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Nitrogen-fixing stem nodules on *Aeschynomene afraspera*

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Summary. *Aeschynomene afraspera* is a wild annual legume growing in periodically waterlogged soils in western Africa. This legume is characterized by a profuse stem nodulation. Nodules are formed on the stem at the emergence of lateral root primordia, called nodulation sites. These sites are irregularly distributed on vertical rows all along the stem and branches. Stem nodules are hemispherically shaped. Their outside is dark green and they contain a red-pigmented central zone. Stem nodules exhibit a high nitrogen-fixing potential. Acetylene reduction assays result in stem nodule activity of 309 μmol C_2H_4 g^{-1} dry nodule h^{-1}. Field-grown stem nodulated *Aeschynomene* accumulated more N (51 g N m^{-2} in 10 weeks) than the root nodulated one. Because of this nitrogen-fixing potential and its ability to grow in waterlogged conditions, *A. afraspera* could probably be introduced into tropical rice cropping systems.

Key words: Stem nodulation – *Aeschynomene afraspera* – Legume – Nitrogen fixation – Acetylene reduction assay (ARA)

The phenomenon of stem nodulation has been reported in three genera of legumes: *Aeschynomene, Neptunia* and *Sesbania*. These plants are hydrophytes, growing wildly in flooded areas, marshes and riversides.

Sesbania rostrata (Dreyfus and Dommergues 1981) and *Neptunia oleracea* (Schaede 1940) are the only stem nodulated plants within their genera, whereas this characteristic is relatively widespread within the genus *Aeschynomene*.

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Aerial stem nodules were first described on *Aeschynomene aspera* L. (Hagerup 1928). Several studies on this topic have since been published (Von Suessenguth and Beyerle 1936; Arora 1954; Barrios and Gonzales 1971; Yatazawa and Yoshida 1979; Eaglesham and Szalay 1983; Alazard 1985). Today 15 species of *Aeschynomene* developing stem nodules are known. *Aeschynomene* spp. fall into three cross-inoculation groups according to their effective nodulation response patterns with strains of *Rhizobium* isolated from stem and root nodules (Alazard 1985).

The present paper refers to earlier investigations on stem nodulation of *A. afraspera*. This plant is characterized by a stem nodulation which is more extensive than that of other stem nodulated *Aeschynomene* species. *A. afraspera* J. Leonard is an annual plant, 1.0–1.5 m tall, which grows well in soils which are subject to seasonal flooding. This plant is widespread in tropical Africa and is described as a closely related species of *A. aspera* L., native of tropical India and Malaysia (Berhaut 1971).

Aeschynomene afraspera shows a high sensitivity to climatic variations, particularly to temperature and photoperiod. At the end of the rainy season in Senegal (13 h photoperiod; day temperature 32 °C), 3 months after seed germination, plants are 2 m tall and at the flowering stage. However, during the cold and dry season, (11 h photoperiod; day temperature 23 °C), flowering takes place when plants are 60 cm in height and 6 weeks old.

The structure of stem nodules of *A. afraspera* is described here. The nitrogen-fixing potential of this plant is evaluated in order to determine its use in tropical agriculture.

Materials and methods

Rhizobium strain. A *Rhizobium* strain, designated as ORS 322, was isolated from stem nodules of *A. afraspera* using standard techniques (Vincent 1970). Its ability to induce stem and root nodules on *A. afraspera* was confirmed. The stock culture was maintained at 4 °C on agar slants containing yeast extract and mannitol (YM). Cultures were grown at 30 °C in YM broth.

Plant culture and inoculation. Seeds of *Aeschynomene afraspera* were surface sterilized by immersion in concentrated H_2SO_4 for 30 min followed by 10 washes in sterile water. This treatment also scarified seeds and improved germination. Seeds were germinated in Petri dishes with 1% water agar.

