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Physiological comparisons of root and stem nodules of *Aeschynomene scabra* and *Sesbania rostrata*

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Abstract

A few legume species possess the ability to form N_2-fixing nodules on stems as well as on roots. Little is known of the functional characteristics of stem nodules, or to what extent they differ from root nodules. Stem and root nodules of greenhouse-grown plants of *Aeschynomene scabra* (inoculated with the photosynthetic rhizobial strain BTAi 1) and *Sesbania rostrata* (inoculated with *Azorhizobium caulinodans* strain BTSr 3) were examined for assimilation of $^{14}CO_2$ in the light and dark, soluble carbohydrate and starch contents, acetylene reduction activity, relative efficiency of nitrogenase in terms of uptake-hydrogenase activity, glutamine synthetase and glutamate synthase, and reduced N and ureide contents. In general, stem nodules possessed higher enzyme activities and metabolite contents than did root nodules, suggesting that they fix N_2 with greater energy efficiency. This greater efficiency correlated with photosynthesis in the cortex of stem nodules. Differences in enzyme activities and metabolite contents between the stem nodules on *A. scabra* and those on *S. rostrata* probably result either from legume-species characteristics or from the photosynthetic capability of strain BTAi 1.

Introduction

Legume species in the genera Aeschynomene and Sesbania can form nodules on stems as well as on roots (Alazard, 1990; Dreyfus and Dommergues, 1981; Dreyfus et al., 1988; Eaglesham and Szalay, 1983; Yatazawa and Yoshida, 1979). Stem nodules differ most strikingly from root nodules in that they have chloroplasts throughout the cortex (Dreyfus and Dommergues, 1981; Legocki et al., 1983) including cells contiguous with the bacteroid zone (A R J Eaglesham, unpublished data). In root nodules, the generation of reducing power and ATP necessary for nitrogenase activity and for growth and maintenance of cells depends on the translocation of photosynthate from leaves (Rawsthorne et al., 1980). Actively fixing legumes partition 30 to 50% of total photosynthate to the root nodules, with losses of 18 to 30% via respiration (Minchin et al., 1981). Any mechanism permitting an economy in photosynthate usage for the process of N_2 fixation is likely to have a positive effect on yield. Consequently, much recent work has focussed on hydrogen recycling via rhizobial uptake-hydrogenase, CO_2 recycling via nodule PEP carboxylase, and ureide-transport compounds that have a lower C:N ration than do amines or amides (Neves and Hungria, 1987).

In stem nodules it would seem likely that C supplied by photosynthesis in the cortical cells would contribute to N_2 fixation in the bacteroids, and to the assimilation of ammonia in the legume cell cytosol. However, little is known of

the functional characteristics of stem nodules or to what extent they differ from those of root nodules. Consequently, in this paper we compare stem and root nodules for physiological parameters related to C and N metabolism. The nodules were induced on *Aeschynomene scabra* and *Sesbania rostrata*. *S. rostrata* was inoculated with rhizobial strain BTSr 3 which has characteristics (Hungria et al., 1991) of the new species *Azorhizobium caulinodans* (Dreyfus et al., 1988). *A. scabra* was inoculated with strain BTAi 1 which was recently shown to synthesize bacteriochlorophyll *a* (Evans et al., 1990), and to be photosynthetic in vitro (Hungria et al., 1991); it has been suggested that this Rhizobium be named *Photorhizobium thompsonianum* (Eaglesham et al., 1990).

Materials and methods

Plant material

Plants of *Aeschynomene scabra* and *Sesbania rostrata* were grown in the greenhouse between May and July, under natural light supplemented with 500-W tungsten-halogen lamps, with day/night temperatures of 30/24°C, and relative humidity of 50 to 80%.

All plants were grown from 30-cm stem cuttings. In the case of *S. rostrata*, root and stem nodules were induced to form on the same plants. Two cuttings were rooted per 15-cm pot containing 2 kg fine silica sand. Each pot contained 500 mL nutrient solution (Eaglesham et al., 1983) with 5.7 m*M* KNO_3 (40 mg N per pot) to encourage vigorous early growth. Once per week each pot was watered with N-free nutrient solution (Eaglesham et al., 1983), otherwise tap water was applied as needed, by application to saucers.

