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## THE *AZOLLA-ANABAENA AZOLLAE* RELATIONSHIP

### X. $^{15}\text{N}_2$ FIXATION AND TRANSPORT IN MAIN STEM AXES\*†

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#### SUMMARY

The fixation of  $^{15}\text{N}_2$  and its transport were studied as a function of leaf development along the main stem axes of *Azolla*.

Main stem axes dissected from *Azolla caroliniana* Willd. plants, and sequential groups of leaves along the axes, had 25 to 35% less nitrogenase activity than the undissected controls when assayed immediately. However, they exhibited activities equivalent to those of the intact controls when allowed to recover for 12 h or more prior to assaying. Nitrogenase activity was determined as a function of leaf age by measuring  $^{15}\text{N}_2$  fixation and  $\text{C}_2\text{H}_2$  reduction in parallel samples. The reduction of both substrates was very low in the apical region, increased rapidly with leaf maturation and plateaued prior to declining as leaves began to senesce. Whereas the nitrogen content and dry matter decreased with increasing leaf age, the C:N ratio increased. These findings are consistent with the demonstration that nitrogen fixed by the endophytic *Anabaena* in mature leaves is transported toward the stem tip where undifferentiated but actively dividing filaments of the endophyte are associated with the apex and leaf primordia.

#### INTRODUCTION

*Azolla* is a genus of aquatic, heterosporous ferns that normally contain a symbiotic, heterocystous cyanobacterium, *Anabaena azollae*, within cavities formed in their aerial dorsal leaf lobes. The endophytic *Anabaena* undergoes a pattern of development and differentiation in parallel to that of the fern (Hill, 1975, 1977; Peters *et al.*, 1978; Calvert and Peters, 1981). Filaments of the endophyte associated with the stem apex are actively dividing and lack heterocysts. As leaf cavities are formed and occupied by the endophyte, heterocysts are rapidly differentiated and their frequencies reach 25 to 30% in mature cavities (Hill, 1975, 1977; Peters, 1975). Heterocyst differentiation in the endophyte is accompanied by diminished cell division, cell enlargement and increased nitrogenase activity as assayed by acetylene reduction (Hill, 1975, 1977; Peters, Ray *et al.*, 1980).

In the present study we have employed  $^{15}\text{N}_2$  reduction to verify the profile of nitrogenase activity observed in excised leaf segments with  $\text{C}_2\text{H}_2$  reduction, and determined the N content, percentage dry matter and C:N ratio as a function of leaf age. In addition, reduction of  $^{15}\text{N}_2$  by main stem axes has been employed in conjunction with a pulse-chase approach to demonstrate the transport of N fixed in mature cavities toward the shoot tip. The results are discussed in relationship to developmental morphology and other aspects of the symbiosis.

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## MATERIALS AND METHODS

*Growth conditions*

*Azolla caroliniana* Willd. was grown on N-free medium (Ray *et al.*, 1978; Peters, Toia *et al.*, 1980) using a constant growth temperature of 24 to 25 °C, and a 16–8 h light–dark cycle. Illumination of approximately 200  $\mu\text{E m}^{-2} \text{s}^{-1}$  was provided by a mixture of cool white fluorescent and incandescent lights. Cultures were maintained by frequent transfer and plant material of uniform culture age was employed in all studies.

*Dissection of stem axes and segments*

Main stem axes and sequential segments, beginning with the shoot tip and progressing through older leaves, were prepared and allowed to recover as described in the Results.

*C<sub>2</sub>H<sub>2</sub> and <sup>15</sup>N<sub>2</sub> reduction*

Acetylene reduction assays were conducted in capped serum vials using 10% C<sub>2</sub>H<sub>2</sub> in air. Incubation periods were 30 to 60 min under the conditions used for growth.