Seedlings were transplanted in plastic pots containing sterile soil 2 days later. The soil used was of a sandy type, pH 7.0 (vernacular name: Dior soil) from Senegal with C, N and P contents of 0.40%, 0.025% and 0.037% respectively. Plants were grown in waterlogged conditions unless otherwise stated in a greenhouse under natural light supplemented with 250 W high-intensity discharge lamps, with a photoperiod of 13 h, day/night temperatures of 35 °C/30 °C and a mean humidity of 90%. To induce root nodulation, a drop of a 3-day-old YM culture (10^8 cells ml^{-1}) of *Rhizobium* strain ORS 322 was added to the soil in each pot. Stems were inoculated by applying a tenfold dilution of the same culture onto the stem with a small spraying bottle or a sterile brush.

Histology of stem nodules and nodulation sites. Stem nodule samples and uninoculated sites were fixed overnight in 3% glutaraldehyde in 0.2*M* cacodylate buffer, pH 7.0, at 4 °C. After washing in cacodylate buffer they were dehydrated in an ethanol series and embedded in Paraplast (+) (Brunswich Co.) or Epon. Serial thin-sections were stained with Regaud's iron-hematoxylin stain or 0.1% toluidine blue.

Acetylene reduction activity (ARA). Plants were grown in 15-cm-diameter plastic pots containing 2 kg sandy soil supplemented with a nutrient solution (Hewitt 1966) containing 30 ppm N. This enables a normal growth until stem and root inoculation, 5 weeks after sowing. Four weeks after inoculation, nodule-bearing stem portions and root systems were assayed separately for nitrogen-fixing activity by the acetylene reduction method (Hardy et al. 1968). Stem and root nodules were carefully excised at the stem and root surface, oven dried over night at 80 °C, cooled in a dessicator and weighed.

Nitrogen accumulation. Plants were grown in plastic cylinders made of commercially available polyvinylchloride drainage tubes (inside diameter, 30 cm; length, 50 cm) vertically driven into the field. These cylinders were bottom closed and contained 35 kg sandy soil supplemented with 1.6 g K_2HPO_4. Three seedlings of *A. afraspera* were transplanted into each cylinder. The soil was moistened for 3 days after transplantation and then kept waterlogged.

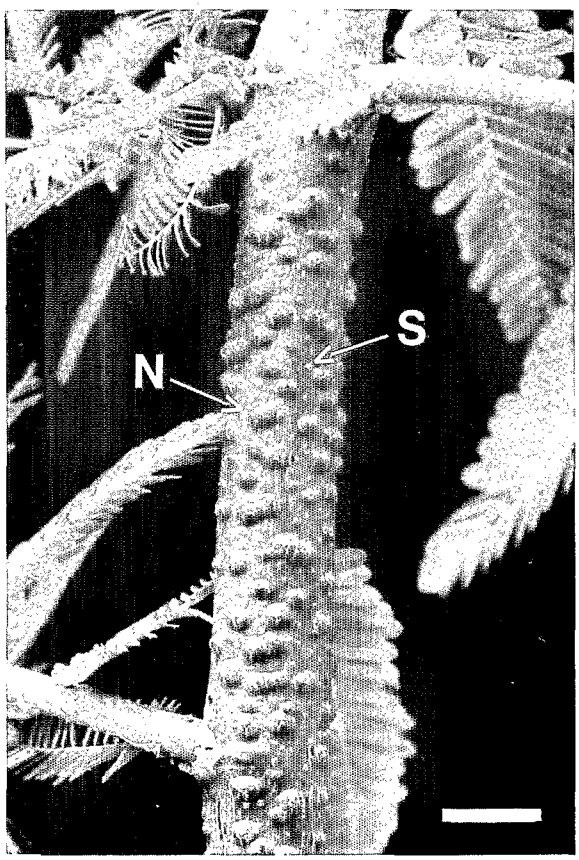

Fig. 1. Nitrogen-fixing nodules (*N*) and uninoculated nodulation sites (*S*) on the stem of *Aeschynomene afraspera*. *Bar* represents 1 cm