In the case of *A. scabra*, root nodules and stem nodules were induced to form on separate groups of plants; poor development of stem nodules has been attributed to prior presence of root nodules (A R J Eaglesham, unpublished). For stem-nodule plants, cuttings were surface-sterilized (by immersion in alcohol for 2 min with thorough rinsing in sterile tap water) and were submerged in glass cylinders in nutrient solution containing 5.7 m*M* KNO_3. After 14 days, the adventitious roots were closely inspected for incipient nodules. Cuttings free of root nodules were transplanted, three per 25-cm sealed ceramic pot. Each ceramic pot contained 6 kg fine silica sand flooded with N-free nutrient solution to a depth of 4 cm, and had been placed in a sealed plastic bag in an oven at 80°C for 7 days to eliminate any contaminating rhizobia. For root-nodulated *A. scabra*, plants were grown as described for *S. rostrata*.

Plants were harvested at 5 weeks after inoculation with rhizobia.

Rhizobia and inoculation

The rhizobial strain for *A. scabra* was BTAi 1. This was isolated at the Boyce Thompson Institute from a submerged stem nodule on *A. indica* (Eaglesham and Szalay, 1983) having originated from the sand in which the plant was grown (Stowers and Eaglesham, 1983). This is now known to be the first photosynthetic rhizobial strain to have been discovered (Eaglesham et al., 1990; Evans et al., 1990; Hungria et al., 1991).

In order to have a non-photosynthetic Rhizobium to compare with BTAi 1, several strains from *Aeschynomene* spp. were obtained from other culture collections; however, all failed to nodulate the stems of *A. scabra*. Therefore, the non-photosynthetic rhizobial strain BTSr 3 was used with its host legume *S. rostrata*. This strain was isolated from a nodule high on the stem of a 3-m high *S. rostrata* plant grown in the field at the International Institute of Tropical Agriculture, Nigeria. The stem of this plant had been inoculated with *Azorhizobium caulinodans* strain ORS 571 (Dreyfus et al., 1988) as a rooted cutting in the greenhouse prior to transplanting in the field, by Dr K Mulongoy. Therefore, strain BTSr 3 is probably the same as ORS 571.

For inocula, the rhizobia were cultured in yeast-extract broths (Vincent, 1970) containing 1% (wt/vol) of either mannitol (BTAi 1) or succinate (BTSr 3). Inoculations were made at 4 weeks after cuttings were transplanted. For root nodules, 2-mL aliquots of inoculum (10^9 cells mL^{-1}) were added to 250 mL of N-free nutrient solution, then applied to the surface of each pot. For stem nodules, inoculum-soaked

cotton swabs were applied to stems on three occasions over a period of 4 days.

Enzyme extractions and analyses

Nitrogenase (EC 1.18.6.1) activity was assayed by the acetylene reduction technique. Stems were cut into 10-cm lengths, and roots excised, and placed separately in 500-mL glass jars fitted with rubber serum stoppers. Acetylene was injected to a partial pressure of 0.015 MPa, and, after incubations at room temperature of 4 and 30 min, samples were taken and assayed for ethylene by gas chromatography (Hardy et al., 1968).

Hydrogen evolution was assayed by gas chromatography without injection of acetylene, and the relative efficiency (RE) of electron allocation was evaluated as follows:

$$RE = 1 - \frac{H_2 \text{ evolved}}{C_2H_2 \text{ reduced}}$$

(Schubert and Evans, 1976).

Nodules were picked off, dried (75°C) and weighed. Total N contents of dried nodules were determined by Kjeldahl digestion and automated NH_4^+-assay by the indophenol-blue method (Keeny and Nelson, 1982). Nodule ureide contents were evaluated after extraction in 50% ethanol in 100 m*M* potassium phosphate buffer (pH 7.0) at 80°C for 5 min. Filtrates were centrifuged and assayed by the phenylhydrazine method (Boddey et al., 1987).

Rates of uptake of $^{14}CO_2$ by excised nodules were determined by modification of previously described methods (Maxwell et al., 1984). Fresh nodules (200 to 400 mg for *S. rostrata*, 80 to 120 mg for *A. scabra*) from stems and roots were placed on moist filter paper in 10-mL glass scintillation vials, and sealed with rubber serum stoppers. In a separate sealed vial containing $NaH^{14}CO_3$ (37 MBq), 4 *M* lactic acid was injected so as to release 10 μCi of $^{14}CO_2$ mL^{-1}. A 1-mL aliquot was injected into each of four replicate reaction vessels, with a 1-h incubation at 28°C and light flux density of 400 μmol m^{-2} sec^{-1}. Also, four replicates were incubated in the dark, covered with black paper. Reactions were stopped by injection of 1.5 mL 50% ethanol (vol/vol) at 70°C. Nodules were homogenized in the ethanol, sonicated for 5 min, extracted for 20 min at 45°C, finally with the addition of 0.2 *M* HCl. Samples were dried in a laminar flow hood, 10 mL of scintillation fluid added to each, and radioactivity determined. Protein contents were determined (Bradford, 1976).

