Stem and root nodules on the tropical wetland legume *Aeschynomene fluminensis*

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**Summary**

*Aeschynomene fluminensis* Vell., originally obtained from flooded areas of the Pantanal Matogrossense region of Brazil, was grown under stem-flooded or non-flooded conditions for 70 d after inoculation with isolates of photosynthetic stem nodule rhizobia obtained from native *A. fluminensis*. Stem nodules formed only on submerged stems of flooded plants (mean of 25 per plant), and did not form on aerial parts, although they were capable of growing and fixing $N_2$ after drainage of the stems. Root nodules formed on both non-flooded and flooded plants but were usually decreased in number by flooding (from means of 124 to 51 per plant, respectively). Flooding (and stem-nodulation) resulted in an increase in shoot (and a decrease in root) dry weight, regardless of rhizobial isolate.

Stem nodules were attached by a wide collar of aerenchymatous tissue at the base of the nodule. There were large air spaces in the stem where nodules were subtended and these were continuous with nodule aerenchyma/outer cortex. In addition, aerenchyma and spongy tissue at the base of the nodule connected both flooded and non-flooded root nodules to large intercellular spaces in the root cortex. The stem and root nodules were ovoid in shape, and essentially aeschynomenoid in type, i.e. the central infected tissue was without uninfected, interstitial cells. Root nodules had a similar structure to stem nodules (although stem nodules were generally larger), and flooded root nodules were approximately twice the size of non-flooded nodules. The infected tissue of root and stem nodules consisted of spherical, bacteroid-containing cells containing one or two rod-shaped bacteroids per peribacteroid unit and prominent organelles. Infection threads were observed in root but not in stem nodules.

The cortex of stem and root nodules had an apparent oxygen diffusion barrier, consisting of concentric layers of small cells with interlocking cell walls and few intercellular spaces. Cell layers external to these consisted of larger cells and intercellular spaces, with some spaces being occluded with an electron-dense material that contained a glycoprotein recognized by the monoclonal antibodies MAC236 and MAC265. The amount of glycoprotein occlusions did not appear to differ between nodule types or treatments, although stem nodules contained intracellular glycoprotein vesicles adjacent to cell walls. The exterior of the nodules consisted of an epidermis of thin flattened cells with occasional lenticels. Amyloplasts were common in lower stem and hypocotyl nodules, but fewer in flooded or non-flooded root nodules. Upper stem nodules (i.e. those within 6 cm of the water surface) differed from more profoundly submerged stem nodules by having chloroplasts throughout the cortex. Root nodules did not contain chloroplasts, and undifferentiated plastids were found mainly in lower stem nodules.

Key words: *Aeschynomene fluminensis*, stem nodules, nitrogen fixation, wetlands, oxygen.

**Introduction**

Many legumes are able to form an $N_2$-fixing symbiosis with soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (collectively termed rhizobia) (Sprent & Sprent, 1990). The organs that are the site of $N_2$-fixation are termed nodules and are normally situated on the roots of the host plant. Nodules provide a low oxygen environment for the optimum expression and operation of nitrogenase, the enzyme responsible for $N_2$-fixation (Witty et al., 1986; Gallon, 1992); they also provide an interface for the exchange of nutrients and metabolites between the symbionts. The plant
supplies photosynthetically-derived carbon to the bacteroids (the symbiotic, N\(_2\)-fixing form of the rhizobia) and, in return, the bacteroids pass fixed nitrogen (ammonium) to the host plant.

In recent years, much attention has been focused on a different form of legume nodulation, that of their stems. Stem nodulation is a relatively rare phenomenon that, until recently, has been confirmed only in some species of the hydrophytic genera *Aeschynomene* and *Sesbania* (Arora, 1954; Yatazawa & Yoshida, 1979; Dreyfus & Dommergues, 1981; Eaglesham & Szalay, 1983; Alazard, 1985; Vaughn & Elmore, 1985). Reports that stem nodules also occur on aquatic *Neptunia* species are likely to be erroneous (James et al., 1992a) and a misquote of Schaede (1940). However, the short list of stem nodulating legumes has just been increased to three genera by the discovery of nodules on stems of *Discolobium* (Loureiro et al., 1994), a relative of *Aeschynomene*.

Stem nodulation apparently allows plants of the above genera to fix N\(_2\) in flooded environments. Nodules formed on the stem above the flooded roots overcome the oxygen constraints afflicting submerged root nodules (Ladha et al., 1992a). Very high N\(_2\) fixation rates are claimed for stem-nodulated legumes such as *Sesbania rostrata* (Ndoye & Dreyfus, 1988; Hungria, Eaglesham & Hardy, 1992; Kwon & Beevers, 1992; Ladha et al., 1992a, b; Parsons, Raven & Sprent, 1992), *Aeschynomene* spp. (Yatazawa & Yoshida, 1979; Eaglesham & Szalay, 1983; Alazard & Duhoux, 1987; Hungria et al., 1992) and *Discolobium pulchellum* (Loureiro et al., 1994). As stem nodules are located so close to the photosynthetic apparatus of the plant, they are ideally placed to receive a constant supply of energy substrates. Moreover, most stem nodules also contain chloroplasts, so they may also supplement their energy supply by their own photosynthetic activity (Eaglesham & Szalay, 1983; de Bruijn, 1989; Hungria et al., 1992; Ladha et al. 1992a; Parsons et al., 1992; Parsons, Sprent & Raven, 1993). This factor might be taken to an extreme in those *Aeschynomene* species that contain photosynthetic rhizobia that are shown to have light-enhanced nitrogenase activity (Evans et al., 1990; Hungria et al., 1993; Lorquin et al., 1993; Loureiro et al., 1993).