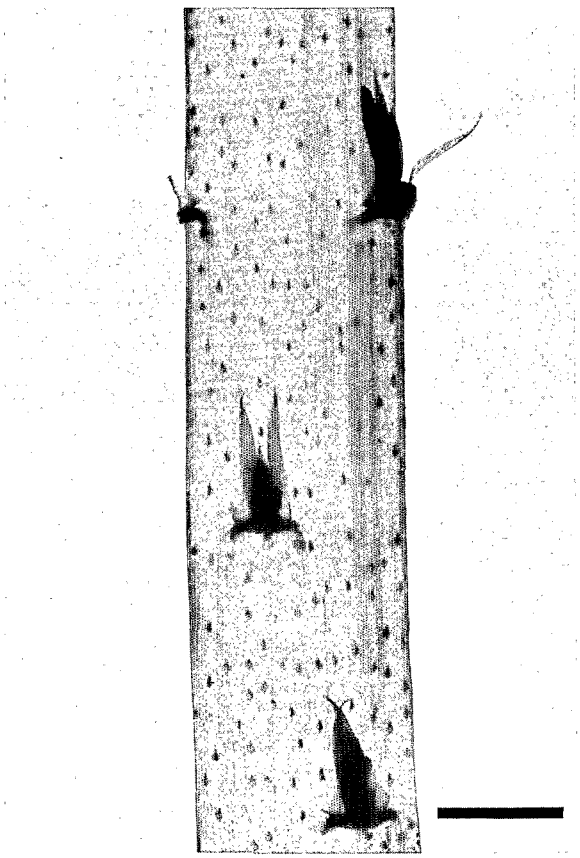

Fig. 2. Unrolled stem of *A. afraspera* showing nodulation sites distributed along dense vertical rows. *Bar* represents 1 cm

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Résumé en Français : (150 mots maximum)

Aeschynomene afraspera est une légumineuse tropicale annuelle qui pousse dans les sols inondés de l'Afrique de l'Ouest. Cette plante est caractérisée par une nodulation caulinaire abondante. Les nodules caulinaires sont formés en des sites prédéterminés appelés sites de nodulation, localisés à l'émergence d'ébauches racinaires adventives. Les nodules caulinaires ont un potentiel fixateur d'azote très élevé. Les plantes d'*Aeschynomene* nodulées sur leur tige peuvent fixer jusqu'à 51 g d'azote par m² en 10 semaines, propriété qui permet d'envisager leur utilisation dans les systèmes de culture du riz en zone tropicale.

Les titres, mots-clés matières et résumés en Anglais sont indispensables pour les documents destinés à entrer dans les Bases AGRIS et ASFA (Aquatic Sciences and Fisheries Abstracts)

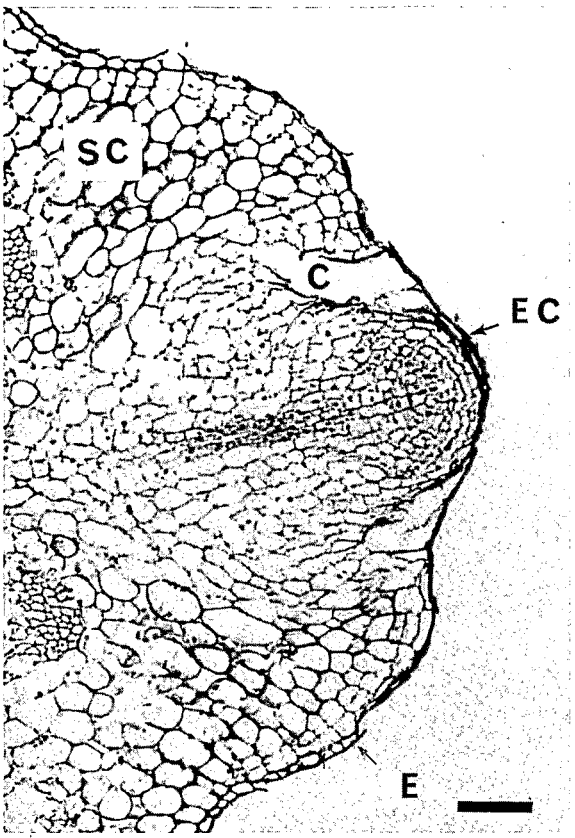

Fig. 3. Transverse section of an uninoculated nodulation site constituted by a root primordium breaking through the stem epidermis. The root primordium forms a circular cavity around it. *C* circular cavity; *E* epidermis; *EC* flattened epidermal cells; *SC* stem cortex. *Bar* represents 0.1 mm