<sup>15</sup>N<sub>2</sub> reduction assays were carried out in 10 ml or 25 ml micro-Fernbach flasks containing 2 or 5 ml of medium and 0.2 or 0.5 g fresh wt of plant material respectively. The flasks were fitted with serum caps and sealed as described previously (Peters, Toia and Lough, 1977). Flasks were evacuated and flushed six times with Ar–0.03% CO<sub>2</sub> prior to filling with 0.2 atm (20.2 kPa) O<sub>2</sub>, 0.0003 atm (0.03 kPa) CO<sub>2</sub> and 0.8 atm (80.8 kPa) N<sub>2</sub> enriched with <sup>15</sup>N<sub>2</sub> as indicated in specific experiments. Measured volumes of the specific gases were added directly to the individual flasks. <sup>15</sup>N<sub>2</sub> was dispensed from a displacement apparatus comparable to that described in Burris (1972). The  $p\text{O}_2$ ,  $p\text{N}_2$  and atom per cent excess <sup>15</sup>N in the gas phase of each flask were determined at the end of the incubation period (Peters *et al.*, 1977). Samples were incubated for 30 or 60 min under conditions employed for growth in the light. The procedures for Kjeldahl digestion, distillation, ammonia determination, hypobromite oxidations and calibration of the mass spectrometer using standards of known atom per cent <sup>15</sup>N were essentially as described previously (Peters *et al.*, 1977).

*<sup>15</sup>N<sub>2</sub> 'pulse-chase' studies*

For the <sup>15</sup>N<sub>2</sub> 'pulse-chase' experiments main stem axes were dissected as described in the Results. Based on total N g<sup>-1</sup> fresh wt, 3 days were required to dissect out a sufficient number of stem axes for the study. The dissected axes were combined and allowed to recovery under normal growth conditions for a minimum of 12 h. Hence, some of the axes were necessarily allowed to recover for up to 72 h. Although not apparent to the naked eye, observation of the stem axes under the dissecting microscope revealed the initiation of new lateral branch apices in the apical region of some stem axes which had been allowed the longest recovery period. No attempt was made to remove these structures. The dissected material was transferred to flasks for <sup>15</sup>N<sub>2</sub> incubations. Each flask contained 0.3 or 0.5 g fresh wt of stem axes and 7 or 10 flasks (three groups in duplicate or triplicate plus a control) were used. Incubations were as described above using an 'air' atmosphere enriched with <sup>15</sup>N<sub>2</sub> and normal growth conditions in the light. At the end of the incubation period ( $T = 0$ ) the flasks were opened and the contents of the control

flask and one group of two or three flasks were frozen using Freon 12 in a bath of liquid N<sub>2</sub>. The other flasks were flushed vigorously with a stream of room air immediately upon opening and, after additional intervals of N<sub>2</sub> fixation under air (chase periods), the contents of the other two groups of flasks were likewise frozen with Freon 12. The frozen samples were lyophilized overnight and placed in a desiccator. Subsequently, the stem axes of the individual samples were dissected into sequential segments as described in the Results. Segments from the same experimental treatments were combined, digested, distilled and analyzed for <sup>15</sup>N enrichment as described above.

#### Dry wt and total N

Samples of known fresh wt were dried at 60°C for 24 h and dry wts determined. Total N was determined after Kjeldahl digestion (Burris, 1972) or by CHN analysis of dried material using a Perkin-Elmer Model 240 Elemental Analyzer equipped with a Control Equipment Corporation MC-341-HA microinjector (Peters, Toia *et al.*, 1980).

### RESULTS

Most of this study involved the use of main stem axes, or sequential segments of them, rather than whole plants. Main stem axes were obtained by dissection, removing the lateral branches and branch apices (Fig. 1) (see also fig. 1(a) in the companion paper, Calvert and Peters, 1981). Sequential segments were cut from the main stem axes, beginning at the stem tip and progressing through older leaves. Depending upon the experimental objective, stem axes were dissected using two different sequences, hereafter designated A and B respectively. In sequence A the segments were comprised of the shoot tip (apical region including leaf primordia and very young leaves), leaves 1 to 4, where leaf 1 is the first clearly discernible