Whether nodules are formed on aerial (above water) or submerged stems, depends on the species (Ladha et al., 1992a; Lorquin et al., 1993). Ladha et al. (1992a) divided stem-nodulating legumes into three groups: (1) *S. rostrata*, *A. afraspera* and *A. nilotica* which have stems with visible adventitious root/nodule primordia (Alazard, 1985), and hence profuse nodulation all over the stem, including aerial portions; (2) *Aeschynomene* species which also have stems with visible nodule/root primordia but form nodules mainly on the submerged stem; and (3) a variety of plants including other *Aeschynomene* and *Sesbania* species, as well as *Neptunia*, *Vicia faba* and *Arachis hypogaea*. Ladha et al. (1992a) suggested that the group 3 plants could not be considered ‘true’ stem-nodulating legumes as nodule primordia were invisible, and nodules form only on the submerged stem. Since adventitious roots arise from these submerged stems, they should be considered as anatomically more similar to roots.

In the present study, we examine nodulation and nodule structure of *A. fluminensis*, an important grazing species for native aquatic fauna, such as capybara (*Hydrochaeris hydrochaeris*), in the Pantanal Matogrossense region of Brazil (Allem & Valls, 1987). As it is a member of the ‘Group 3’ stem nodulators of Ladha et al. (1992a) we determine whether *A. fluminensis* has ‘genuine’ stem nodules (i.e. that they are connected vascularity to the aerial or submerged stem) according to the criteria of James et al. (1992a) and Loureiro et al. (1994), and under what conditions and with which rhizobia they form. We also investigate root nodulation in this species and determine whether root and stem nodules can grow and fix N\(_2\) under submerged conditions as has been recently demonstrated with *Sesbania rostrata* (Ladha et al., 1992b), *Neptunia plena* (James et al., 1992a; James, Minchin & Sprent, 1992b), *Discolobium* (Loureiro et al., 1994) and *Trifolium repens* (Pugh et al., 1995).

**MATERIALS AND METHODS**

**Plant growth**

*A. fluminensis* seeds were obtained from flooded areas of the Pantanal Matogrossense, Mato Grosso, Brazil, and were germinated after surface sterilization and scarification. Three days after germination, seedlings (4 cm in height) were transferred to pots filled with a sterilized soil/vermiculite (3:1) mixture in a greenhouse at EMBRAPA/CNPAB, Rio de Janeiro, under conditions described by Loureiro et al. (1994). Roots were then inoculated with one of 19 isolates of rhizobia (UFMT1–19), originally obtained from stem nodules on native *A. fluminensis*, all of which contained bacterio-chlorophyll *a* (Loureiro et al., 1993). At 20 d after germination the roots and lower stems of half the plants were flooded (to a final depth of 9 cm above the hypocotyl at the time of harvest). At this time (20 d), stems of all (flooded and non-flooded) plants were inoculated by rubbing rhizobia-containing broth onto them with sterile cotton wool; this was repeated every 15 d until harvest at 90 d after germination.

**Nitrogenase activity and microscopy**

Ninety days after germination mature nodules had formed on the roots and, in some cases, on the stems.
Table 1. Effect of 70 d flooding on stem and root nodulation, plant dry weights and nitrogenase activity (ARA) of Aeschynomene fluminensis after inoculation of stems and roots by 10 isolates of rhizobia obtained from native A. fluminensis