Glutamine synthetase (GS, EC 6.3.1.2) was extracted by grinding nodules (approximately 500 mg fresh weight) with a mortar and pestle in the medium of Kendall et al. (1986). After centrifugation at 20,000 *g* for 30 min, low molecular weight components of the crude extract were removed by Sephadex G-25 gel filtration with 50 m*M* Tris-HCl buffer (pH 7.8) containing 10 m*M* $MgSO_4$, 10% ethanediol and 5 m*M* dithiothreitol. All extraction procedures were at 4°C. The transferase assay was performed with 50-μL samples according to Cullimore and Sims (1980), and the semi-biosynthetic-hydroxylamine assay according to Cullimore et al. (1982).

Glutamate synthase (GOGAT, EC 1.2.1.14) was extracted by grinding approximately 500 mg fresh nodules with a mortar and pestle, in 50 m*M* potassium phosphate buffer (pH 7.5) containing 5 m*M* EDTA, phenylmethyl sulphonyl fluoride and 0.05% Triton X-100. The filtered homogenate was centrifuged at 20,000 *g* for 30 min, and low molecular-weight components of the crude extract were removed by Sephadex G-25 gel filtration with the extraction buffer. All extraction procedures were at 4°C. The enzyme was assayed using 50-μL aliquots of nodule extract and the following reaction mixture in a total of 1 mL: 100 m*M* potassium phosphate pH 7.5, 1% mercaptoethanol, 1 m*M* 2-oxoglutarate, 0.1 m*M* NADH, and 0.1 m*M* EDTA. Activity was determined spectrophotometrically at 30°C by monitoring the oxidation of NADH at 340 nm after the addition of 5 m*M* L-glutamine.

Soluble carbohydrate was extracted from frozen nodules as follows. Aliquots of 200 mg were placed in 5 ml ethanol at 70°C, sonicated for 10 sec and allowed to stand without heat for 10 min. After centrifugation at 1,400 *g* for 10 min, the supernatant was collected. The pellet was washed twice in 5 mL of 100% ethanol and supernatants collected. Soluble carbohydrate in

the combined supernatant fraction was determined by the anthrone method (modified from Ou-Lee and Setter, 1985). The pellet was assayed for starch after gelling by the addition of 200 μL 95% ethanol (vol/vol) and 4 mL of acetate-fluoride buffer (0.1 *M* sodium acetate, 20 m*M* NaF, with 100 m*M* acetic acid added dropwise to a pH of 4.5). After 15 min in a boiling-water bath, samples were cooled quickly on ice. To each sample was added 1 mL of buffered amyloglucosidase (250 mg amyloglucosidase at 6 U mg^{-1} (Sigma Chemical Co.) in 100 mL of acetate-fluoride buffer), followed by mixing, capping and incubation at 50°C for 24 h. Samples were then vortexed and centrifuged at 1,400 *g* for 10 min, and 200 μL of supernatant added to 300 μL acetate-fluoride buffer. Glucose was assayed by conversion via glucose oxidase (Sigma Chemical Co.) to gluconate and hydrogen peroxide, and the latter produced a color in combination with o-dianisidine and peroxidase; this was achieved by the addition of 5 mL of Sigma Kit 510-A and 1.6 mL o-dianisidine reagent. After mixing and incubation at 37°C for 30 min, samples were read at 450 nm. Glucose and starch standards were analyzed with each set of determinations.

Results and discussion

In the dark, the rates of $^{14}CO_2$ assimilation (putative PEP carboxylase activity) by stem and root nodules from *Aeschynomene scabra* and *Sesbania rostrata* were in the range 76 to 88 nmol mg^{-1} protein min^{-1} (64 to 77 μmol g^{-1} dry nodule h^{-1}) (Table 1). In soybean nodules it was calculated that 66% of PEP carboxylase activity (of 102 μmol g^{-1} dry nodule h^{-1}) was involved in the production of organic acids, the oxidation of which would provide a 48% saving in ATP equivalents (King et al., 1986). Therefore, the rates we observed contributed significantly to C economy.