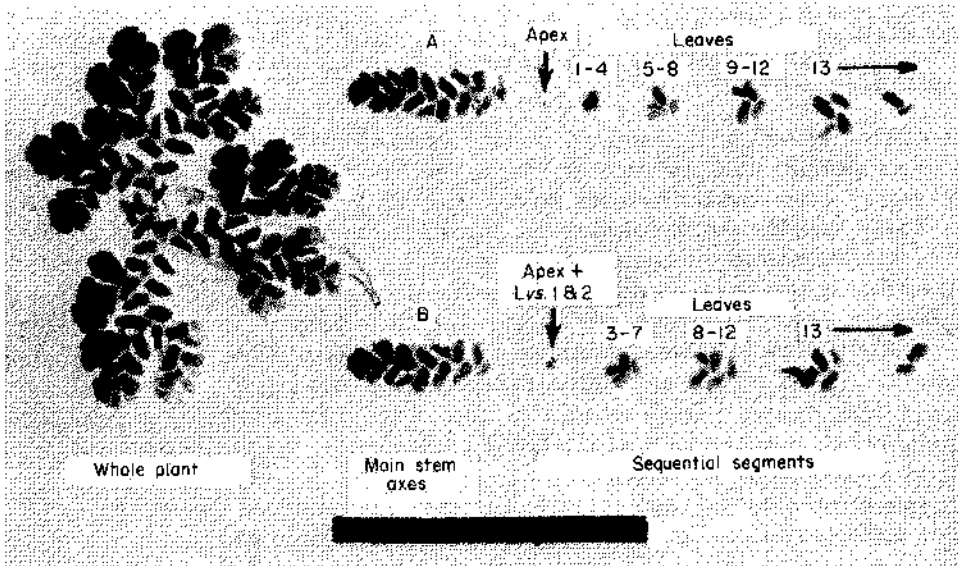


Fig. 1. A demonstration of the relationship between the whole plant, main stem axes and the sequential segments. A and B represent the two sequences of dissection used.

dorsal leaf lobe and has a completely defined leaf cavity (Calvert and Peters, 1981), leaves 5 to 8, and so on (Fig. 1, A). In sequence B the first segment contained the shoot tip pulse leaves 1 and 2, the next, leaves 3 to 7, and so on (Fig. 1, B).

The effect of dissection on nitrogenase activity was determined using the  $C_2H_2$  reduction assay. When assayed immediately after dissection, the rates obtained for the main stem axes were 65 to 75% of those obtained with whole plants. Nitrogenase activity equivalent to that of whole plants was restored when axes were allowed to incubate for 4 h or more, under normal growth conditions prior to assaying (Table 1, experiment A). Furthermore, the axes could be maintained under normal growth conditions for up to 72 h prior to assaying without any appreciable change in nitrogenase activity (Table 1, experiment B). Cutting the main stem axes into segments also resulted in decrease in  $C_2H_2$  reduction activity (not shown). Although the recovery period (see Table 1, footnote) was longer, the

Table 1. Comparison of the acetylene reduction rate in whole plants and main axes as a function of recovery period

	Recovery period† (h)	Acetylene reduction (nmol $C_2H_4$ formed $g^{-1}$ fresh wt $min^{-1}$ )		
		Whole plant control	Axes	% of control
Expt A*	0	33.23 ± 6.06	22.05 ± 1.91	66
	4	24.52 ± 2.05	23.67 ± 0.50	97
	6	26.44 ± 2.03	29.49 ± 2.27	111
	24	28.10 ± 5.36	29.28 ± 1.57	104
Expt B	24	17.56 ± 0.24	18.28 ± 1.77	104
	48	12.54 ± 0.56	15.00 ± 1.65	120
	72	16.26 ± 1.09	18.33 ± 1.16	113

\* Expt A, short term recovery; Expt B, long term recovery.