| Isolate/treatment | Nodule no. per plant | Nodule dry weight (mg per plant) | Dry weight (g per plant) | ARA (μmol C₂H₄ h⁻¹ per plant) |
|-------------------|----------------------|---------------------------------|--------------------------|-------------------------------|
|                   |                      | Root (mg)                      | Stem (mg)                | Root (g)                      | Stem (g)                      | Root (μmol) | Stem (μmol) |
| UFMT1             | Non-flooded          | 69 ghijklmnop                   | 31 jklmnopq              | 2.99 abcd                    | 632 ghij                     | 67-92 defg   | —           |
|                   | Flooded              | 42 mnop                        | 30 jklmnopq              | 1.88 bcde                    | 626 abcde                    | 68-09 defg   | 77-98 bc    |
| UFMT3             | Non-flooded          | 114 bcdefghijk                 | 31 jklmnopq              | 3.21 abcd                    | 4515 efghi                   | 17-32 hijk   | —           |
|                   | Flooded              | 29 pqrs                        | 66 efgijklm              | 2.65 abcd                    | 621 bcdef                    | 33-49 fhijk  | 128-40 a    |
| UFMT5             | Non-flooded          | 97 bcdefghijklm                | 39 iklmnopq              | 4.38 a                       | 435 efghi                    | 23-23 ghijk  | —           |
|                   | Flooded              | 52 lmnop                       | 39 iklmnopq              | 3.74 ab                       | 587 abcdef                   | 17-60 hijk   | 27-64 efghi |
| UFMT6             | Non-flooded          | 123 abcdefghij                 | 81 cdefghi               | 2.44 bcde                    | 626 abcde                    | 34-86 efghi  | —           |
|                   | Flooded              | 43 mnop                        | 52 fghijklmnop            | 2.13 bcde                    | 721 ab                        | 53-18 defghi  | 68-34 bcd   |
| UFMT7             | Non-flooded          | 213 a                          | 57 fghijklmn             | 2.19 bcde                    | 413 efghi                    | 98-79 bcd    | —           |
|                   | Flooded              | 70 hijklmnop                   | 157 b                    | 2.87 abcde                   | 737 a                        | 15-10 hijk   | 21-80 efghi |
| UFMT9             | Non-flooded          | 137 abcddefg                  | 209 a                    | 2.70 abcd                    | 486 bcdefgh                 | 138-36 b     | —           |
|                   | Flooded              | 54 klmnop                      | 61 efgijklmn             | 3.18 abcd                    | 566 abcdefgh                | 30-93 fhijk  | 88-99 b     |
| UFMT11            | Non-flooded          | 155 abcd                      | 73 efghijk               | 3.04 abcd                    | 515 abcdefgh                | 84-60 bcdef  | —           |
|                   | Flooded              | 62 jklmnop                     | 42 jklmnopq              | 1.59 cde                     | 626 abcde                    | 16-37 hijk   | 48-08 cdefg |
| UFMT15            | Non-flooded          | 158 abcd                      | 70 efghijkl              | 2.13 bcde                    | 593 abcdefgh                | 278-34 a     | —           |
|                   | Flooded              | 110 bcdefghijkl               | 121 bcde                 | 2.66 abcde                   | 609 abcdef                   | 19-07 hijk   | 42-42 cdefgh |
| UFMT16            | Non-flooded          | 151 abcd                      | 86 cdefgh                | 2.66 abcde                   | 471 cdefghi                 | 134-23 bc    | —           |
|                   | Flooded              | 39 nopq                       | 16 nopq                  | 2.65 abcd                    | 627 abcde                    | 44-23 defghi  | 56-41 bcd   |
| UFMT17            | Non-flooded          | 203 a                         | 105 cde                  | 3.05 abcde                   | 543 abcdefgh                | 116-36 b     | —           |
|                   | Flooded              | 73 ghijklmnop                 | 55 fghijklmn             | 2.33 bcde                    | 709 abc                      | 30-32 fhijk  | 92-43 ab    |
| Control           | Non-flooded          | 08 qrst                       | 06 pq                    | 1.33 de                      | 150 j                       | 1-93 jk      | —           |
|                   | Flooded              | 03 t                          | 09 pg                    | 1.23 e                       | 269 ij                      | 1-08 k       | 3-10 i     |

Data are means of three replicates. Values within columns followed by the same letter are not significantly different at P = 0.05.
Figure 1. (a) Aeschynomene fluminensis plant inoculated with photosynthetic rhizobial strain UFMT3 and grown for 70 d under flooding to a depth of 9 cm above the hypocotyl. Stem nodules have formed all over the flooded stem (white arrows) to within 1 cm of the surface of the water (9 cm above the hypocotyl; not shown). Root nodules are also visible (black arrows) and there is aerenchyma (a) where the tap root joins the hypocotyl (bar, 1 cm). (b) Longitudinal section (LS) of a piece of stem with an upper stem nodule. The nodule (N) is attached to the stem (S) by a short stalk of spongy cells (asterisk). Note the vascular bundles in the nodule cortex (arrows) and the lenticel (large arrow) (bar 250 μm). (c) Transverse section (TS) of stem/nodule junction.
Pieces of stem with stem nodules and nodulated root systems were taken from the plants and tested for nitrogenase activity using the acetylene reduction assay (ARA) according to Loureiro et al. (1994). Active nodules from stems and roots were then excised and prepared for light and transmission electron microscopy (TEM) according to James et al. (1992a). Immunogold labelling of nodules with the monoclonal antibodies MAC236 and MAC265 (gifts from N. J. Brewin, John Innes Institute, Norwich, UK) for TEM and light microscopy was performed using the methods of James et al. (1991). Some of the nodules were still attached to stems or roots and all had pink central tissue, indicating leghaemoglobin.

Plant dry weights, nodule dry weights and the number of root and stem nodules per plant were also determined.

**Statistical analysis**

Where necessary, results were square-root transformed to give a normal distribution. Analysis of variance was made using MSTAT-C, and Tukey’s test was used to determine significant differences between treatments.

