Effect of Debudding of Faba bean on the Soluble Nitrogen and Ammonia Concentration of the Root Nodules and Associated Nitrogenase Activity.

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Abstract

The feedback control mechanism in which the demand for fixed N by the plant regulates nitrogenase activity (NA) in legumes as proposed by Salisbury (1987) was investigated in a debudding experiment. Faba beans were inoculated and grown without nitrogen. At 35 days when plants had eight fully opened leaves, were nodulated and actively fixing N_2, the apical portions of 24 plants were removed (debudded). Debudding had the effect of restricting the growth of plants and was expected to cause the accumulation of organic nitrogen in the plant with a resultant decline in NA. Eight days after debudding, 8 of the debudded plants were allowed to resume growth for a further eight days. Total soluble N (total amino acid), soluble carbohydrate, ammonium and malate concentrations in the nodules were measured at 8 and 16 days after debudding. Nitrogenase activity (measured as ARA) of debudded plants was 54% and 18% of controls at 8 and 16 days after debudding. Debudded plants allowed to resume growth had 7% and 21% more dry matter in shoot and nodules compared to those debudded continuously; ARA had also increased to 171% of that of continuously debudded plants. Total amino acid in nodules of debudded plants was 30% more than in controls at day 8 and 120% at day 16 after debudding. In controls and plants allowed to resume growth, total amino acid in their nodules was similar at the end of the experiment. In both control and debudded plants, asparagine was the predominant amino acid (over 75%) throughout the study. There were no significant differences in ammonium content of nodules between treatments at any time. A clear role for malate in the regulation of ARA was not established. It is suggested that the overall increase in the N status of debudded plants was due to their inability to utilise this N and therefore, increased N translocated from the shoot to the nodules. This may have increased total amino acid content of the nodules exerting a feedback control on nodule function. The results of this experiment are consistent with the feedback mechanism advanced to explain the inhibition of nitrogenase activity by combined nitrogen.

Key words: Acetylene reduction activity, faba bean, feedback control, legume, nitrogenase activity, nitrogen fixation, nodules

Introduction

There have been several reports of the inhibition of nitrogenase activity when inorganic N is supplied to nodulated roots (Schuller & Werner, 1993; Silsbury, 1987). While the mechanism responsible for this is largely unresolved, a feedback control mechanism proposed by Silsbury (1987), in which the demand for fixed N by the plant has received considerable support (Hartwig *et al.*, 1993; Parsons *et al.*, 1993; Oti-Boateng & Silsbury, 1993; Oti- Boateng *et al.*, 1994). The evolution of mechanisms by plants to regulate the net intake of mineral N to correspond with the plant's nitrogen need has also been proposed (Imsade & Tourraine, 1994). They reported that nitrate uptake rates are regulated by plant biomass production and this was independent of plant species.

Oti-Boateng *et al.* (1994) showed that removal of both apical meristems and all lateral buds of faba bean caused soluble N to increase in the nodules because the plants were restricted in their capacity to utiilise fixed N. Coupled with the increase in soluble

N was a decline in nitrogenase activity. It was proposed that the increase in nodule and plant soluble N could have caused N_2 fixation to decline. Similar results were obtained by Fujita *et al.* (1991) when soybean pods were removed and by Hartwig *et al.* (1994) with defoliated white clover. Jacobsen (1984) compared the effects of nitrate on a wild type pea and a nitrate reductase deficient mutant and also found that the reduced products of nitrate rather than nitrate *per se* was responsible for the inhibition of N_2 fixation by nitrate suggesting a role for soluble N in the inhibition.

In this study a more detailed investigation was undertaken on the soluble nitrogen and ammonium composition of the faba bean nodule in relation to the inhibition of nitrogenase activity following debudding. The ammonium produced in the bacteroid is assimilated in the nodule cytosol and does not normally accumulate in plant tissues (Bergersen & Turner, 1967). Malate level in the nodule was also measured since it has been reported to accumulate in the nodule tissue following nitrate application to nodulated roots of soybean and pea and considered to be linked to the inhibition of nitrogenase (Nelson and Edie, 1988).