† This refers to a post-dissection time interval during which the nitrogenase activity measured in dissected material has, within experimental error and sample variability, returned to pre-dissection levels measured in the appropriate controls. It does not necessarily imply that other metabolic activities respond in an analogous manner.

segments regained 85 to 95% of the activity of the main stem axes after a 12 h recovery period. The recovery of the segments relative to the whole axes was necessarily determined from the combined  $C_2H_2$  reduction per unit time and combined weights of the individual segments, due to the gradient in  $C_2H_2$  reduction activity as a function of leaf age along the stem axes. For example, in one of the studies the rates of acetylene reduction for whole plants and recovered main stem axes were  $35.01 \pm 1.90$  and  $37.82 \pm 1.34$  nmol  $g^{-1}$  fresh wt  $min^{-1}$  respectively. The combined fresh wt of the segments from these axes, using sequence A, was 0.145 g and the total nmol  $C_2H_2$  reduced  $min^{-1}$  were 4.73 for a rate of  $32.62$  nmol  $g^{-1}$  fresh wt  $min^{-1}$ . Thus, after a 24 h recovery period, these segments had regained 93% of the activity measured in the whole plant and 86% of that measured in the axes.

Using sequential leaf segments (sequence A), the profile of nitrogenase activity along the axis was verified by  $^{15}N_2$  reduction (Table 2). In addition to the results of parallel determinations  $^{15}N_2$  fixation and  $C_2H_2$  reduction in the segments, Table 2 shows that the N content  $g^{-1}$  fresh wt decreases in progressively older segments of the stem axes. It should be noted that approximately 35 main stem axes (0.1 g

fresh wt of material) contained 0.22 mg N. Since 0.4 to 0.6 mg N were usually used for analysis of  $^{15}\text{N}$ , 70 to 100 main stem axes were required for each sample. Moreover, analysis of the segments from sequence A actually required combining segments from more than five times this number of axes. A case in point is the apical segment. Although its N content was greater than that of subsequent segments (Table 2), its actual fresh wt was much less than the other segments (see Fig. 1, A). Hence, the number of apical segments required for each analysis was much greater than the number of subsequent segments.

Table 2. Acetylene reduction  $^{15}\text{N}_2$  fixation and total Kjeldahl nitrogen in sequential segments of the Azolla main stem axes (Sequence A)

Segment	Relative $\text{C}_2\text{H}_2$ reduction	Relative $^{15}\text{N}_2$ fixation	Nitrogen ( $\text{mg g}^{-1}$ fresh wt)
Apical region	10	11	3.25
lvs. 1-4	79	80	2.80
lvs. 5-8	100*	100†	2.22
lvs. 9-12	69	62	1.85
lvs. 13 →	55	65	1.62

\* 100%  $\text{C}_2\text{H}_2$  reduction = 23.16 nmol  $\text{C}_2\text{H}_4$  formed  $\text{g}^{-1}$  fresh wt  $\text{min}^{-1}$ .

† 100%  $^{15}\text{N}_2$  fixation = 6.30 nmol  $^{15}\text{N}_2$  fixed  $\text{g}^{-1}$  fresh wt  $\text{min}^{-1}$ .

From the profile of  $\text{N}_2$  fixation as well as the N content of the individual segments and morphology of the association (Calvert and Peters, 1981) it was apparent that  $\text{N}_2$  fixed by the endophyte in mature leaves had to be transported toward the stem tip. In order to demonstrate the occurrence of this, we employed a pulse-chase approach with  $^{15}\text{N}_2$ . The atom per cent excess  $^{15}\text{N}$  in segments of the main stem axes was determined after incubation of the stem axes under  $^{15}\text{N}_2$ -enriched air as well as after subsequent intervals of incubation under room air. In order to increase the amount of plant material and total N in all segments, but especially in the apical region, the segments from dissection sequence B were employed. The use of this sequence did not significantly alter the  $\text{C}_2\text{H}_2$  reduction profile (Table 3). Comparison of Tables 2 and 3 indicates that the majority of the nitrogenase activity associated with leaves 1 to 4 in sequence A was confined to leaves 3 and 4. In accord with Table 2, Table 3 shows that the percentage N on a dry wt basis (determined with a CHN analyzer), decreased in progressively older segments from sequence B. Table 3 also shows that progressively older segments decrease in percentage dry matter but have an increasing C:N ratio.