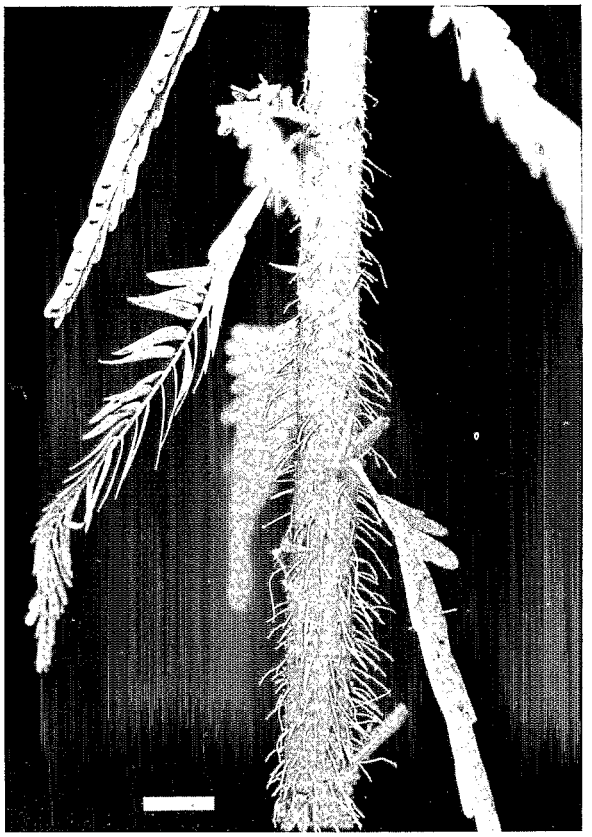

Fig. 4. Stem of young *A. afraspera* plant cultivated and immersed in water for 10 days. Most root primordia have evolved into adventitious rootlets. *Bar* represents 1 cm

All the plants were root inoculated 1 week later. Half of the plants was stem inoculated 3 and 5 weeks after sowing. The other half was not stem inoculated (control). At harvest, 5 weeks after the last inoculation, all plant material was oven dried for 48 h at 80 °C and weighed. The dry material was ground and mixed, and the total nitrogen content was determined by Kjeldahl digestion (Bremner 1960) using a Büchi automated Kjeldahl system.

Results and discussion

Stem inoculation of Aeschynomene afraspera

Artificial stem inoculation of *Aeschynomene afraspera* by a single application of rhizobia onto the stem produced a regular nodulation (Fig. 1). In controlled conditions, nodules appeared only on those parts of the stem where inoculum had been externally applied. Infection did not develop from inoculated parts of the plant to uninoculated parts through the stem vascular bundles. Repeated stem inoculation (4 times over 2 months) of a growing plant until 1.5 m in height resulted in a mean weight of nodules of about 12 g fresh weight (data not shown). Waterlogging was not a prerequisite for stem nodulation (Eaglesham and Szalay 1983).

Aeschynomene afraspera growing in natural habitats exhibits a considerable number of aerial stem nodules distributed at random along the principal stem and lateral branches. Dust, rain and wind seem to play a significant role in natural stem inoculation. A few nodules occur also on the roots of the plants. Because of the poor root nodulation, the presence of stem nodules can be viewed as an alternative adaptation to wet environmental conditions (Hagerup 1928).

The nodulation sites

The stem of *A. afraspera* bore round, small swellings, no larger than 1 mm in diameter, distributed along dense vertical rows (Fig. 2). These swellings were called the nodulation sites. On the same row, nodulation sites were gradually distributed, more frequently at the base of the stem than at the top. At half-height, a 1.20-m-long plant bore about 10 sites cm^{-2}.