Materials and methods

Plant culture

Forty eight plants of faba bean cv. Fiord were grown as described by Oti-Boateng & Silsbury (1993) in pots of coarse river sand in a sunlit glasshouse. They were irrigated with deionised water until seedling emergence after which a half strength Hoagland's solution (0 m*M* for nitrate) as described by Oti-Boateng *et al.* (1994) was flushed through the sand each day. Fifteen days after emergence, plants were transferred from the sunlit glasshouse to a growth room at 20 ^{o}C± 1 ^{o}C under a 12-h photoperiod. High pressure sodium vapour lamps (GTE Sylvania) provided a photon irradiance of approximately 500 µ mol quanta m^{-2} s^{-2} at leaf level.

Treatments

Thirty-five days after sowing when plants had nodu- lated and were actively fixing nitrogen, the apical portions of 24 of the plants were removed (debudded). All lateral buds which subsequently developed on debudded plants were removed daily. Eight days after debudding eight of the debudded plants were allowed to resume growth for a further 8 days.

Experimental design

The experiment was a randomised block design with four replicates and three debudding treatments. Harvests were made in two series, the first for acetylene reduction and dry weight determination and the second for chemical analysis on days 0, 8 and 16 after debudding. Harvests for acetylene reduction assay were made as described by Oti-Boateng *et al.* (1994). For chemical analysis, one plant from each replicate was separated from sand in ice cold water. Nodules were then picked from roots kept on ice and placed in glass vials also on ice. These were topped with liquid nitrogen and later stored at 20 ^{o}C until required for analysis.

Nitrogen fixation

Nitrogen fixation was estimated by the acetylene reduction assay on whole plants using a closed system as described by Oti-Boateng & Salisbury (1993). Despite the obvious limitations of this method (Minchin *et al.*, 1994) the use of the method for relative comparison of treatments still appears justified (Vessey *et al.*, 1994).

Soluble nitrogen-amino acid and ammonium

A hundred milligram of nodule sample was ground with 2 ml 80% ethanol: 20% 0.5*M* potassium acetate buffer (pH 5.5) and centrifuged for 15 min at 1800 g. The supernatant was collected and the procedure repeated with 2 ml and then 1 ml ethanol/acetate buffer. Supernatants were pooled and made up to 5 ml for total amino acid, ammonium and soluble carbohydrate determinations.

Ammonium was determined by the indophenol procedure using the Sigma Diagnostics procedure (1989). A 0.2 ml aliquot of the nodule extract was mixed with 0.5 ml of Phenol-Nitroprusside Solution Sigma 640-1 (reagent 1), 0.5ml of Alkaline Hypochlorite Solution-Sigma 640-3 (reagent 2) and 2.5 ml water. The solution was mixed thoroughly after the addition of each reagent and left at room temperature for 30 min. The absorbance of the solution was then read at 630 nm. Ammonia in the sample was calculated from a standard calibration curve for the estimation of ammonia by the indophenol reaction. In a test extraction with 0.5 mm NH_4NO_3 included in the extraction medium the recovery was 91%.

Total amino acid content was measured as the sum of all amino acids in the extract. The relative amounts of amino acids in the extract were determined based on a HPLC separation of ortho-phthaladehyde by the Hewlett Packard Amino Quant Series II analyser and 9-fluorenzylmethyl chloroformate derivatives of primary and secondary amino acids. Two hundred micro litres of the extract obtained for soluble N determination was filtered using Ultrafree-MC millipore obtained from Surgical and Medical Supplies and loaded onto the aminoquant.

Soluble carbohydrate

Soluble carbohydrate was determined as described by Oti-Boateng *et al.* (1994) using the extract prepared for soluble N determination.

Malate content

Malate was measured by the Boehringer Mannheim procedure (Ca. no. 139068) which is based on a coupled malate dehydrogenase/glutamate oxaloacetate transaminase reaction. Tests were made on the nodule extracts. When 1.5 mmol of malate was added to such an extract the recovery was 94%.

Results

The effect of debudding on nitrogenase activity, soluble N, ammonium and soluble carbohydrate content of nodules.

Nitrogenase activity (ARA) of debudded plants (Table 1) was 54% of that of controls at day 8 and 18 % at day 16 after debudding (AD). Debudded plants allowed to resume growth 8 days after debud- ding had 7% and 21% more dry matter in shoot and nodules compared to those debudded continuously (data not shown) and ARA had increased by 71% compared to that of continuously debudded plants at day 16 AD. Total amino acid content of nodules of continuously debudded plants was 30% more at day 8 AD and 120% more at day 16AD compared to the non-debudded controls.

