J. Mycopathol, Res, ***49(1)*** *: 65—70 (2011)*

Stem nodules development in *Aeschynomene aspera* L.

W. RADHAPIYARI DEVI* AND N. IRABANTA SINGH
Aerobiology, Microbiology, Biotechnology and Plant Pathology Laboratory, Department of Life Sciences, Manipur University, Canchipur, Imphal, 795 003, Manipur

Received : 05.08.2010 Accepted : 29.10.2010 Published : 25.04.2011

Aeschynomene aspera L. can be nodulated by *Rhizobium* sp.on both stem and its root system. The process of stem nodulation showed that rhizobial infection occurred at the site of emergence of adventitious root primordium on the stem of *A. aspera.* At the base of root primordium rhizobia invaded cortical cells when first infected cell enlarged. Rhizobial penetration into the deeper cortex was by progressive collapse of infected cell towards the meristematic zone by invagination of the host cell wall. Soon after infection, the cell collapsed progressively forming infection strand-like structure which developed up to the meristematic zone. When infection reached the meristematic zone, the invaded host cells ceased to collapse but divided repeatedly to form the nodule. Nodules were connected to the stem and aeschynomenoid. There were no uninfection cells in the central core of the infected tissue and true infection threads were never observed during the nodule development stage.

Key words: *Aeschynomene aspera* L., *Rhizobium,* root primordium, meristematic zone, nodule formation

INTRODUCTION

A few tropical legumes, belonging to the genera *Aeschynomene, Neptunia, Discolobium* and *Sesbania*, are able to form nitrogen-fixing nodules both on stems and roots. Stem nodulation in the genus *Aeschynomene* has been first reported on *A. aspera* L (Hagerup, 1928). Stem nodulation, restricted to date to only one species of *Sesbania* (*S. rostrata*) (Dreyfus and Dommergues, 1981), one species in *Neptunia* (*N. oleracea*) (Schaede, 1940), also *Discolobium pulchellum* (Loureiro *et al.*, 1994), and much more widespread within the genus *Aeschynomene* (Alazard, 1985).

Aeschynomene aspera L., is characterized by profuse stem nodulation. The evaluation of different *Rhizobium* isolates from root nodules of *A. aspera* for nodulation ability on selected legumes has been studied by Subba Rao *et al.* (1980). Moreover, A. *afraspera* can be successfully used as a green manure in rice cultivation, increasing the rice grain yield by 80% (Alazard and Becker, 1987; Rinaudo, *et al.,* 1988; Ladha *et al.,* 1992).

Infection and nodule development process in root nodulated legumes can occur (Dart, 1977 and Bauer, 1981). In temperate legumes, the formation of infection threads involves within root hairs followed by the release of rhizobia in the cortical cells of the host plant. In tropical legumes, *Arachis* (Allen, and Allen, 1940; Chandler, 1978) and *Stylosanthes* (Ranga Rao, 1977; Chandler *et al.,* 1982) processes by the absence of infection threads and involves direct invasion of cortical cells at the site of emerging lateral roots. Intercellular infection is initiated at the basal junction between the root hair cells and adjacent epidermal cells.

Development of the nodule occurs by repeated division of the infected host cells by rhizobia invading the host cell through cell wall degradation. Nodules of *Aeschynomene indica* (Arora, 1954; Yatazawa and Yoshida, 1979; 1984), *A. afraspera*

* corresponding e-mail- randhawang@gmail.com

(Alazard and Duhoux, 1987; 1990) are known to arise near emerging lateral roots. When root hairs are absent, it is presumed that the mode of entry is through epidermal cells of the cortex. No infection threads are formed in the nodules; hence rhizobia are spread by cell division. Studies by Tsien *et al.*, (1983) and Duhoux (1984) on *Sesbania rostrata* are the first reports on the infection process and nodule morphogenesis in stem nodulated legumes where rhizobial infection initiated intercellularly between the basal cells of the protruding root primordium forming infection pockets in which rhizobia multiply in large numbers. At the same time, a meristematic zone arises in the adjacent cortical cells of the root primordium. Infection threads then originate from the intercellular pockets of infection and divide into several branches that invade the meristematic cells.