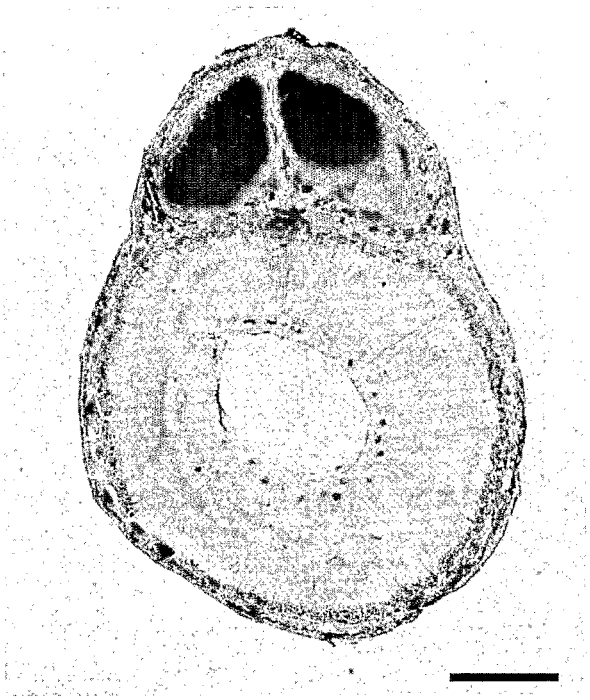

Fig. 5. Cross section of a 15-day-old stem nodule showing two dark areas of infected tissue on both sides of remaining vascular bundles of the root primordium. Note the hemispherical shape of the nodule with a broad attachment to the stem. Toluidine blue 0.1%; Paraplast (+) block. *Bar* represents 1 mm

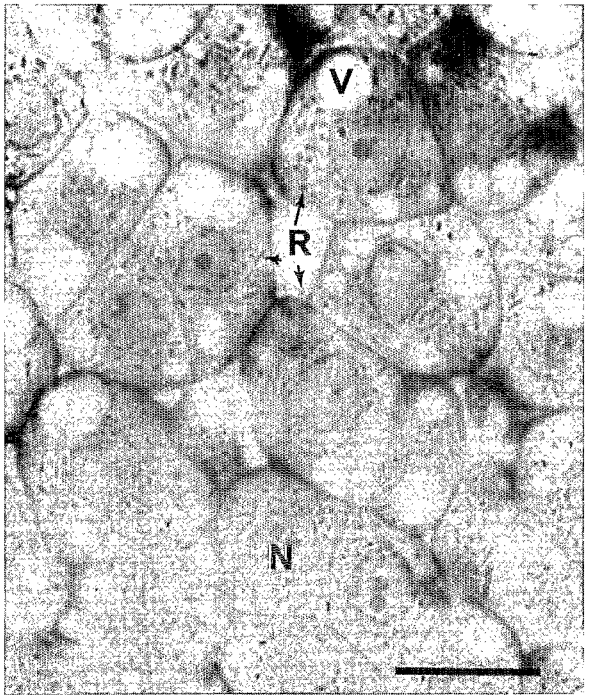

Fig. 6. Part of the infected zone of a 10-day-old nodule. Infected cells contain numerous rhizobia (*R*), prominent nucleus (*N*) and vacuoles (*V*). Regaud's iron-haematoxylin stain; Epon block; *Bar* represents 10 μm

Nodulation sites of *A. afraspera* always included an adventitious root primordium. Sites located below the two upper internodes were generally mature for rhizobial infection. Thin sections showed that the root primordium broke down through the epidermal dome (Fig. 3). The apex of the root primordium was overlayed with a thin skin of flattened epidermal cells (Fig. 3). The large base of the epidermal dome was continuous with the stem cortex. In immature sites, at the top of the plant, the root primordium was still embedded in the cortical tissues of the stem and nodule development was never observed.

When immersed in water the cuttings of stem or branches developed abundant adventitious roots (Fig. 4).

