosarum* bv. *viciae*, NA 533 (supplied by Dr A. Gibson of the Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra, Australia), SU 319 (from Nodulaid, group E; Agricultural Laboratories Pty Ltd, N.S.W., Australia) or CC 305 (obtained from Mr J. Brockwell of the Division of Plant Industry, CSIRO, Canberra, Australia) and two nitrogen sources, asparagine and nitrate at four rates.

At 35 days after sowing, excess amounts of 0, 2.5, 5.0, and 10.0m*M* solutions of nitrate and asparagine prepared with a half strength Hoagland's solution (Table 1) were randomly applied to the pots, drained for 60 min and plugged. The procedure was repeated daily for the next 8 days. The antibiotic Securopen which had previously been found by Oti-Boateng & Silsbury (1993) to control bacteria in asparagine solutions without affecting nodule activity, was added at 500 µg/ml to prevent the growth of bacteria in the asparagine solutions.

Plants were harvested every 2 days (3 h after the start of the photoperiod), assayed for ARA as described below, before being partitioned into shoot, root and nodule, dried at 85 °C for 48 h and weighed. Nodules were not separated from roots at harvests 2 and 4. Total nitrogen was determined by micro-Kjeldahl analysis (Eastin, 1978) on dried samples from the first and final harvests. Analyses of variance were performed on the data to determine the interactions and main effects of treatments.

Estimation of nitrogenase activity

Nitrogenase activity was estimated by the acetylene reduction assay using four plants in a closed system as described by Oti-Boateng & Silsbury (1993). Assays commenced at 11.00 h when pots were taken from the growth room and plants extracted by gently washing off the sand with water at 20 °C. Excess water was blotted from the nodulated roots and whole plants incubated in 1.06 litre glass jars with screw-down metal lids penetrated with a subaseal. 110 ml of industrial acetylene was injected and excess gas allowed to escape through an extra needle. Samples of 500 µl were withdrawn at 10 and 40 min after exposure to C_2H_2 and injected into a gas chromatograph, equipped with a flame ionisation detector and an 80-100 mesh Porapak R column, and calibrated against standards of C_2H_4 in 10% C_2H_2.

Results and discussion

Dry weight of plant and plant parts

When the nitrogen treatments were imposed on day 35, the dry weight of plants inoculated with strain NA 533 was significantly greater than those inoculated with the other two strains. This difference persisted throughout the experiment (Fig. 1). There were no significant interactions between *Rhizobium* and source of N, and between *Rhizobium*

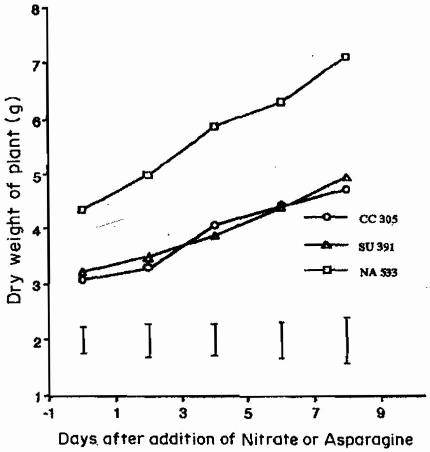

Fig. 1 Total plant dry weight (g) of 'Fiord' faba bean inoculated with three strains of *Rhizobium leguminosarum* bv. *Viciae* (CC 305, SU 391 and NA 533) and measured 35 days after sowing for 8 days. Bars show LSD at 1%

TABLE 1.
Composition of nutrient solution

	NO_3 Level (mgl^{-1})			
	0.0mM	*2.5 mM*	*5mM*	*10mM*
KNO_3	-	84.17	168.34	336.68
$Ca(NO_3)_2.4H_2O$	-	196.59	393.18	786.36
$MgS0_4.7H_2O$	246.38	246.38	246.38	246.38
KH_2PO_4	34.00	34.00	34.00	34.00
H_3BO_3	2.86	2.86	2.86	2.86
$MnCl_2.4H_20$	1.81	1.81	1.81	1.81
$ZnSO_4.7H_2O$	0.22	0.22	0.22	0.22
$CuSO_4.5H_2O$	0.08	0.08	0.08	0.08
$Na_2Mo0_4.2H_20$	0.12	0.12	0.12	0.12
EDTA	23.82	23.82	23.82	23.82
$FeSO_4.7H_2O$	19.92	19.92	19.92	19.92
K_2SO_4	217.75	145.02	72.51	-
$CaSO_4.2H_2O$	430.00	287.00	143.50	-