In plants allowed to resume growth 8 days AD, total amino acid content of the nodules did not differ significantly from controls at the end of the experiment. No differences in the ammonium content of the nodules was found between controls, continuously debudded plants and debudded plants allowed to resume growth at any time. There was no treatment effect on soluble carbohydrate content of the nodules at day 8 AD but plants allowed to resume vegetative

TABLE 1

Effect of debudding on nitrogenase activity (AR), soluble carbohydrate and nitrogen and ammonium content of nodules of faba bean. Nitrogenase Activity (Acetylene reduction activity) was determined on whole plant material. Other data shown for nodules are expressed per g fresh weight.

Treatment	*Nitrogenase activity (μ mol C_2H_4 $pl^{-1}h^{-1}$)*			*Total amino acid (μg)*			*Soluble carbohydrate (mg)*			*Ammonium (μg)*		
Days after debudding	0	8	16	0	8	16	0	8	16	0	8	16
Control	10.6	25.0	57.7	-	4402	2801		12.9	11.2	-	19.6	27.4
Debudded (continuous)		13.4	10.1	-	5729	6247		11.1	9.9	-	22.2	32.0
Debudded (8 days)			17.3			2848			15.3	-	-	28.9
SE ±	-	3.4	3.3		1622	864	-	1.5	1.3		1.2	-
LSD 5%		10.8	10.5	-	-	2744	-		4.1		-	-
1%	-		19.3									

growth 8 days AD had significantly higher ($P<0.05$) soluble carbohydrate levels at day 16 AD than both control and continuously debudded plants (Table 1).

Composition of nodule soluble N

Asparagine was the predominant amino acid (about 80%) with the others being glutamate (6.7%), aspartic acid (3.5%) glutamine (2%), alanine (1%), methionine (1.5%) and serine and arginine (1%). The concentrations of the principal amino acids, although marginally higher in debudded plants than controls did not differ significantly at day 8 AD. By the end of the experiment, however, the concentrations of aspartate, asparagine, serine, alanine, arginine and tyrosine were significantly higher in nodules of plants debudded continuously than in control and debudded plants allowed to resume growth 8 days AD. Two amino acids, tyrosine and valine which were not found at day 8 AD in all treatments were found in the nodules of plants which were continuously debudded (Table 2).

Malate content of the nodules

Malate content was 39% higher in debudded plants compared to controls at day 8 AD. At day 16 AD, malate levels were similar in controls and continuously debudded plants but 37% and 42% lower in debudded plants allowed to resume growth compared to controls and continuously debudded plants respectively. The differences were not significant at both times (Table 3).

Discussion

The results of this study confirm earlier findings by Heim *et al.* (1993), Hartwig *et al.* (1994) and Oti-Boateng *et al.*, (1994) which suggest plant biomass and the ability to utilise soluble N as the factors controlling dinitrogen fixation. The decline in nitrogenase activity of nodules of debudded plants and its resumption in

TABLE 2

Amino acid composition of the nodules of faba bean grown for 35d and debudded for either 8d and then allowed to resume normal growth or debudded continuously for 16d. Amino acid data are expressed as µg/g fresh weight. Values in brackets represent percentages of total amino acid.

Amino acid	*Day*	*Control (continuous)*	*Debudded (8 days)*	*Debudded*	*SE ±*	*LSD5%*
Asp	8	172(3.9)	205(3.6)	-	87	ns
	16	116(4.1)	310(5.0)	160(5.6)	38	121
Glu	8	370(8.4)	377(6.6)	-	200	ns
	16	192(6.9)	442(7.1)	273(9.6)	81	ns
Asn	8	3520(80)	4683(81.7)	-	790	ns
	16	2282(81)	4787(76.6)	2139(75.0)	682	2171
Ser	8	29(0.7)	59(1.0)	-	19	ns
	16	0	96(1.5)	50(1.8)	26	82
Gln	8	130(3.0)	149(2.6)	-	11	ns
	16	59(2.1)	0	38(1.3)	58	ns
Ala	8	64(1.5)	78(1.4)	-	60	ns
	16	21(0.7)	140(2.2)	51(1.8)	88	ns
Arg	8	39(0.9)	87(1.5)	-	42	ns
	16	73(0.8)	253(4.0)	44(1.5)	59	187
Tyr	8	0	0	-	-	-
	16	0	78(1.2)	15(0.5)	20	64
Met	8	78(1.8)	91(1.6)	-	22	ns
	16	58(2.1)	93(1.4)	75(2.6)	18	ns
Val	8	0	0	-		
	16	0	48(0.8)	0		-