Description of stem nodules

Stem nodules were visible 4–5 days after inoculation. They were fully developed 3 weeks later, forming prominent swellings under the stem epidermis. Nodules were very numerous and were sometimes contiguous (Fig. 1). The overall morphology of *A. afraspera* nodules was that of the determinate type (Goodchild 1977). Mature nodules were hemispherically shaped with a broad attachment to the stem and appeared flattened against the stem. They were 3–5 mm in transverse diameter and 2–3 mm high. Nodules were dark green in external appearance and contained a red-pigmented central tissue, suggesting the presence of leghemoglobin. Cross-sections of young nodules showed two distinct infected areas on both sides of vascular connections of the root primordium (Fig. 5), indicating that each of these infected areas results probably from a separate infection origin within the infection site. The central tissue of the nodule never contained any uninfected cells. Mature infected cells presented a prominent nucleus and vacuoles (Fig. 6).

Nitrogen-fixing potential of A. afraspera

Acetylene reduction activity (ARA) values of stem and root nodules of A. afraspera are shown in Table 1. Activities in both cases were linear over a 1.5-h assay period and depended on the age of the nodules. Nodules were assayed 4 weeks after inoculation when nodules had higher specific activities. *Aeschynomene afraspera* exhibited a high nitrogen-fixing capacity. Specific ARA of stem nodules was 309 μmol C_2H_4 g^{-1} nodule dry wt. h^{-1}, an efficiency similar to that of other stem nodules, *Sesbania rostrata* (Dreyfus and Dommergues 1981) and *Aeschynomene scabra* (Eaglesham and Szalay 1983), which have ARAs of 259

Table 1. Nodulation and acetylene reduction activity (ARA) of 9-week-old plants of *A. afraspera* inoculated with *Rhizobium* ORS 322. The nodule-bearing stem portion was that between the second and the fourth node above cotyledons

Part of plant	Nodules per plant		ARA (μmol C_2H_4 plant^{-1} h^{-1})	Specific ARA (μmol C_2H_4 g^{-1} nodule dry wt. h^{-1})
	Dry wt. (mg)	Number		
Stem portion	605 ± 57	145 ± 12	187 ± 13	309 ± 27
Root system	43 ± 11	19 ± 4	5 ± 2.5	116 ± 23

All data are means of five replicates ± standard deviation

Table 2. Effect of stem inoculation upon nitrogen fixation by *A. afraspera* grown in field conditions

Treatment	Plant dry weight (g plant^{-1})	Nitrogen content of plants	
		(% per dry wt.)	(mg N plant^{-1})
Stem and root inoculated	46 ± 3.5	2.97 ± 0.15	1366 ± 125
Root inoculated only	8.0 ± 1.3	1.97 ± 0.10	158 ± 18

All data are means of five replicates ± standard deviation

and 327 μmol C_2H_4 g^{-1} nodule dry wt. h^{-1} respectively. The specific ARA of stem nodules is higher than that of root nodules of field-grown grain legumes such as *Vigna, Vicia* or *Lupinus*, which range from 180 to 250 μmol C_2H_4 g^{-1} nodule dry wt. h^{-1} (Graham and Chatel 1983). *Aeschynomene* root nodulation was poor in waterlogged conditions as shown in Table 1. Root nodules were small and their specific ARA significantly lower than that of the stem nodules. The development of root nodules and their nitrogen-fixing activity was probably affected by waterlogging.

An estimation of nitrogen fixation by stem nodules of *A. afraspera* using the difference method (Williams et al. 1977) is presented in Table 2. When harvested, stem nodulated plants were about 1.2 m in height while plants without stem nodules were only 0.5 m tall. *A. afraspera* bearing stem nodules had a relatively high nitrogen content of about 3% (dry wt.). The total N content of stem inoculated plants was 1366 mg N whereas that of plants without stem nodules was only 158 mg N. By difference, the net fixation by stem nodules was calculated as 1208 mg N plant^{-1}, representing about 90% of the total N content of the stem inoculated plant. It can be noted that the nitrogen fixation value may be an overestimate because the difference method assumes that the root systems are comparable in the two treatments. Stem inoculation could provide sufficient nitrogen to support additional root growth, resulting in a higher soil nitrogen uptake. In that case, the difference method would overestimate nitrogen fixation.