NB. Appropriate concentrations of asparagine were prepared with 0mM nitrate solutions

TABLE 2.
Nodule dry weight (mg) of 'Fiord' faba bean supplied with four levels of nitrate or asparagine (0, 2.5, 5.0 and 10mM) starting from 35 days after sowing and for 8 days. Results were averaged for all strains of Rhizobium

Source of nitrogen	*Rate (mM)*	*Days after imposition of treatment*		
		0	*4*	*8*
Nitrate	0	97	153	207
	2.5	126		176
	5.0		132	144
	10.0		114	117
Asparagine				
	0	97	143	203
	2.5		98	139
	5.0		112	117
	10.0		100	102
SE±			20.0	17.0
LSD 5%			36.2	30.8

TABLE 3.

Acetylene reduction activity (ARA) μ mol C_2H_4 per 4 plants per hour of 'Fiord' faba bean supplied with four levels of nitrate or asparagine starting from 35 days after sowing and for 8 days. Results were averaged for all strains of Rhizobium

		Days after imposition of treatment				
Source of nitrogen	*Rate(mM)*	*0*	*2*	*4*	*6*	*8*
Nitrate						
	0	10.3	12.3	14.1	17.1	20.8
	2.5		9.3	10.8	13.7	14.8
	5.0		9.0	8.4	9.1	10.7
	10.0		8.1	7.7	7.4	7.2
Asparagine						
	0	10.3	13.6	13.6	15.9	18.7
	2.5		8.6	7.8	10.0	10.4
	5.0		8.3	6.9	6.7	7.1
	10.0		6.0	6.8	3.9	2.6
SE±			1.7	1.6	2.1	1.8
LSD 5%			3.1	2.9	3.8	3.3

and concentration of nitrate or asparagine showing that plants used all sources of N, including fixation, equally well for growth during the experimental period.

Dry weight of nodule

Nodule dry weight in the N-treated plants (for both nitrate and asparagine) was significantly lower ($P<0.05$) than the no-N controls at the end of the experiment but was not affected by the strain of *Rhizobium* used. The effect of asparagine on nodule dry weight was greater than that for nitrate (Table 2). Higher concentrations of nitrate or asparagiine caused higher reductions in nodule dry weight.

Acetylene reduction activity (ARA)

The strain of *Rhizobium* used had a significant effect on ARA ($P<0.05$) on the minus N controls at the first harvest on day 35 and the order was : NA 533 > SU 391 > CC 305. However, these differences disappeared when treatments were imposed, *Rhizobum* strain had no further effect on

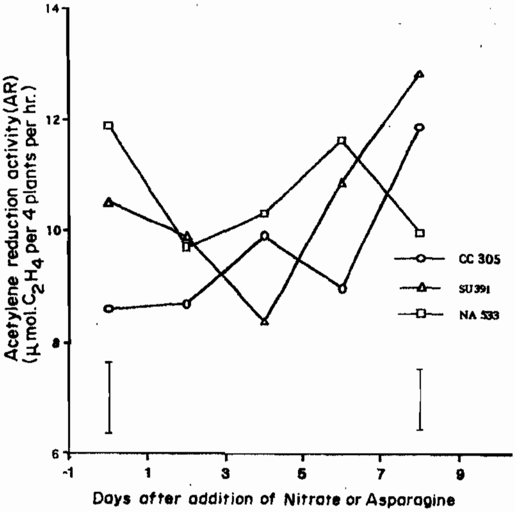

Fig. 2 Acetylene reduction activity (AR) (u mol C2h4/ 4pl/hr) of 'Fiord' faba bean inoculated at sowing with three strains of Rhizobium *leguminosarum* bv. *Viciae* (CC 305, SU 391 and NA 533) and measured 35 days after sowing for 8 days. Significance at 5% is shown only where bars are shown.