In his description of stem nodules on *A. aspera* 60 years ago, Hagerup (1928) remarked on their green color and on the presence of starch grains in their cortical cells, and he suggested the likelihood of 'efficient' photosynthesis. The increased incorporation of $^{14}CO_2$ in the light demonstrated that stem nodules are, indeed, photosynthetic organs (Table 1). Increases of 10- and 33-fold were obtained with *S. rostrata* and *A. scabra*, respectively, whereas light had no effect on root nodules. The responses of stem nodules were doubtless the result of the presence of chloroplasts, but the greater response in *A. scabra* correlates with the photosynthetic capability of the rhizobial endophyte. Reports that BTAi 1 synthesizes bacteriochlorophyll *a* and photosynthetic reaction centers (Evans et al., 1990), and exhibits light-driven assimilation of $^{14}CO_2$ and light-decreased O_2 uptake (Hungria et al., 1991) demonstrate that it is the first photosynthetic Rhizobium to have been discovered. Moreover, there is evidence that stem-nodule bacteroids of BTAi 1 can photosynthesize in situ (Evans et al., 1990). Inability to synthesize bacteriochlorophyll in the dark (Evans et al., 1990) explains the lack of stimulation of CO_2 fixation by light in *A. scabra* root nodules.

Table 1. The effects of light (400 μmol m^{-2} sec^{-1}) on rate of $^{14}CO_2$ assimilation by 5-week-old nodules of *Sesbania rostrata* and *Aeschynomene scabra*

Legume species	Nodule type	Light/ dark	$^{14}CO_2$ assimilation rate (nmol mg^{-1} protein min^{-1})
S. rostrata	Stem	Light	941a[a]
		Dark	88.6b
	Root	Light	75.2b
		Dark	88.5b
A. scabra	Stem	Light	2618a
		Dark	75.7b
	Root	Light	81.5b
		Dark	82.6b

[a] Within legume species, means of five replicates followed by the same letter are not significantly different ($p = 0.05$, Tukey's test).

The higher contents of soluble carbohydrate and of starch in stem nodules than in root nodules (Table 2) are explained in terms of the photosynthetic capability of the former. With the greater availability of C, specific acetylene reduction activities of stem nodules were higher than those of root nodules, by 50% in *S. rostrata* and by 8-fold in *A. scabra* (Table 3). Very high specific activity has been observed before with stem nodules of *A. scabra* (Eaglesham and Szalay, 1983); these activities (300 to

Table 2. Soluble carbohydrate and starch contents of 5-week-old root and stem nodules of *Sesbania rostrata* and *Aeschynomene scabra*

Legume species	Nodule type	Soluble carbohydrate (mg glucose equiv g^{-1} FW)	Starch (mg g^{-1} FW)
S. rostrata	Stem	16.1a[a]	29.7a
	Root	11.7b	16.6b
A. scabra	Stem	19.4a	50.2a
	Root	9.87b	17.8b

[a] Within legume species, means of five replicates followed by the same letter are not significantly different ($p = 0.05$, Tukey's test).

400 μmol g^{-1} nodule h^{-1}) are higher than values commonly reported for alfalfa, soybean or cowpea. Statistical comparison of stem nodules of *A. scabra* and *S. rostrata* revealed that the specific acetylene reduction activity of the former was significantly greater than that of the latter. Whether the acetylene-induced inhibition of acetylene reduction activity of root nodules (Minchin et al., 1983; 1986) occurs also with stem nodules remains to be determined.

Dixon (1972) suggested that the recycling of hydrogen in root nodules by uptake-hydrogenase is a mechanism of protection of nitrogenase from O_2. The higher RE of stem nodules in comparison with root nodules of *A. scabra* (Table 3) may correlate with higher levels of O_2 diffusion to the bacteroid zone from the photosynthetic cortex. However, it is interesting to note that no such higher RE was found in the stem nodules of *S. rostrata*. Differences in leghemoglobin components in stem and root nodules of both *S. rostrata* (Bogusz, 1984) and *A. scabra* (Legocki et al., 1983) may correlate with higher levels of O_2 in the bacteroid zone of stem nodules. On the other hand, model system studies with bacteroids from *S. rostrata* stem nodules have suggested that O_2 levels are not significantly different from those in root nodules (Bergersen et al., 1988).