In one of the pulse-chase type experiments, main stem axes were incubated for 30 min under the equivalent of an air atmosphere enriched to 90 atom per cent excess  $^{15}\text{N}$  (see Methods). Stem axes which had been immediately frozen and lyophilized at the end of the 30 min 'pulse', as well as stem axes which had been 'chased' for 60 or 120 min with room air prior to freezing and lyophilization, were cut into segments (sequence B). The results are expressed using the atom per cent excess  $^{15}\text{N}$  (Fig. 2) and the  $\mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt (Table 4) in the individual segments at the end of the 30 min incubation under  $^{15}\text{N}_2$  and after the two chase periods. These findings clearly demonstrate the transport of fixed nitrogen from mature leaves toward the apex. The relatively high amount of fixed  $^{15}\text{N}_2$  in the first segment from the stem axes receiving no air chase is attributed to transport occurring within the main stem axes during the 30 min incubation

period. Previous studies (Tables 2 and 3) had clearly shown that fixation rates were very low in these segments. No attempt was made to correct the data in Table 4 for the  $^{14}\text{N}_2$  fixed during the air chase periods. Assuming that the rate of  $^{14}\text{N}_2$  fixation was equivalent to that of the  $^{15}\text{N}_2$  during the 30 min period, our calculations indicated that it accounted for only 1.7% of the total  $^{14}\text{N}$  content of

Table 3. Acetylene reduction rates, percentage nitrogen on a dry wt basis, percentage dry matter and C:N ratio, in sequential segments of the *Azolla* main stem axes (sequence B)

Segment	Relative $\text{C}_2\text{H}_2$ reduction	Percentage N	Percentage dry matter	C:N ratio
Apical $\pm$ 1 vs. 1-2	15	6.18	12	7.05
1 vs. 3-7	100*	5.49	10	7.74
1 vs. 8-12	100	4.76	.5	8.48
1 vs. 13 $\rightarrow$	30	3.75	4	10.60

\* 100% = 23.85 nmol  $\text{C}_2\text{H}_4$  formed  $\text{g}^{-1}$  fresh wt  $\text{min}^{-1}$ .

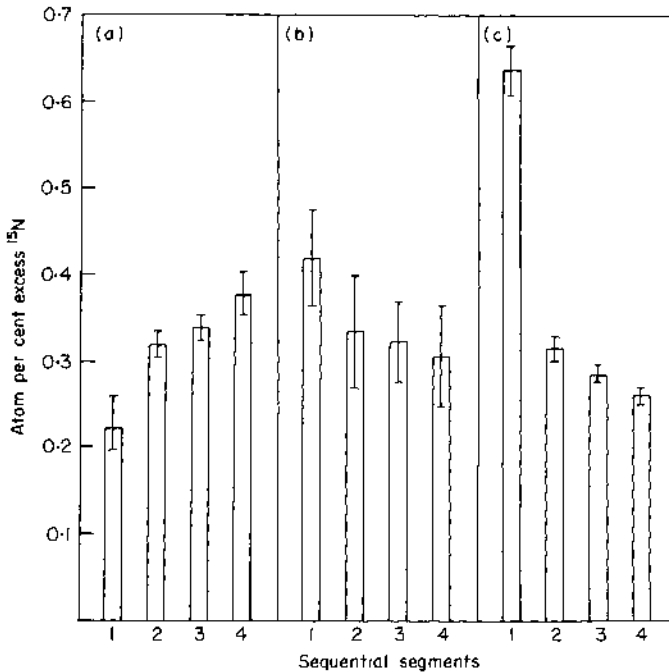


Fig. 2. Atom per cent excess  $^{15}\text{N}$  in sequential segments after (a) 30 min pulse under air enriched to 90 atom per cent excess  $^{15}\text{N}$  (0 time); (b) 30 min pulse followed by a 60 min chase in air; (c) 30 min pulse followed by a 120 min chase in air. Dissection according to sequence B. 1 = apical region and leaves 1-2, 2 = leaves 3-7, 3 = leaves 8-12 and 4 = leaves 13 and older.