Most studies in *Aeschynomene* sp. have dealt with the ultra structure of established root or stem nodules (Arora, 1954; Yatazawa and Yoshida, 1979; Yatazawa *et al.*, 1984; Vaughn and Elmore, 1985; Alazard and Duhoux, 1990; Loureiro *et al.*, 1995). To our knowledge, there is no report on the infection and stem nodule development in *A. aspera* and hence reported in this paper.

MATERIALS AND METHODS

Isolation of Rhizobium

Vincent (1970) standard procedures outlined were used for isolation of a *Rhizobium* from stem nodules of *Aeschynomene aspera*. The strain was maintained on Yeast Extract Mannitol Agar Slope (Vincent, 1970).

Plant culture and inoculation

For stem nodulation tests, seeds were surface sterilized with 0.1N sulphuric acid for 3 minutes and washed three times with sterile distilled water. Surface sterilized seeds were germinated at 30°C on sterile 1% water agar in Petridishes for 24 to 48 hr. Then seedling were transferred into plastic pots (15 cm diameter) containing sterile water logged, 2 kg sandy soil, pH 7.0 for stem nodulation trail in the greenhouse at 23-35°C (day-night temperatures). Stems were inoculated by being sprayed with a 10-fold dilution of the *Rhizobium* spp. MTCC-10058 of the broth culture (Alazard, 1985: Alazard and Duhoux, 1987; 1990).

Histology of stem nodules and nodulation sites

Nodules were fixed for both light and electron microscopy (both scanning and transmission electron microscopy) 24 hr after inoculation and for 30 days so that successive stages of stem nodule development could be noted.

Surface features of stem nodules of *A. aspera* were examined by scanning electron microscopy (SEM). For SEM, tissues were fixed overnight in 2.5% glutaraldehyde in 0.1M sodium-cacodylate buffer (pH 7.2) at 4°C, and again rinsed in 0.1 M Na-cacodylate buffer 3 times of 15 minutes each at 4°C. They were then dehydrated progressively in an acetone series, critical point dried with liquid CO_2 at 31.5°C and immersed in Tetra Methyl Silane for 5-10 min 2 changes at 4°C and dried, mounted on Brass stubs, and sputter-coated with 35 nm of gold (JSM 6360).

Anatomy of nodules was examined by light microscopy and transmission electron microscopy (TEM). For TEM, nodules were fixed in 2.5% glutaraldehyde in 0.1M Na-cacodylate buffer, pH 7.0 for 15 min at 4°C. The fixed material was washed 3 times in the same buffer, post-fixed in 1% Os04 in 0.1 M Na-cacodylate buffer at 4°C. Fixed nodules were rinsed 3 times with 0.1M Na-cacodylate of 15 minutes each at 4°C. They were then dehydrated progressively in an acetone series and cleared out using propylene oxide at room temperature and embedded in BEEM capsules in the embedding oven set at 50°C for 12-24 hrs. Then they were polymerized at 60°C for 24-48 hrs. 60-90 nm ultra-thin sections were immersed under the copper grip and double stained in uranyl acetate and then lead citrate (Reynolds, 1963) and examined by transmission electron microscopy (JEM 100 CX-II)

Materials for light microscopy (Labophot-2) were fixed overnight in a solution containing 32.5% ethanol, 5% acetic acid and 5% formaldehyde (v/v), and were cut from Epon blocks prepared. Sections were stained with Regaud's iron-haematoxylin stain (Lison, 1960).

Measurement of nodule diameter

Fixed nodules or nodulation sites were sliced in half under a dissecting microscope, and the diameter of the specimens was measured to 0.3 mm. Ten

nodules were measured at each sampling date. Results are reported as mean ± S.T.D.