Nevertheless, the maximum estimate of nitrogen fixation by stem nodulated plants based on the cylinder experiment (3 plants 0.07 m^{-2}) was 51.3 g N m^{-2}, a higher value than that obtained with *Sesbania rostrata*, 30 g N m^{-2}, for 2 months (Rinaudo and Moudiongui 1985), indicating that *Aeschynomene afraspera* is one of the most active nitrogen-fixing legumes.

These data suggest that *A. afraspera* might have an important agronomic value as a green manure crop, particularly in the rice production system of tropical regions. *A. afraspera* is a rapidly growing plant which could grow in paddy fields of tropical Africa at the beginning of the rice season while rice seedlings are still in the nursery beds. Furthermore, this plant is able to fix nitrogen under waterlogged or flooded soil conditions, which are usually unfavourable for legumes. Studies in this direction are in progress.

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References

Alazard D (1985) Stem and root nodulation in *Aeschynomene* spp. Appl Environ Microbiol 50:732–734

Arora N (1954) Morphological development of the root and stem nodules of *Aeschynomene indica* L. Phytomorphology 4:211–216

Barrios S, Gonzalez V (1971) Rhizobial symbiosis on Venezuelan savannas. Plant and Soil 34:707–719

Berhaut J (1971) Flore illustrée du Sénégal. Dicotylédones. Tome V. Légumineuses papillonacées. Clairafrique, Dakar

Bremner JM (1960) Determination of nitrogen in soil by the Kjeldahl method. J Agri Sci 55:11–33

Dreyfus BL, Dommergues YR (1981) Nitrogen-fixing nodules

induced by *Rhizobium* on the stems of the tropical legume *Sesbania rostrata*. FEMS Microbiol Lett 10:313 – 317
Eaglesham ARJ, Szalay AA (1983) Aerial stem nodules on *Aeschynomene* spp. Plant Sci Lett 29:265 – 272
Graham PH, Chatel DL (1983) Agronomy. In: Broughton WJ (ed) Nitrogen fixation, vol 3. Oxford University Press, Oxford, pp 56 – 98
Goodchild DJ (1977) The ultrastructure of root nodules in relation to nitrogen fixation. Int Rev Cytol [Suppl] 6:125 – 139
Hagerup O (1928) En hygrofil baelplante (*Aeschynomene aspera* L.) med bakterieknolde paa staengelen. Dan Bot Ark 15:1 – 9
Hardy RWF, Hosten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N-fixation: laboratory and field evaluation. Plant Physiol 43:1185 – 1207
Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Tec Comm N 22. Commonwealth Agricultural Bureaux, Farnham Royal, England
Lison M (1960) Histochimie et cytologie animale. Principes et méthodes. Gauthier-Villars, Paris
Rinaudo G, Moudiongui A (1985) Fixation de l'azote par *Sesbania rostrata*: son utilisation comme engrais vert. Bull Res Agr Gembloux (in press)
Schaede R (1940) Die Knöllchen der adventiven Wasserwurzeln von *Neptunia oleraced* und ihre Bakteriensymbiose. Planta 31:1 – 21
Von Suessenguth K, Beyerle R (1936) Über Bakterienknöllchen am Spross von *Aeschynomene paniculata*. Willd Hedwigia 75: 234 – 237
Vincent JM (1970) A manual for the practical study of root nodule bacteria. IBP Handbook N 15, Blackwell, Oxford
Williams WA, Jones MB, Delwiche CC (1977) Clover N-fixation measurement by total N-difference and NA-values in lysimeters. Agron J 69:1023 – 1024
Yatazawa M, Yoshida S (1979) Stem nodules in *Aeschynomene indica* and their capacity of nitrogen fixation. Plant Physiol 45: 293 – 295

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