the plant material at the end of the 2 h chase. That the  $^{14}\text{N}_2$  fixed was negligible in comparison to that already present in the tissue is also indicated by the fact that while the distribution of the amount of fixed  $^{15}\text{N}_2$  changes dramatically among the segments during the time course, the total amount of  $^{15}\text{N}_2$  fixed by the combined segments at each interval, i.e. the main stem axes, is constant within the confines

of the experimental error. In another study which is not shown, a 1 h incubation was followed by 2 h and 4 h chase periods. The  $^{15}\text{N}$  enrichment of the apical region was higher at the end of the 1 h incubation than at the end of the 30 min incubation, again indicating the occurrence of transport during the incubation under  $^{15}\text{N}_2$ . The change in the distribution of  $^{15}\text{N}$  in the segments during the air 'chase' periods after the 1 h incubation was consistent with the study shown in demonstrating transport of fixed N from the mature leaves toward the actively growing shoot tip.

Table 4. The  $^{15}\text{N}_2$  ( $\mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt\*) content of sequential leaf segments excised from the main stem axes after a 30 min pulse under 20%  $\text{O}_2$ , 0.03%  $\text{CO}_2$  and 80%  $\text{N}_2$  enriched to 90 atom per cent excess  $^{15}\text{N}$  (0 time) and excised after the pulse followed by 60 min and 120 min chase period in air

Segment	Chase period (min)		
	0	60	120
Apical + 1 vs. 1-2	5.04	8.67	10.10
1 vs. 3-7	5.58	5.67	4.31
1 vs. 8-12	5.44	3.47	4.06
1 vs. 13 →	4.20	3.94	2.81
Total	20.26	21.75	21.26

Main axes contained  $5.65 \mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt and a rate of  $188 \text{ nmol } ^{15}\text{N}_2 \text{ fixed g}^{-1} \text{ dry wt min}^{-1}$  at the end of the 30 min incubation under  $^{15}\text{N}_2$ .

\* For dry wt as a percentage of the fresh wt of the segments see Table 3.

## DISCUSSION

In *Azolla-Anabaena* associations the endophytic *Anabaena* undergoes a pattern of development and differentiation which parallels that of the fern (Konar and Kapoor, 1972; Hill, 1977; Peters *et al.*, 1978). While filaments of the endophyte associated with stem apices are comprised almost entirely of actively dividing vegetative cells, heterocysts are rapidly differentiated as leaf cavities are formed (Hill, 1977; Peters *et al.*, 1978). In sequential leaves, or groups of leaves excised from main stem axes of *A. filiculoides* (Hill, 1977), *A. caroliniana* (Peters, Ray *et al.*, 1980) and *A. pinnata* as well as *A. filiculoides* (Shi *et al.*, 1981), nitrogenase-catalyzed acetylene reduction was low or negligible in the apical portion, increased rapidly as leaves matured, leveled off and then declined as leaves senesced. The rapidity of this sequence is dependent upon the *Azolla* species and/or varieties and, to a lesser extent, the culture conditions.

Although not addressed in the previous studies, dissection of the plant to obtain stem axes and the subsequent excision of leaves results in decreased nitrogenase activity. However, this loss of activity is temporary. Activities equivalent to those present in the intact plant are regained if a recovery period under normal growth conditions is employed prior to assaying. Of equal importance to the present study was the demonstration that the activities of stem axes remained constant through 72 h. This enabled us to prepare and pool amounts of material sufficient for  $^{15}\text{N}$  studies.