**Results**

**Effect of flooding and isolate of rhizobia on stem/root nodulation, nitrogenase activity and plant dry weight**

Inoculation with the 19 photosynthetic isolates originally obtained from field-grown *A. fluminensis* (UFMT1–19) resulted in stem and root nodule formation, and Table 1 presents results from the 10 ‘best’ strains. Significant ($P < 0.01$) positive correlations were seen between flooding, stem nodulation and shoot dry weight. Significant ($P < 0.01$) negative correlations were seen between flooding, root nodulation (number and dry weight per plant) and root dry weight. However, these correlations mask some interesting and significant results with individual rhizobial strains. For instance, although with most strains/isolates flooding resulted in a c. 50% reduction in root nodule number, their dry weights were not generally reduced by flooding to the same extent, suggesting that the flooded nodules were bigger than non-flooded nodules. In particular the root nodules on plants inoculated with UFMT7 increased their total dry weight three-fold with flooding compared with non-flooded plants (Table 1), but each flooded plant had only one third the number of nodules, i.e. an eight-fold increase in dry weight of individual nodules with flooding.

In all treatments/nodule types there was significant acetylene reduction activity (ARA), indicating active nitrogenase, except for the uninoculated controls (Table 1). However, the ARA results might not give accurate estimates of nodule activity as the method used has been shown to give underestimates of nitrogenase activities in legume nodules (Witty & Minchin, 1988). On the other hand, they are useful for general comparisons. For example, root nodule ARA was negatively correlated with flooding treatment ($P < 0.01$) and stem nodule number/dry weight ($P < 0.01$), whereas stem nodule ARA was positively correlated with flooding treatment ($P < 0.01$) and shoot dry weight ($P < 0.01$), but negatively correlated with number of root nodules per flooded plant ($P < 0.01$) and root nodule ARA ($P < 0.01$).

Inoculation with isolate UFMT3 produced the greatest number of stem nodules with the highest stem nodule acetylene reduction activity per plant, although inoculation with UFMT16 resulted in the greatest dry weight of stem nodules per plant (Table 1). However, isolate UFMT15 on non-flooded plants produced the highest acetylene reduction activity of any treatment/nodule type, with the root nodules being considerably greater in activity than either flooded root or stem nodules on the same set of plants. The highest flooded root nodule activity was shown by those plants inoculated with UFMT1. This was equal to the activity of non-flooded nodules on the same plants (Table 1).

Stem nodules formed only on submerged parts of flooded plants (Fig. 1a), to within 1 cm of the water surface, and flooding appeared to be essential for their formation. However, when the water around the stems was drained the nodules were able to continue growing and fixing $N_2$. With most isolates of rhizobia, flooding and stem nodulation resulted in an increase in shoot dry weight, accompanied by a decrease in root dry weight, with a resultant decrease in root/shoot ratio (not shown). The most efficient strain/treatment combination as regards shoot growth was inoculation with the strain UFMT7 showing the vascular connection (arrow) of the stem (S) to the nodule (N) (bar 100 μm). (d) TS of the base of stem (S) with a nodule (N) attached. This region corresponds to that indicated by the asterisk in Fig. 1b. There are large cavities (C) where the nodule subtends the stem, and the nodule tissue contains numerous intercellular spaces (bar, 50 μm). (e) TS cross-cortex section of an upper stem nodule. Immediately external to the infected cells (I), is a distribution zone (DZ) of cells with intercellular spaces adjacent to the infected cells. External to the DZ is a boundary layer of interlocking cells with few intercellular spaces (BL). The mid-cortex (MC) comprises the larger cells external to the BL and contains numerous chloroplasts (arrows), with fewer chloroplasts being present in the outer cortex (OC) and hypodermal cells (not shown). Chloroplasts nearer the infected tissue (in the DZ and BL) tend to be less distinct in appearance (bar, 10 μm).
Figure 2. (a) Transmission electron micrograph (TEM) of a chloroplast from the mid-cortex of an upper stem nodule. Note the starch grains (S) and the stacked grana (G), indicating active photosynthesis. There is a plasmodesma in the cell wall adjacent to the chloroplast (arrow) (bar, 500 nm). (b) TEM of DZ cells from an upper stem nodule. The cortical cells contain numerous organelles such as mitochondria (M), nuclei (N) and ill-defined chloroplasts (arrows). Note the large intercellular spaces adjacent to the infected cells, and throughout the DZ (S) (bar, 2 μm). (c) TEM of infected cells from an upper stem nodule. There are typically
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which resulted in a doubling of shoot dry weight under flooded conditions, with root growth unaffected (Table 1).

Stem nodule structure

There were no obvious differences in structure between nodules on plants inoculated with the different UFMT isolates, and the data presented here, and in the subsequent sections, are from representative samples of all the isolate/treatment combinations.

Nodules were connected to the stem via a short broad stalk of large-celled tissue with numerous intercellular spaces (Fig. 1b, c). This spongy tissue was continuous with possible aeration pathways in the form of large air spaces in the stem adjacent to where nodules were connected (Fig. 1d), and also in the pith (not shown). Often, there were adventitious roots associated with the nodules (Fig. 1a) but vascular traces ran directly from the stem vascular system to the nodule (Fig. 1e) as in all other true stem nodules (James et al., 1992a).