RESULTS AND DISCUSSION

Nodulation sites on the stem of A. aspera

Stem nodulation of A. aspera occurred at pre-determined sites independently of bacterial infection (Alazard and Duhoux, 1987, 1990; Legocki, *et al.*, 1983). Beneath epidermal cells, stem nodulating site of *A. aspera* consisted of a protruding root primordium. Rhizobial infection became susceptible only between the central root primordium and the surrounding cortical tissue when a circular cavity was formed (Fig.1). Due to mechanical forces

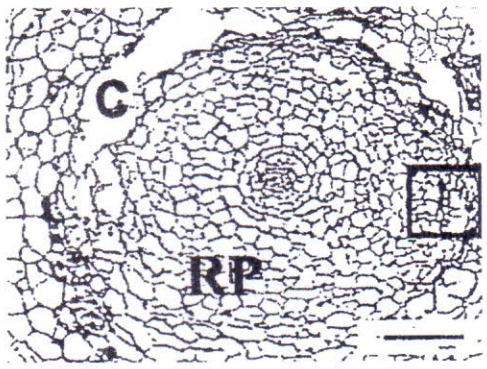

Fig. 1. Light micrograph of a TS of *A.aspera* through and inoculated nodulation site at a later stage in development showing the collapsed cell (boxed area) located in the outer cortical cells of the root primordium. The circular gravity (C) encircling the root primodium (RP). Epon block; iron-haematoxylin stain. xlOO (Bar = 0.1 mm)

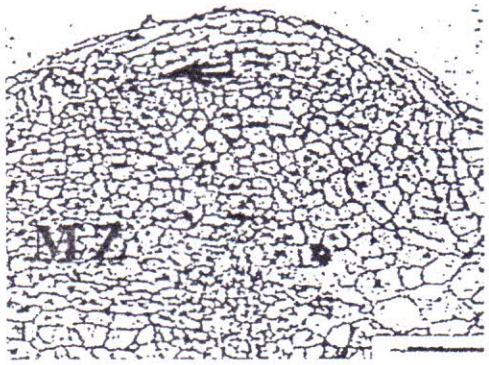

Fig. 2. Light micrograph of a LS section through a nodulation site, 2-day after inoculation, showing an enlarged infected cell (arrow) in the cortex near the circular gravity. The meristematic zone (MZ) induced beneath the infected cell. Epon block; iron-haematoxylin stain, x 80 (Bar = 100 μm)

associated with the developing root primordium only a thin layer of flattened epidermal cells overlaid which allowed rhizobial access to the inside of the stem nodulation site.

Infection of the stem nodulation site

Two days after inoculation, stem infection was the presence evidence near the epidermal cell layer. A meristematic zone arose in the inner cortex at a distance (Fig. 2) that rhizobia could trigger meristem initiation by means of a diffusible chemical signal as described in temperate legumes (Truchet *et al.*, 1980). A slightly later stage of development revealed that infection was initiated in the cortex of the root primordium by transverse sections through the stem nodulation site (Fig. 1) and collapsed of the initially infected cell filling with rhizobia.

Infection development

Rhizobia entered the host cell by invagination of the cell wall through intracellular invasion of host cell in the development of infection. By plasma membrane, rhizobia were embedded in new cell wall material at the entry point. The newly formed wall material at this point was reminiscent of that observed for infection thread initiation in root hairs (Callaham and Torrey, 1981). However, the infection structures described were restricted to and did not extend beyond the boundaries of the host cell. In addition, rhizobia were not surrounded by electron-transparent matrix as found in true infection threads (Callaham and Torrey, 1981; Turgeon and Bauer, 1985). Cell invasion appeared to have a deleterious effect on the host cell. It created disorganization of the cytoplasm (Fig. 3) and soon after, the cell

Fig. 3. TEM showing intracellular penetration of rhizobia (R) embedded in the host cell wall material (HCW) surrounded by plasma membrane (PM). x 11000 (Bar = 1 μm)