The developmental profile of nitrogenase activity was verified using  $^{15}\text{N}_2$  reduction in parallel with assays of  $\text{C}_2\text{H}_2$  reduction. It was also shown that the Kjeldahl N  $\text{g}^{-1}$  fresh wt of plant tissue was highest in the apical region and



decreased as a function of leaf age. Although consistent with the more compact organization of the tissues in the apical region and subsequent leaves (Duckett, Toth and Soni, 1975; Peters *et al.*, 1978; Calvert and Peters, 1981), the increase in nitrogenase activity but decrease in N content as a function of leaf age necessarily implied the transfer of fixed nitrogen from mature leaves toward the shoot tip.

Altering the excision sequence of main stem axes such that the first segment included leaves 1 and 2 in addition to the apical region had little effect on the profile of nitrogenase activity but significantly increased the N content of this segment for  $^{15}\text{N}$  analysis. Using this sequence, it was shown that the N content  $\text{g}^{-1}$  dry wt and the percentage dry matter decreased as a function of leaf age while the C:N ratio increased. The values for the C:N ratio, which increased from approximately 7:1 to 10:1, include contributions from the fern and endophyte in the respective leaf segments. However, it is worth noting that in the free-living cyanobacterium *Anabaena cylindrica*, proheterocysts were observed at a C:N ratio of 7:1 but there was no nitrogenase activity until the C:N ratio reached 8:1 (Kulasooriya, Lang and Fay, 1972).

The decreasing N content and increasing C:N ratio as a function of leaf age support the concepts of Hill (1977). Based on the profile of acetylene reduction and differentiation of the endophyte he suggested that  $\text{N}_2$  fixed by the endophyte in mature leaves might be used by the fern to prevent heterocyst formation in the apex and that nitrogen depletion could be a factor which causes heterocysts to develop once the leaves have been colonized. That  $\text{N}_2$  fixed in mature leaves is in fact transported to the apical region was shown here using  $^{15}\text{N}_2$  fixation by main stem axes in conjunction with air chase periods and analysis of  $^{15}\text{N}$  content of sequential leaf segments. These results strongly suggest that the fern supplies the cyanophyte in the apical region with a combined nitrogen source which maintains its growth and prevents heterocyst differentiation. However, direct evidence for this, the mechanism by which it occurs and the nitrogen source which is provided have not been resolved.

An apparent transport rate of the nitrogenous compound was calculated from the data in Tables 3 and 4. Assuming amount of  $\text{N}_2$  fixed in segment 1 was 15% of that in main stem axes (Table 3), segment 1 should have contained  $0.85 \mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt at the end of the 30 min pulse. However, it was found to contain  $5.04 \mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt (Table 4). This indicated that  $4.19 \mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt were transported to this segment during the 30 min pulse. This translates to  $140 \text{ nmol g}^{-1}$  dry wt  $\text{min}^{-1}$ . Since the average distance from the midpoint of segment 1 to the midpoint of segment 2 and 3 was 1.6 mm, we estimate transport toward the apex at a rate of  $87 \text{ nmol fixed } \text{N}_2 \text{ g}^{-1}$  dry wt  $\text{min}^{-1} \text{ mm}^{-1}$ .

The data in Tables 3 and 4 at  $T = 0$ , can also be interpreted to indicate some transport from the actively fixing region (segments 2 and 3), to leaves 13 and older (segment 4). However, this interpretation must be qualified. Unlike the other segments which always contained the same number of leaves, segment 4 included the remaining leaves on the stem axes. Thus this segment was comprised from as few as 4 leaves, i.e. leaves 13 to 16, to as many as 10 leaves, i.e. leaves 13 to 22, depending upon the length of the stem axes. This resulted in some variability in  $\text{C}_2\text{H}_2$  reduction activity by this segment. This variability is indirectly indicated by a comparison of the relative  $\text{C}_2\text{H}_2$  reduction by leaves 13 and older in Tables 2 and 3 and arises in part from the fact that the older leaves of this final segment have less nitrogenase activity than the younger. Thus qualified, the data from Tables 2, 3 and 4 indicate that from  $0.9$  to  $2.52 \mu\text{mol } ^{15}\text{N}_2 \text{ g}^{-1}$  dry wt might be

transported from the actively fixing leaves, i.e. leaves 3 to 12, to the older leaves. (The variable length of the final segments preclude calculation of a transport rate). However, the change in distribution of the  $^{15}\text{N}$  label during the chase periods clearly demonstrates that the majority of the fixed N is transported toward the shoot tip.