The assimilation of ammonia produced in nodules occurs via the combined functions of GS and GOGAT in the host-cell cytoplasm (Miflin and Cullimore, 1984). Probably as a consequence of higher nitrogenase activity and higher availability of carbohydrate, GS transferase and GOGAT activities were also higher in stem than in root nodules of both species (Table 4). The transferase:synthetase ratios for GS were similar for stem and root nodules within both species, i.e. 46 for *A. scabra*, and 45 for *S. rostrata*. The stem:root ratios for GS and GOGAT activities were larger in *A. scabra* (4.5 and 2.8, respectively) than in *S. rostrata* (1.5 and 1.3, respectively) (Table 4).

Detached nodules did not exude xylem sap, therefore ureide contents were analyzed in nodule homogenates. Ureide concentrations in *A. scabra* nodules were higher than those in *S. rostrata* (Table 5) and similar to those found in *Phaseolus vulgaris* nodules from which 90% of N was transported as ureide (M Hungria and A R J Eaglesham, unpublished). As a general rule, nodulated tropical legume species transport N mainly as ureides, and it is believed that the lower C:N ratio (as compared to amide and amine transported by temperate legumes) contributes a 14% economy in C usage, although it is unclear what energy costs are involved in ureide

Table 3. Relative efficiencies and acetylene reduction activities of nitrogenase of 5-week-old root and stem nodules of *Sesbania rostrata* and *Aeschynomene scabra*

Legume species	Nodule type	Relative efficiency	Acetylene reduction (μmol g^{-1} nod. h^{-1})
S. rostrata	Stem	0.85a[a]	93.4a
	Root	0.85a	62.4b
A. scabra	Stem	0.86a	363a
	Root	0.71b	40.2b

[a] Within legume species, means of five (relative efficiency) or six (acetylene reduction) replicates followed by the same letter are not significantly different ($p = 0.05$, Tukey's test).

Table 4. Activities of glutamine synthetase (GS) and glutamate synthase (GOGAT) of 5-week-old root and stem nodules of *Sesbania rostrata* and *Aeschynomene scabra*

Legume species	Nodule type	GS	GOGAT
		(μmol mg^{-1} protein h^{-1})	
S. rostrata	Stem	3.01a[a]	7.44a
	Root	2.03b	5.58b
A. scabra	Stem	5.47a	13.5a
	Root	1.22b	4.73b

[a] Within legume species, means of five replicates followed by the same letter are not significantly different ($p = 0.05$, Tukey's test).

Table 5. Dry weight, percent N, and ureide contents of 5-week-old root and stem nodules of *Sesbania rostrata* and *Aeschynomene scabra*

Legume species	Nodule type	Dry weight (mg nodule^{-1})	N conc. (%)	Ureide (μgN g^{-1} nod.)
S. rostrata	Stem	2.30a[a]	4.46a	724a
	Root	2.15a	4.12b	577b
A. scabra	Stem	0.39a	4.99a	1774a
	Root	0.23a	4.04b	1515b

[a] Within legume species, means of six replicates followed by the same letter are not significantly different ($p = 0.05$, Tukey's test).

utilization (Neves and Hungria, 1987). Consistent with the higher specific nitrogenase activities in stem nodules as compared with root nodules, concentrations of reduced N and ureides were higher in the former than in the latter (Table 5).

Both stem and root nodules of *A. scabra* and *S. rostrata* possess mechanisms for economizing on C requirements, i.e. PEP carboxylase and ureide synthesis. In both species, stem nodules apparently benefit from photosynthesis in cortical cells by the provision of C compounds in situ for nitrogenase, GS and GOGAT and for ureide synthesis. The differences in enzyme activities between stem nodules on *A. scabra* and *S. rostrata* are consistent with the previously observed energy-sufficiency of Aeschynomene stem nodules (Eardly and Eaglesham, 1985), and can be explained in terms of the photosynthetic capability of rhizobial strain BTAi 1. However, pending comparisons of photosynthetic and non-photosynthetic rhizobial strains able to nodulate the same host legume, it cannot be ruled out that observed differences resulted from contrasting characteristics of stem nodules on *A. scabra* vs. *S. rostrata*.

In conclusion, the characteristics of stem nodules over root nodules would seem to justify an effort to induce stem nodules on agriculturally important legumes. If successful, it would be advantageous to have the infecting Rhizobium express photosynthesis genes.

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