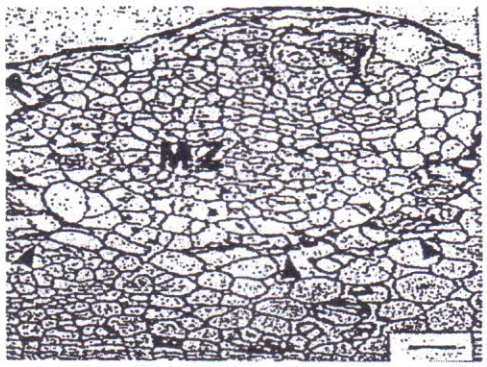

Fig. 4. Light micrograph of LS through a 4 day old nodule showing two initially infected cells which have collapsed surrounded by a layer of cells containing darkly stained tannins (large arrow heads) in the meristematic zone (MZ) Epon block; iron haematoxylin stain. xl20 (Bar = 50 μm)

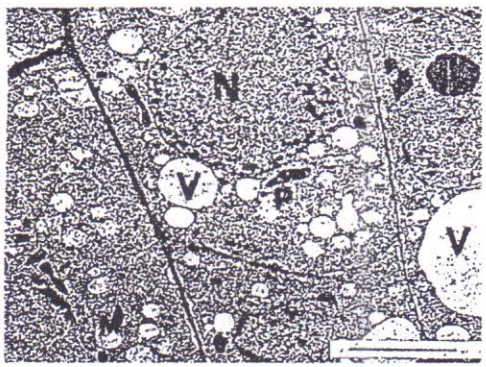

Fig. 5. TEM in the meristematic zone 3 day after inoculation. Uninffected cells have a large central located nucleus (N) in vacuolated (V) cytoplasm, plastids (P), mitochondria (M). x 5000 (Bar = 5 μm)

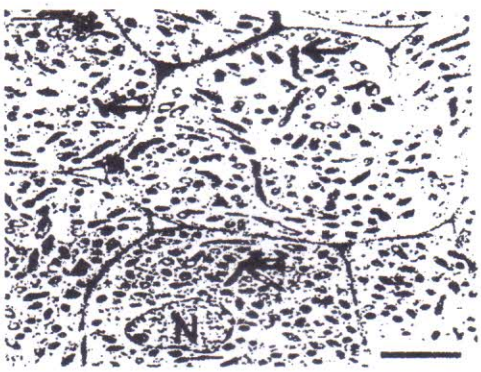

Fig. 6. TEM of infected cells from the upper stem nodule. There are one or two rod-shaped bacteroids per peribacteriod unit (PBU) (arrows). Host cell nucleus (N) and mitochondria (M) are visible. x7000 (Bar = 2 μm).

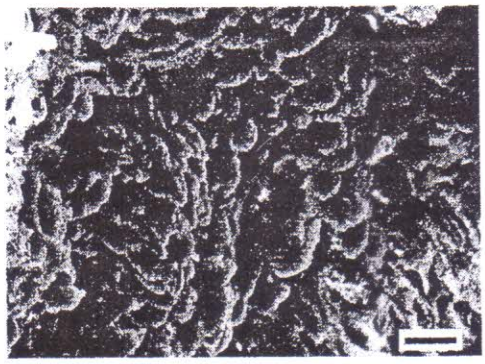

Fig. 7. SEM of infected cells from *A. aspera* stem nodule. The bacteroids are simple rods and are confined to the cortex of the host cells. x400 (Bar = 50 μm)

collapsed (Alazard and Duhoux, 1990). Infection advanced from cell to cell of the host cell wall in the adjacent cell by internal invaginations. Then rhizobia penetrated into the deeper stem cortex by the successive collapse of invaded cells and intercellular infection strand-like structures were formed in the resulting spaces.