TABLE 4

Total N (mg) at 35 days and at 43 days after sowing of total plant and plant parts (shoot, root, nodule) of 'Fiord' faba bean inoculated with three strains of Rhizobium leguminosarum bv. viciae and supplied with nitrate or asparagine (0mM or 10mM) from 35 days AD

	Shoot			*Root*			*Nodule*			*Total plant*		
Days after sowing	*35*	*43*		*35*	*43*		*35*	*43*		*35*	*43*	
Level of N (mM)	*0*	*0*	*10*	*0*	*0*	*10*	*0*	*0*	*10*	*0*	*0*	*10*
Rhizobium												
						Nitrate						
CC305	48.7	118.3	134.7	35.9	48.3	54.5	7.2	17.9	7.7	91.9	184.5	196.9
SU391	51.2	92.0	152.8	42.5	42.7	66.1	9.3	17.5	9.1	98.8	152.2	237.5
NA533	105.0	193.9	197.6	75.8	98.0	89.7	9.5	16.2	8.5	190.3	296.3	294.0
Mean		134.7	161.7		63.0	69.7		17.2	8.5	211.0	24	
						Asparagine						
CC305		91.1	148.1		58.0	72.9		18.1	6.2		167.1	227.2
SU391		80.4	142.6		49.4	106.6		15.9	5.9		145.7	255.2
NA533		190.1	218.9		82.1	118.8		16.5	7.1		282.6	344.8
Mean		120.5	169.9		62.3	99.4		16.8	6.4		198.5	275.7

Analysis of variance for the response of total N (mg) to strain of Rhizobium and nitrate or asparagine (0mM or 10mM)

Treatment	*Shoot*		*Root*		*Nodule*		*Total plant*	
	35	*43*	*35*	*43*	*35*	*43*	*35*	*43*
Rhizobium(Rh)	***	***	***	***	ns	ns	***	***
Source of N		***		***		***	***	***
Rh x Source of N		ns		ns		ns		ns

*** Denotes Significance at 0.01%

ARA until the end of the experiment when plants inoculated with strain SU 391 and CC305 had significantly higher ARA than those inoculated with NA 533 (Fig. 2).

Overall, both nitrate and asparagine depressed RA rates significantly ($P < 0.05$), the decline induced by asparagine being greater than that caused by nitrate (Table 5). Concentration also exerted a highly significant effect on ARA ($P < 0.05$) at 48 h + and this persisted throughout the experiment (Table 3).

Total N in plant and plant parts

Plants inoculated with strain NA 533 contained significantly more N ($P < 0.01$) than plants inoculated with either strain SU 391 or CC 305 at day 35 (Table 4). After this the asparagine and nitrate treated plants had alternative sources of N apart from fixation of N_2. Table 4 shows the total N in plants at 35 and 43 days. The strain of *Rhizobium* did not interact with nitrate or asparagine in influencing the N content of any of the plant

TABLE 5

N accumulation (mg) at day 43 in total plant and plant parts (shoot, root, nodule) and RA (mol C2H4/4 plants/h) of 'Fiord' faba bean inoculated by three strains of Rhizobium leguminosarum bv. viciae and supplied with nitrate asparagine (0 or 10mM)

	Shoot		Root		Nodule		Total plant		ARA	
Level of N	*0*	*10*	*0*	*10*	*0*	*10*	*0*	*10*	*0*	*10*
Treatment					**Nitrate**					
CC 305	69.6	86.0	12.4	18.5	10.6	0.5	92.8	105.2	22.2	7.2
SU 391	40.7	101.6	0.2	27.1	8.2	-0.2	53.4	145.2	23.5	7.2
NA 533	88.9	92.6	17.5	12.1	6.7	-1.0	93.8	104.6	16.9	7.2
Mean	66.4	93.4	10.0	19.3	8.5	-0.3	80.0	118.0	20.8	7.2
					Asparagine					
CC 305	42.3	111.7	22.1	37.0	10.8	-1.0	75.4	135.4	20.7	3.0
SU 391	40.7	101.6	6.9	64.1	6.7	-3.4	46.9	56.4	21.0	2.3
NA 533	85.1	113.9	1.6	43.0	7.0	-2.4	80.1	154.5	14.5	2.1
Mean	56.0	109.1	10.2	48.0	8.2	-2.3	67.5	149.1	18.7	2.6

Analysis of variance for the accumulation of N in plant and plant parts.