Stem nodules were typically 'aeschynomenoid' in appearance (Corby, 1988), i.e. round to oblate in shape and without uninfected interstitial cells in the infected tissue (Figs 1b, e, 2c, d). However, most of the stem nodules examined had lenticels (Fig. 1b), which are a feature not of aeschynomenoid, but rather of desmodioid nodules (Corby, 1988). Stem nodules on A. fluminensis could be divided into two types: those on the lower stem near the hypocotyl with few (or no) chloroplasts in the inner cortex (Fig. 2d) and those on the upper stem, near the surface of the water, which had numerous chloroplasts (Figs 1e, 2a, b).

In both types, the infected tissue was similar consisting of round, bacteroid-containing cells with large spaces between them (Figs 1e, 2c, d), although some were occluded (Fig. 2e). Central nuclei were often prominent within the infected cells and there were no vacuoles evident (Figs 1e, 2b–d), except in cells which were senescing (not shown). In healthy cells there were only 1–2 bacteroids per peribacteroid unit (PBU) and bacteroids tended to be rod-shaped with some electron-transparent deposits which might be poly-β-hydroxybutyrate (PHB) (Fig. 2b, c).

The cortex of upper and lower stem nodules was also broadly similar and roughly followed the pattern reported in a number of nodule types (Parsons & Day, 1990; James et al., 1991, 1992a; Brown & Walsh, 1994; Iannetta et al., 1995). In the two to three cell layers immediately adjacent to the infected tissue there were large intercellular spaces, and the small cells within these layers contained numerous organelles and amyloplasts (Figs 1e, 2b, d, 3a). These cell layers are analogous to the 'distribution zone' described in other nodules (Witty et al., 1986; Parsons & Day, 1990). External to the distribution zone (DZ) the cells were larger and more close-packed and often had interlocking cell walls with few intercellular spaces (Figs 1e, 2d, 3a); these cell layers are similar to that name as a 'boundary cell layer' in soybean nodules (Parsons & Day, 1990) and which has since been recognized in other legume nodules (James et al., 1992a; Brown & Walsh, 1994; Iannetta et al., 1995).

In upper stem nodules (Fig. 1b), chloroplasts were present throughout the cortex, especially near intercellular spaces (Figs 1e, 2a, b). They were even seen in distribution zone (DZ) cells immediately adjacent to infected cells (Figs 1e, 2b), although these chloroplasts appeared to be largely inactive with no distinct grana (2b). Chloroplasts were particularly prominent in the boundary cell layers and the mid-cortex (Fig. 1e) where they contained stacked grana and amyloplasts, indicating photosynthetic activity (Fig. 2a). In lower stem nodules chloroplasts were not often seen, although there were plastids, and possibly ineffective chloroplasts, which were either undeveloped or had degenerated into amyloplasts (Fig. 2d). In general, cells in the inner cortex of lower stem nodules (Figs 2d, 3a) contained greater numbers of amyloplasts than did those of upper stem nodules (Fig. 1e).

The mid and outer cortex of all stem nodules was similar, consisting of progressively larger cells towards the exterior of the nodules, and with large (often occluded) intercellular spaces between them (Figs 1e, 2d, 3a–c). Vascular bundles were located within the mid-cortex (Fig. 1b), just external to the DZ. Surrounding the cortex of both stem and root nodules was a corky hypodermis of thin, flattened cells (Figs 1b, 4a, c) which was occasionally interrupted by lenticels made up of expanded hypodermal and/or outer cortical cells (Figs 1b, 4a); these could be profuse in lower stem nodules (not shown).

Root nodule structure

Root nodules were generally smaller than stem nodules (only 3 mm in diameter compared with
Figure 3. (a) TS of a lower stem nodule after immunogold labelling (plus silver enhancement) with the monoclonal antibody MAC236. MAC236 and MAC265 recognize an intercellular glycoprotein involved in the regulation of oxygen diffusion in legume root nodules (VandenBosch et al., 1989; James et al., 1991), and the MAC236 antigen can be seen in intercellular spaces in this section (arrows). Note that the glycoprotein is confined to the mid cortex (MC), and is not apparent in the inner cortex (DZ and BL). There are numerous amyloplasts (a) in the mid and inner cortex (bar, 10 μm). (b) TEM of immunogold labelled (with MAC236) intercellular space from the mid-cortex of a lower stem nodule (bar, 500 nm). (c) TEM of intercellular space (S) from the mid-cortex of a lower stem nodule. Close to the space, and adjacent to cell walls are intracellular vesicles (arrows) that are immunogold labelled with MAC265 (Fig. 3d). The intercellular space is much more
4 mm for stem nodules) and lighter (0.5–1 mg d.wt per root nodule compared with 2–4 mg d.wt per stem nodule), regardless of flooding (Table 1). However, they were similar in morphology and structure to stem nodules (Figs 1a, 4a). Nodules were often observed attached to the roots at lateral root junctions but not always (Fig. 4a). They were connected to the root by a wide base of spongy cells, and large air spaces were observed in the root cortex adjacent to the nodule bases (Fig. 4a, b). These spaces were presumably a continuation of the air pathways observed in the stems (Fig. 1d) and were present in both flooded and non-flooded roots. Some lenticellular tissue was also present on flooded roots (Fig. 1a).