It is suggested that the transported compound may be ammonium or glutamine. Previous studies with  $^{15}\text{N}_2$  showed that the endophyte isolated from *Azolla* leaves in all stages of development released up to 50 % of the fixed  $\text{N}_2$  as ammonia (Peters, 1977; Peters, Ray *et al.*, 1980). Undifferentiated filaments in these preparations may well have assimilated some of the released ammonia. Thus, it was suggested that those filaments of the endophyte actively fixing  $\text{N}_2$  might not be able to assimilate it. Within the association, fixed  $^{15}\text{N}_2$  was shown to be rapidly assimilated and incorporated into amino acids and protein (Peters *et al.*, 1979). In accord with this, while both the fern and endophyte exhibited glutamine synthetase, glutamate synthase and glutamate dehydrogenase activities, 80 % of the glutamate dehydrogenase and 90 % of the glutamine synthetase activities were attributable to the fern (Ray *et al.*, 1978). Further, it was suggested that the endophytic glutamine synthetase might well be associated with the undifferentiated filaments in the apex. Because of the normal correlation of glutamine synthetase with heterocysts (Stewart, 1977; Haselkorn, 1978) this implied the existence of a control mechanism whereby heterocysts differentiated in *Azolla* would lack glutamine synthetase and release ammonia to the plant (Ray *et al.*, 1978; Peters *et al.*, 1979). This was consistent with the suggestion (Stewart, 1977; Haselkorn, 1978) that in plant-cyanophyte associations the plant modifies the cyanophytes' ammonia assimilating pathway by producing substances which inhibit either glutamine synthetase activity or synthesis in the endophyte. Thus, in the *Azolla-Anabaena* associations it is possible that ammonia released by the endophyte in the cavities of mature leaves is being transported, or that the ammonia is metabolized to glutamine by the plant glutamine synthetase and then transported, or that both are occurring.

The mixed population of *Anabaena* filaments obtained upon its isolation from all stages of leaf development (Peters and Mayne, 1974; Peters, Ray *et al.*, 1980) fixes  $\text{CO}_2$  by the Calvin cycle and the action spectrum for photosynthesis has a maximum in the region of phycobilin absorption (Ray *et al.*, 1979). However, within the leaf cavities the endophyte might exhibit photoheterotrophic (Peters and Mayne, 1974) or mixotrophic metabolism (Peters, 1975). Hill (1977), in fact, suggested that the  $\text{CO}_2$  fixation in the endophyte preparations reported by Peters (1975) might be largely associated with generative filaments from the apical region.  $\text{CO}_2$  fixation by the endophyte as a function of leaf age has not yet been determined. However, heterocysts lack Photosystem II (PSII) and  $\text{CO}_2$  fixation capability (Haselkorn, 1978). Thus the high heterocyst frequencies of the endophyte in mature cavities would indeed seem to implicate a dependence upon fixed carbon compounds from the fern as a source of reductant to maintain the high rates of  $\text{N}_2$  fixation (Peters *et al.* 1979; Peters, Ray *et al.*, 1980).

Calvert and Peters (1981) have shown that two distinct classes of epidermal hairs (trichomes), termed simple and branched, are associated with the development of the leaf cavity. Based on the distribution and frequencies of these two hair types and the developmental physiology, we suggest that the simple hairs may be involved with the transfer of fixed carbon from the *Azolla* to the endophyte in mature cavities and the branched hairs with the transfer of fixed nitrogen.

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