Treatment	*Shoot*	*Root*	*Nodule*	*Total plant*
Rhizobium (Rh)	ns	ns	ns	ns
Source of nitrogen	***	***	***	***
Rh x Source of N	ns	ns	ns	ns

*** Denotes significance at 0.1%

parts at day 43, but over all, plants inoculated with NA 533 had significantly more N ($P < 0.01$) in shoot and root than had plants inoculated by either SU 391 or CC 305. Total N in the nodule did not differ between strains of *Rhizobium* but treatments with both nitrate and asparagine led to significant reduction in total N($P < 0.01$) of the nodules. Plants exposed to asparagine had significantly higher total N ($P < 0.01$) than those exposed to nitrate.

Although total N was significantly higher ($P < 0.01$) in shoot and root of plants inoculated with strain NA 533 than SU 391 or CC 305, whether assimilating dinitrogen, nitrate or asparagine, the three symbioses differed in the amounts of N accumulated from NO_3^- and from asparagine (Table 5). The amount of N in SU 391 plants was higher than for either NA533 or CC305 inoculated plants. This probably reflects greater external N requirements by SU 391 plants since they assimilated the least N from N_2. Acetylene reduction activity was, however, depressed to the same level in all symbioses by nitrate. All plants supplied with asparagine accumulated more N than those supplied with nitrate and ARA in these plants was significantly lower ($P<0.01$) than that of plants assimilating nitrate (Table 5).

Relative AR activity

Although there were no significant interactions between symbioses and N treatments,

TABLE 6

Relative decline in acetylene reduction activity of 'Fiord' faba bean plants inoculated by three different strains of Rhizobium (CC 305, SU 391 and NA 533) and then supplied with nitrate or asparagine 35 days after sowing.

	Days after imposition of treatment				
Treatment	*0*	*2*	*4*	*6*	*8*
			Nitrate		
CC 305	100	68.8	49.9	66.1	47.8
SU 391	100	64.0	63.1	47.8	49.3
NA 533	100	82.1	81.6	65.7	61.8
Mean	100	71.6	64.9	59.9	53.0
			Asparagine		
CC305	100	53.3	57.8	46.7	32.0
SU 391	100	72.9	47.4	39.7	37.1
NA 533	100	46.2	49.8	44.4	38.6
Means	100	57.4	51.6	43.6	36.0
LSD 5%					
Rhizobium (Rh)	-	ns	8.4	ns	5.9
N Source	-	7.1	ns	6.8	5.1
Rh x Source of N	-	ns	ns	ns	ns

calculation of the extent of decline in ARA when nitrate or asparagine was supplied to the three symbioses showed that ARA of plants inoculated by strain NA 533 declined at a lower rate than those of the other symbioses (Table 6). When nitrate and asparagine were supplied to fababean nodulated by three strains of *Rhizobium legumino-sarum* bv *viciae* dry weight of nodules and ARA were both significantly depressed by nitrate and asparagine in all the symbioses. Higher concentration elicited the greatest depression. McNeil (1982) obtained similar results when 10m*M* nitrate was supplied to soybean cv. Davis, inoculated with four strains of *Bradyrhizobium japonicum* after effective symbioses had been established. He found that both the size and the number of nodules were reduced while ARA declined rapidly in all symbioses. Substantial differences in the ability of strains to nodulate the host in the presence of nitrate were, however, found but they existed only when moderate levels of nitrate (0.2 - 2.0 m *M*) were supplied; at 10m*M* concentration all symbioses were affected. This suggests that once a symbiotic association has been established, N irrespective of its source is likely to affect N_2 fixation adversely.

Based on the assimilate diversion hypothesis it was expected that N_2 fixation would be depressed to a higher degree by nitrate than asparagine because the latter is an already reduced form of N and assimilate requirement for its utilisation by the plant would be lower, allowing N_2 fixation to proceed at a higher rate than when nitrate was supplied. Since this was not the case the assimilate diversion hypothesis may not completely explain the adverse effects of combined N on nodule function.