Infected cells of root nodules (Fig. 4c, d) were similar to stem nodules and there were no obvious differences in bacteroid frequency, organelles, vacuoles etc. However, in contrast to stem nodules, intercellular spaces in root nodules could be very large and infection threads were occasionally seen within the cells (Fig. 4d). The cortex of root nodules (Fig. 4c) was also similar to stem nodules, especially to those nodules without chloroplasts on the lower stem (Fig. 2d), with amyloplasts being abundant in the DZ. There were no obvious structural differences between flooded and non-flooded root nodules.

**Immunogold silver enhancement of the antigens recognized by these antibodies is concentrated mainly within large intercellular spaces in the mid to outer cortex and accumulates little in occluded spaces internal to the mid-cortex. This pattern of distribution was confirmed using immunogold labelling under TEM (Fig. 3b, c). However, TEM also showed that adjacent to sparsely-labelled spaces in the mid-cortex of both stem and root nodules were numerous intracellular vesicles that contained the glycoprotein (Fig. 3c, d); the vesicles usually being more intensely labelled by MAC265 than the intercellular spaces in this region. In general, labelling by MAC236 was more intense in *A. fluminensis* nodules than that by MAC265 (compare Fig. 3b with Fig. 3c, d).

**Occurrence of intercellular space glycoprotein**

**Immunogold silver enhancement of the antigens recognized by the monoclonal antibodies MAC236 and MAC265 (VandenBosch et al., 1989) revealed that the pattern of expression of these antigens differed little between flooding treatment and nodule types. Figure 3a shows a cross-cortex view of a lower stem nodule and demonstrates that the glycoprotein recognized by these antibodies is concentrated mainly within large intercellular spaces in the mid to outer cortex and accumulates little in occluded spaces internal to the mid-cortex. This pattern of distribution was confirmed using immunogold labelling under TEM (Fig. 3b, c). However, TEM also showed that adjacent to sparsely-labelled spaces in the mid-cortex of both stem and root nodules were numerous intracellular vesicles that contained the glycoprotein (Fig. 3c, d); the vesicles usually being more intensely labelled by MAC265 than the intercellular spaces in this region. In general, labelling by MAC236 was more intense in *A. fluminensis* nodules than that by MAC265 (compare Fig. 3b with Fig. 3c, d).**

**DISCUSSION**

**Effect of flooding on stem and root nodulation of *A. fluminensis***

We have shown that *A. fluminensis* is capable of forming genuine stem nodules, i.e. they are connected vascularly to the stem and not to adventitious roots arising from the stem (James et al., 1992a) and, when growing close to the surface of the water, they contain chloroplasts in the nodule cortex. Before the present study *A. fluminensis* was considered not 'truly' stem-nodulated but with nodules on a 'root-type' submerged stem or on adventitious roots (Ladha et al., 1992a), a definition also supported by Stegink & Vaughn (1988) and Alazard (1985).

We have also shown that the stem nodules require submergence for their formation. This is unlike the aerial stem nodules on *S. rostrata*, *A. indica*, *A. scabra*, *A. afraspera* and *A. nilotica* (Yatazawa & Yoshida, 1979; Dreyfus & Dommergues, 1981; Alazard & Duhoux, 1987; Ladha et al., 1992a) which require high humidity around, but not actual inundation of, the stem for formation of stem nodules (Eaglesham & Szalay, 1983; Alazard & Duhoux, 1987; Ladha et al., 1992a; Parsons et al., 1993). Therefore, *A. fluminensis* is similar to *A. elaphroxylon*, *A. pfundii* and other stem-nodulators of Group 3 (Ladha et al., 1992a) which form submerged stem nodules only. However, although *A. fluminensis* stem nodules form only under submerged conditions, once formed they are capable of functioning under non-flooded conditions, unlike stem nodules on *Discolobium pulchellum* which obligately require inundation for formation and function (Loureiro et al., 1994).

The decrease in the number of root nodules on flooded *A. fluminensis* is similar to that reported in other stem-nodulated species (Eaglesham & Szalay, 1983; Alazard & Duhoux, 1988; Ladha et al., 1992b; Parsons et al., 1992), although this is not always the case with *S. rostrata* (Ladha et al., 1992b). The decline in number and activity of root nodules, along with a parallel increase in stem nodulation, has been attributed to a lack of oxygen available to the roots under flooded conditions. This causes the production of aerial stem nodules which do not normally have such oxygen constraints (Dreyfus & Dommergues, 1981; Eaglesham & Szalay, 1983; Alazard & Duhoux, 1987; Ndoye & Dreyfus, 1988). However, the decline in root nodules has also been attributed to the preferential production of stem nodules (and the actual suppression of root nodulation), regardless of flooding. Stem nodules might be more efficient at