plants allowed to resume growth, coupled with a decrease in total amino acid levels in these nodules lend further support to this assertion. Pate *et al.* (1969) reported that the export of N from nodules was a selective process and that asparagine, aspartate, glutamine and aspartate are the major compounds exported from the nodules of *Vicia faba*. They reported the inability of nodules to accumulate abnormally large amounts of nitrogenous solutes as a result of fixation.

Under conditions where the phloem sap had elevated levels of N due to an inability of the shoot to utilise N, the excess was returned to the roots and nodules elevating recycled N

TABLE 3

Effect of debudding on malate content of fresh nodules of faba bean. Data shown is expressed

Treatment	*Malate content (ng)*	
	Days after debudding	
	8	16
Control	84	78
Debudded (Continuous)	117	81
Debudded (8 days)		57
SE ±	35	12

concentrations. From pea nodule xylem sap analysis, Minchin & Pate (1973) also found asparagine to be the major amino acid in the nodules and computed that 30% of current N_2 exported to the shoot was returned to the root. Recycled N may, therefore, be the medium through which a feedback mechanism operates. Differences in nodule amino acid content were mainly due to differences in asparagine levels and suggest asparagine to be the regulatory factor controlling nitrogenase function in nodules actively fixing nitrogen.

Pate *et al.* (1981) also found that about 56% of fixed N exported from the nodules of lupin was asparagine with 29% being glutamine. This may, however, be an underestimation since their measurements were based on the difference in xylem sap composition from above and below the nodulated region. The results also show that under normal growth conditions the concentration of nodule soluble N, particularly asparagine is fairly constant although total amino acid levels may differ under different growth conditions. The absence of glutamine in the the nodules of plants 16 days after continuous debudding and the presence of tyrosine and valine may reflect the synthesis of other amino acids from others already present under abnornal conditions of high soluble N accumulation.

In contrast to soluble N, the concentration of ammonium in the nodules did not differ between controls, continuously debudded and debudded plants allowed to resume vegetative growth 8 days AD. The results are consistent with previous studies which suggest ammonia concentration in the nod- dules to be fairly constant (Bergersen & Turner 1967, Boland *et al.*, 1980 and Heim *et al.*, 1993).

Ammonia concentration in the nodule may therefore not be involved in the regulation of nitrogenase function.

Recent evidence suggests that bacteroids are dependent on the host plant for respiratory substrates to support nitrogen fixation and malate is the principal substrate taken up by bacteroids (Schuller & Warner, 1993). Nelson & Edie (1988) also reported higher malate pools (46%) in less effective nodules of *Pisum sativum* compared to more effective ones. The results of this experiment did not clearly show the role of malate in nodule function although 8 days after debudding when nitrogenase activity had started declining in debudded plants, malate concentration in nodules of these plants was 39% higher than those of controls in which nitrogenase activity was increasing to support higher vegetative growth. By day 16 AD, the concentration of malate in the nodules of control plants and continuously debudded plants was similar probably because some of the nodules of debudded plants had senesced. By contrast malate level in nodules of debudded plants allowed to resume growth was about 70% of controls and continuously debudded plants suggesting that the new nodule growth and the increase in ARA to support new vegetative growth required higher levels of malate.

Conclusion

This study provides further evidence that the demand for fixed N_2 by a nodulated legume is the regu- latory factor controlling nodule function. Recycled soluble N, predominantly asparagine rather than the direct effects of the initial products of N_2 fixation appears to be more important in the regulatory mechanism. As suggested by Heim *et al.* (1993) and Hartwig *et al.*(1994) the increase in nodular diffusion resistance proposed as an alternative hypothesis to explain the inhibition of nitrogenase act- ivity by combined N may rather be a consequence of increased nodule soluble N. The decrease in the plant's

ability to use fixed N reduces bacteroid function with a concomitant reduction in respiratory requirements. Nodular O_2 diffusion resistance therefore increases. The results support the feedback mechanism.

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