As a result, infection strand-like structures were observed in the cortex leading away from the fissure at the base of the root primordium up to the meristematic zone partially surrounded by the layer of tannin-containing cell (Fig.4). Meristematic cells were constituted by small uninfected thin-walled cells containing a large centrally nucleus plus numerous vacuoles and plastids in a dense cytoplasm (Fig.5). Infected cells from the upper stem nodule had one or two rod-shaped bacteroid per peribacteroid unit (PBU) with host cell nucleus, vacuole and mitochondria (Fig. 6) and bacteroids were simple rods and confined to the cortex of the host cells (Fig. 7)

Development of nodule

First to second day after the first infection, cell could be observed when the infection strand reached the meristematic zone, the infected meristematic cells did not collapse which enclosed a few rhizobia in a peribacteriod membrane. At this stage, the presence of rhizobia did not induce the collapse of the host cell. At the entry point into the meristematic cells, rhizobia were not embedded within the cell wall material (Fig. 8). A phase of rapid multiplication of rhizobia occurred while the infected cells divided repeatedly, leading to an increase in the volume of the nodule.

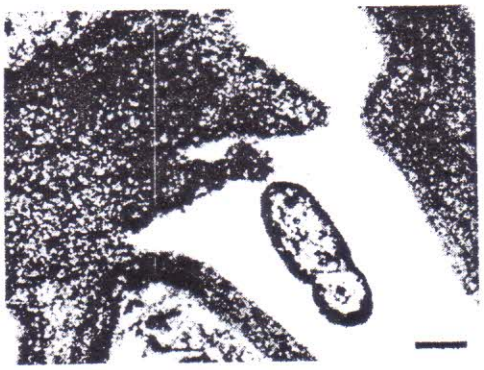

Fig. 8. TEM. Rhizobium (R) can be seen in direct contact (arrow heads) with the cell wall of the meristematic cells suggesting that they are penetrating into the meristematic cells and rod shaped in the cell. x 14 (Bar = 0.2 μm)

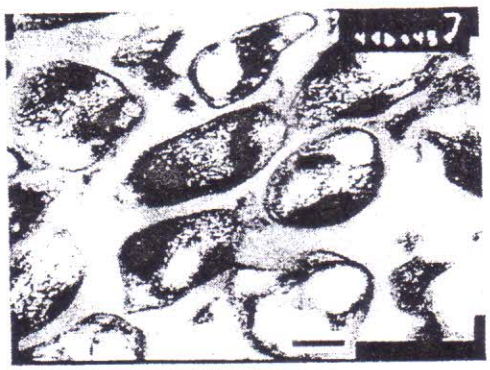

Fig. 9. TEM showing rod shaped rhizobia in the central tissue of the nodule enclosed singly in a peribacteroid membrane with poly-b-hydroxybutyrate. x 40000 (Bar = 0.5 μm)

There were no uninfected cells in the central core of the infected tissue and true infection threads were never observed during the nodule development stage. Rhizobia in the central tissue of the nodule were rod shaped or elongated, similar to their free-living form (Fig. 9). They were singly enclosed in a peribacteroid membrane probably originating from the plasma membrane. They had a dense fibrillar nuclear material and some electron-transparent regions at the polar end, probably corresponding to poly-3-hydroxybutyric acid inclusions (Fig. 1).

The infection process leading to stem nodule formation in *A. aspera* was quite different from that described for the other stem nodulated legume *A. fluminensis* (Loureiro *et al.*, 1995). On the other hand, it appeared closely related to that of alternate modes of infection described in thread less legumes, particularly in *Stylosanthes* spp. (Chandler *et al.*, 1982), *A. afraspera* (Alazard and Duhoux, 1990) where early invaded cells collapsed (Chandler *et al.*, 1982). The situation in *Arachis* (Chandler, 1978) was rather different, in that the penetration of rhizobia in the deeper cortex was via progression by separating the cells at the middle lamellae. *Aeschynomene*, *Arachis*, and *Stylosanthes* which belongs to the legume tribe *Aeschynomeneae* (PolhilL *et al.*; 1981) form nodules of the aeschynomenoid type that was located in the axils of lateral roots (Corby, 1981). In addition, the aeschynomenoid nodules were also found in most genera of the tribe Daibergieae and in the monogeneric tribe Adesmieae (Faria *et al.*, 1984). It was likely that all these plants could be sharing a common mode of entry (Sprent, 1989).