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sparsely labelled than that in Fig. 3b where MAC236 was used (bar, 1 μm). (d) High magnification of an intracellular glycoprotein vesicle (V) from Fig. 4c (asterisk). The vesicle is labelled with MAC265, and there is also some labelling in the cytoplasm (arrows). Note the unusual protuberances (P) on the vesicle (bar, 200 nm).
Figure 4. (a) TS of a root from a flooded plant with a nodule (N) attached by a broad stalk of spongy cells (S). Note the prominent lenticel (arrow) on the nodule (bar, 100 μm). (b) TS of the base of a non-flooded root nodule (N) where it subtends the root (R). The nodule tissue contains numerous intercellular spaces, and there are large cavities (C) in the root which appear to be a continuation of an aeration pathway down from the stem (Fig. 1c) (bar, 50 μm). (c) Cortex and infected tissue of a flooded root nodule with numerous amyloplasts
N₂-fixing than are root nodules (Hungria et al., 1992; Ladha et al., 1992a).

As aerial stem nodules cannot be formed on *A. fluminensis*, it is probable that the formation of stem nodules and the decrease in the number of root nodules with flooding (and their clustering at the hypocotyl/taproot base), is a means of keeping the N₂-fixing capacity of the plant as close to the water surface as possible, thus reducing the pathlength for passive oxygen diffusion down the stem (James et al., 1992b). The reluctance of *A. fluminensis*, *A. elaphroxylon* (Vaughn & Elmore, 1985), and other Group 3 species (Ladha et al., 1992a), to form aerial stem nodules might be a result of the unexposed adventitious root primordia on the stems of these plants. By not being exposed they are inaccessible to invading rhizobia, except when inundated (Alazard & Duhoux, 1987). By contrast, other stem-nodulating legumes such as *S. rostrata* and *A. afraspera* possess protruding root primordia that allow for aerial rhizobial infection without prior flooding of stems (Tsien, Dreyfus & Schmidt, 1983; Alazard & Duhoux, 1988).

Nitrogenase activity of stem nodules has been reported to be much greater than that of corresponding root nodules on *S. rostrata*, *Discolobium pulchellum* and *Aeschynomene* spp. (Dreyfus & Dommergues, 1981; Ladha et al., 1992b; Hungria et al., 1992; Loureiro et al., 1994). However, recent results by Parsons et al. (1992) with *S. rostrata* showed little difference between root and stem nodules when a 'flow-through' ARA was used. Flow-through systems allow a more accurate estimate of nitrogenase activity from ARA (Witty & Minchin, 1988) as, unlike the closed assay system used in the present study, they do not mask the acetylene-induced decline in nitrogenase activity that occurs in many symbioses. Therefore, at this stage we can say only that *A. fluminensis* stem and root nodules under flooded and non-flooded conditions exhibited significant nitrogenase activity. We cannot compare them with results from other studies of legumes with stem nodules and/or submerged nodules. On the other hand, the present study has shown that flooding and stem-nodulation of *A. fluminensis* resulted in enhanced shoot growth compared with non-flooded, purely root-nodulated plants, and this was generally achieved with fewer nodules. It is possible, therefore, that the fewer, larger nodules on stems and roots of flooded plants are more efficient at N₂-fixation than are the numerous small root nodules on non-flooded plants, though direct experimental evidence for this is lacking.

**Nodule structure**

As in many *Aeschynomene* species, stem and root nodules on *A. fluminensis* were very similar in morphology and structure (Stegink & Vaughn, 1988). Stem and root nodules were in most respects of the typical aeschynomenoid-type (Corby, 1988; Sprent, Sutherland & de Faria, 1989). However, *A. fluminensis* root nodules differ from the usual aeschynomenoid-type in having occasional infection threads; hence it is likely that infection threads are involved in entry of rhizobia into the host cells of *A. fluminensis* rather than via host cell wall dissolution as suggested for other *Aeschynomene* species (Vaughn & Elmore, 1985; Stegink & Vaughn, 1988; Alazard & Duhoux, 1990). This, plus the recent report of infection threads in *Discolobium* nodules by Loureiro et al. (1994), makes it tempting to suggest that infection threads may be more widespread in aeschynomenoid-type nodules than has hitherto been suspected (Corby, 1988; Sprent et al., 1989) and requires further investigation.

*A. fluminensis* stem and root nodule bacteroids were rod-shaped and similar to those in *A. afraspera* nodules (Alazard & Duhoux, 1990). This differs from the spherical/ovoid bacteroids reported in other aeschynomenoid types (Yatazawa, Yoshida & Maeda, 1984; Vaughn & Elmore, 1985; Evans et al., 1990), including an earlier report on *A. fluminensis* root nodules (Stegink & Vaughn, 1988).

Also seen in *A. fluminensis* lower stem and root nodules were ‘oleosome-type’ bodies in the innermost cell layers of the inner cortex, similar to the oleosomes that are particularly prominent in peanut nodules (Jayaram & Bal, 1991). Oleosomes are lipid storage organelles and are hence involved in storing and then supplying photosynthate for nodule metabolism during the night, especially for nitrogen fixation (Jayaram & Bal, 1991). It is interesting therefore that the ‘oleosomes’ seen in *A. fluminensis* appeared to be confined to the lower stem nodules and the root nodules, where there were also apparently increased amounts of starch compared with upper stem nodules.