Differences in the ability of strains of *Rhizobium* to nodulate the host in the presence of moderate levels of N have however been found by others who have examined host-*Rhizobium* interactions in the presence of N (Pate & Dart 1961; Heichel & Vance, 1979; Harper & Gibson 1984). Pate & Dart (1961) from studies with barrel medic and vetch divided symbioses into (a) those in which addition of inorganic N depressed symbiotic efficiency measured in terms of nodule numbers and N_2 fixation; and b) those in which the symbiosis was stimulated to some degree by added N. Where symbiotic efficiency was stimulated by added N, combined N inhibited nodulation on primary roots but an unusually extensive and effective nodulation of later formed parts of the root system enabled the symbiosis to recover. The findings of the present study, together with those of McNeil (1982), and the studies of Pate & Dart (1961) suggest that the selection of *Rhizobium* strains for differential tolerance to soil nitrate may be achieved through their selection in the presence of moderate levels of combined or soil N at sowing, rather than when symbioses have been established.

The results of this experiment also suggest that plant total N and biomass may influence N_2 fixation on and/or uptake of N from exogenous sources. At the end of the experiment the symbioses with strain SU 391 and CC 305 had higher rates of ARA than strain NA 533. This was because the total N content of plants inoculated with strain SU 391 or CC 305 was low and these plants were apparently under nitrogen stress. This was confirmed by the fact that these plants took up significantly more N from exogenous sources than those inoculated with NA 533 whether supplied nitrate or not, most probably because the symbiosis was capable of supplying the required N for growth. Acetylene reduction activity however, declined in all symbioses even though the amount of N taken up from exogenous sources was different in each symbiosis. Although the closed system of assay has obvious limitations (Minchin *et al.*, 1994), its use in assessing relative differences in nitrogenase activity legume/*Rhizobium* symbioses is still advocated (Vessey 1994).

The relative ARA in response to nitrate was, however, highest in the most 'effective' symbiosis, suggesting that N_2 fixation declined in proportion to the supply of reduced N. No differences were observed for asparagine which caused a more rapid decline in all symbioses. The results of Herridge *et al.* (1984) support these findings. They showed with soybean that symbiotic deficiencies were compensated for by a more efficient exploitation of soil N by plants, and that soil N and N from fixation were complementary in meeting the requirement of the crop. Gibson (1967) also suggested that the rate of N_2 fixation was so regulated that an overall balance was maintained between N_2 fixation and weight increase. Below a particular plant N percentage, resources are diverted to increase the rate of N_2 fixation while above it, the opposite occurred. El-Sherbeeny *et al.* (1977) and Oti-Boateng *et al.* (1994) have also shown strong correlation between total N and dry matter yield of faba bean plants.

The experiment also confirms earlier findings (El-Sherbeeny *et al.*, 1977 & Silsbury, 1991) that under similar conditions and without mineral N, symbiotic efficiency measured as plant dry weight, nodule dry weight, ARA, total N and grain yield of plants, varies when different strains of *Rhizobium leguminosarum* bv. *viciae* are used to nodulate faba bean. At the time treatments were imposed, the symbiosis with one strain (NA 533) had proved to be better than the others.

Conclusion

The experiment has shown that the adverse effects of exogenous nitrogen on N_2 fixation was not overcome by altering the source of N. The three strains of *Rhizobium* that nodulated 'Fiord' faba bean did not differ in response to combined N. However, the symbiosis with NA 533 was 'marginally' less sensitive to nitrate than SU 391 and CC 305 probably due to early establishment of the symbiosis in NA 533 plants, resulting in larger plants at the time of imposition of treatments. Responses to combined N may, therefore, be influenced by plant size because 'large' plants will require more reduced N than 'small' plants to influence the internal concentration of N and, thus, may appear to be more tolerant of exogenously applied combined N. It is, therefore, likely that plant species with potentially high growth rates will have a high demand for N whether from the soil or by fixation. Such plants may also show an 'apparent' low sensitivity to nitrate because higher amounts of reduced N will be required to change the concentration of N in these plants.

Acknowledgement

The authors are grateful to Mr. J. Brockwell and the late Dr Alan Gibson, both of the CSIRO, Canberra, Australia, for the supply of *Rhizobium* and again to the late Dr Alan Gibson for his useful suggestions in the preparation of this manuscript.

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