Mode of entry in ways other than through root hairs might be more common in legumes, since the infection process had been described in only a small number of leguminous plants, particularly in tropical legume genera where even nodulation had not yet been examined (Faria *et al.*, 1984).

ACKNOWLEDGMENTS

The authors thank to Dr. Sudip Dey and Joston P. Nongkynoih, S.A.I.F., North East Hill University, Shillong for providing the electron microscopy facilities. One of the authors (W. Radhapiyari Devi) thanks HOD, Life Sciences, Manipur University, Canchipur for providing fellowship during the course of the present work.

REFERENCES

Alazard, D., 1985. Stem and root nodulation in *Aeschynomene* spp. *Applied* and *Environmental Microbiology*, **50:** 732-734

Alazard, D. and Becker, M., 1987. *Aeschynomene* as green manure for rice. Plant and Soil, **101:** 141-143

Alazard, D. and Duhoux, E., 1987. Nitrogen-fixing stem nodules on *Aeschynomene afraspera. Biology and Fertility of Soils*, **4:** 61-68

Alazard, D. and Duhoux, E., 1990. Development of stem nodules in a tropical forage legume, *Aeschynomene afraspera. Journal of Experimental Botany*, **41**: 1199-1206

Allen, O. N. and Allen, E. K., 1940. Response of the peanut plant to inoculation with rhizobia with special reference to morphological development of the nodules. *Botanical Gazette*, **102:** 121-142

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

Bauer, W. D., 1981. Infection of legumes by rhizobia. *Annual Review of Plant Physiology*, **32:** 407-419

Callaham, D.A., and Torrey, J.C., 1981. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. *Canadian Journal of Botany*, **59:** 1647-1664

Chandler, M.R., 1978. Some observations on infection of *Arachis hypogaea* L. by *Rhizobium*. *Journal of Experimental Botany*, **29:** 749-755

Chandler, M.R., Date, R.A., and Roughley, R.J., 1982. Infection and root nodule development in *Stylosanthes* species by *Rhizobium*. *Ibid.* **33:** 47-57

Corby, H.D.L., 1981. The systematic value of leguminous root nodules. In *Advances in legume systematics, Part 2.* (Eds R. M. Polhill and P. H. Raven) Royal Botanic Gardens, Kew, 657-669

Dart, P., 1977. Infection and development of leguminous nodules. In *A treatise on dinitrogen fixation. Section III, biology.* (Eds R. W. F. Hardy and W. S. Silver.) J. Wiley and Sons, New York, PP. 367-417

Dreyfus, B. L., and Dommergues, Y., 1981. Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. *FEMS Microbiology Letters*, **10:** 313-317

Duhoux, E., 1984. Ontogenese des nodules caulinaires du *Sesbania rostrata* (legumineuse). *Canadian Journal of Botany*, **62:** 982-994

Faria, S. M.; De, Francao, A. A.; Jesus, R . M.; Menandro, M. De S.; Baitello, J. B,; Mucci, E. S. F.; Dobereiner, J., and Sprent, J. I., 1984. New nodulating legume trees from south-east Brazil. *New Phytologist*, **98:** 317-328

Hagerup, O., 1928. Enhrofel haelplante (*Aeschynomene aspera* L.) med bakteiekno' de paa staengelen. *Dan. Bot. Ark.*, **15:** 1-9

Ladha, J.K.; Pareek, R.P. and Becker, M., 1992. Stem-nodulating legumes *Rhizobium* symbiosis and its agronomic use in lowland rice. *Advances in Soil Science.* **20:** 148-192

Legocki, R.P.; Eaglesham, A.R.J, and Szalay, A.A., 1983. Stem nodulation in *Aeschynomene*: a model system for *bacterium*-plant interaction. (Ed. A. Puhler), Molecular genetics of the bacteria-plant interaction. Springer-Verlag KG, Berlin, pp. 210-219.