On the other hand, despite the fact that they were even more profoundly submerged than were stem nodules, there was less starch in flooded root nodules. Moreover, bacteroids also contained less poly-β-
hydroxybutyrate (PHB), a carbon storage compound that accumulates in bacteroids under decreased pO₂ (Stem et al., 1986). Flooded root nodules also did not appear to have more starch or PHB than did non-flooded root nodules, suggesting that flooded stem and root nodules were possibly no more oxygen-limited than were their terrestrial counterparts. The large starch deposits seen in lower stem nodules might have resulted from the closeness of the nodules to the supply of photosynthate from the stem and hence could indicate a surplus of carbohydrate.

Photosynthesis in A. fluminensis stem nodules

Aeschynomene fluminensis was typical of a number of stem-nodulated Aeschynomene species in having chloroplasts in the cortex of upper nodules (Vaughn & Elmore, 1985; Hungria et al., 1992). The more profoundly submerged lower stem nodules had few chloroplasts and mainly undifferentiated plastids, whereas upper stem nodules had fully-differentiated chloroplasts. This difference is possibly related to different light intensities experienced at different depths.

Stem nodules of Sesbania rostrata are also rich in chloroplasts (Dreyfus & Dommergues, 1981; Duhoux, 1984; De Brujin, 1989). It has been suggested that these fix significant carbon and might enhance N₂-fixation in stem nodules to a level above that in root nodules (de Brujin 1989; Evans et al., 1990; Ladha et al., 1992a; Hungria et al., 1992; Parsons et al., 1992, 1993). In the case of A. fluminensis it appeared that most of the active chloroplasts were in the mid-cortex rather than in the inner cortex adjacent to the infected cells. Those chloroplasts that were in the distribution zone cells appeared to be little more than undifferentiated plastids, similar to those seen in lower stem nodules. This was also observed in A. indica nodules by Vaughn & Elmore (1985) and might result from light penetration being reduced by passage through several cortical cell layers.

Oxygen supply to submerged nodules

The submerged nature of the flooded A. fluminensis nodules does not appear to have affected their ability to fix N₂; indeed increased shoot growth under flooded conditions suggests that N₂ fixation might actually be enhanced, as shown recently in flooded Trifolium repens (Pugh et al., 1995). Aeschynomene fluminensis has neither floating nor extremely submerged nodules. It is therefore likely that the oxygen needs of submerged A. fluminensis nodules are in large part supplied by diffusion down a stem pathway, and the occurrence of large air spaces in the stem and roots where nodules subtend them with spongy aeration tissue provides structural evidence for this. This was also suggested for nodules of Neptunia and Discolobium (James et al., 1992a, b; Loureiro et al., 1994), where there was also extensive aerenchyma from the stem down to the nodules. However, unlike Neptunia and Discolobium, aerenchyma did not appear to be present on or within A. fluminensis stems (although it was visible at the base of tap roots, Fig. 1 a), and most nodules had only a few lenticels, suggesting that the internal stem-root aeration pathway to the submerged stem nodules is most important. In addition, the lack of external aerenchyma would decrease any effects of increased nodule aeration via water currents such as those shown with Neptunia (James et al., 1992b).

Although these three hydrophytic species are native to the Pantanal Matogrossense, they might differ slightly in habitat as A. fluminensis might be more adapted to living in stagnant or still water rather than in the gently-flowing backwaters beside which Neptunia and Discolobium grow (Loureiro et al., 1994).

The stem and root nodule structure of A. fluminensis shown in the present study suggests that an oxygen diffusion barrier (DB) also exists in these nodules, in common with other aquatic and flooding tolerant nodules (James et al., 1992a, b; Loureiro et al., 1994). The structure of the oxygen diffusion barrier in most legume nodules so far examined (Brown & Walsh, 1994; James et al., 1994) is considered to be similar to the general type described in soybean nodules by Parsons & Day (1990) and James et al. (1991). In conventional terrestrial legume nodules such as those on soybean (Glycine max) a DB is necessary to protect nitrogenase from oxygen concentrations above microaerobic (Witty et al., 1986; Gallon, 1992). Any fluctuations in the demand for oxygen by the bacteroids are offset by adjustments in the resistance of the cortical oxygen diffusion barrier and by rapid bacteroid respiration (Witty et al., 1986).

In A. fluminensis there were no apparent differences in diffusion barrier structure, e.g. the extent of glycoprotein occlusions (James et al., 1991) and intracellular vesicles (Iannetta et al., 1995) between stem and root nodules, or between submerged and non-flooded root nodules. This suggests that oxygen diffusion resistance varied little between nodule types, regardless of flooding, although this remains to be tested. These structural observations, plus the enhanced shoot growth with flooding, suggest that oxygen supply to submerged stem and root nodules was adequate and did not adversely affect N₂ fixation.

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