Lison, M., 1960. In *Histochimie et cytochimie animate. Principes et methodes.* Gauthier-Villars, Paris

Loureiro, M.F.; De Faria, S.; James, E.K.; Pott, A., and Franco, A.A., 1994. Nitrogen-fixing nodules of the legume, *Discolobium pulchellum* Benth. *New Phytol*, **128:** 283-295

Loureiro, M.F.; James, E.K.; Sprent, J.I. and Franco, A.A., 1995. Stem and root nodules on the tropical wetland legume *Aeschynomene fluminensis*. **Ibid. 130:** 531-544

Polhill, RM.; Raven, P.H., and Stirton, C.H., 1981. Evolution systematics of the leguminosae. In *Advances in legume systematics.* (Eds R. M. Polhill and P. H. Raven) Royal Botanic Gardens, Kew. pp. I-26

Ranga Rao, V., 1977. Effect of root temperature on the infection processes on *Lotus* and *Stylosanthes*. *Journal of Experimental Botany*, **28:** 241-259

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology*, **17:** 208-212

Rinaudo, G.; Alazard, D. and Moudiongui, A., 1988. Stem-nodulating legumes as green manure for rice in West Africa. In *Sustainable Agriculture.* Green Manure in Rice Farming: 97-109. International Rice Research Institute. P.O. Box 993, Manila Philippines

Rinaudo, G.; Dreyfus, B. and Dommergues, Y.R., 1983. *Sesbania rostrata*, green manure and the nitrogen content of rice crop and *soil. Soil Biology Biochemistry*, **15:** 111-193

Schaede, R. 1940. Die Knollchen der adventiven Wasserwurzein von *Neptunia oleracea* und ihre Bakteriensymbiose. *Planta.* **31**: 1-21

Sprent, J.I., 1989. Transley review N 15. Why steps are essential for the formation of 'functional legume nodules? *New Phytologist*, **111:** 129-153

Subba Rao, N.S,; Tilak, K.V.B.R. and Singh, C.S., 1980. Root nodulation studies in *Aeschynomene aspera. Plant and Soil*, **56:** 491-494

Truchet, G.; Michel, M., and Denarie, J., 1980. Sequential analysis of the organogenesis of Lucerne (*Medicago sativa*) root nodules using symbiotically-defective mutants of *Rhizobium meliloti. Differentiation*, **16:** 163-172

Tsien, H.C.; Dreyfus, B.L., and Schmidt, E.L., 1983. Initial stages in the morphogenesis of the nitrogen-fixing stem nodules of *Sesbania rostrata. Journal of Bacteriology*, **156:** 888-897

Turgeon, B.G., and Bauer, W.D., 1985. Ultrastructure or infection-thread development during the infection of soybean by *Rhizobium japonicum. Planta*, **163:** 328-349

Vaughn, K.C., and Elmore, C.D., 1985. Ultrastructural characterization of nitrogen-fixing stem nodules on *Aeschynomene indica. Cytobios*, **42:** 49-62

Vincent, J.M., 1970. *A manual for the practical study of root nodule bacteria.* IBP Handbook N 15, Blackwell, Oxford

Yatazawa, M. and Yoshida, S., 1979. Stem Nodules in *Aeschynomene indica* and Their Capacity of Nitrogen Fixation. *Physiologia Plantarum*, **45:** 293 - 295

Yatazawa, M.; Yoshida, S., and Maeda, E., 1984. Fine structure of root nodules of *Aeschynomene indica* L. *Soil Science and Plant Nutrition*, **30:** 405-416