

## A Structural and Histochemical Study of Actinorhizal Nodules of *Casuarina equisetifolia* Linn.

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**ABSTRACT:** *Casuarina equisetifolia* Linn. is an introduced actinorhizal plant grown as roadside ornamental tree and as forest plantation in Pakistan. Actinorhizal nodules are formed on the roots of *C. equisetifolia* as a response to infection by *Frankia*. Structure of *C. equisetifolia* nodules is described using histochemical methods to elucidate the nature of micro-symbiont within the nodules. *C. equisetifolia* nodules were similar to a root in general anatomical structure and resembled with *Myrica*-type nodules. The nodule consisted of a distinct periderm enclosing the cortex. The cortex showed patches of infected cells interspersed among uninfected ones. Inside the cortex there was a stele bounded by an endodermis, which contained tannins. The phloem-xylem relationship was amphicribal. A poorly defined meristem was also observed. Apart from simple nodules, dichotomously branched and coralloid nodules were also observed. The stele in simple and branched nodules reached up to the tip of the nodules. The micro-symbiont was observed in the nodule tissues in hyphal form only. The histochemical tests revealed absence of vacuoles and starch grains, and presence of tannins and lignin.

**KEY WORDS:** *Casuarina*, *Frankia*, Actinorhizae, Hyphae.

### INTRODUCTION

Casuarinas are fast growing multipurpose trees and have various uses ranging from environmental protection to pulp and paper production. They form actinorhizal symbioses with *Frankia* and contribute substantially to the nitrogen economy of forest plantations. *Casuarina equisetifolia* Linn. is an introduced plant grown as roadside ornamental tree and as forest plantation in Pakistan. Relatively high cellulose and low lignin contents in the wood of *C. equisetifolia* make it a suitable alternative for use in pulp and paper industry of Pakistan (Mahmood, 1993). Athar and Mahmood (1983) observed actinorhizal nodules in *C. equisetifolia* plants. Studies on the initiation, development and structure of *Casuarina* nodules have been made by various workers (Torrey, 1976; Kant and Narayana, 1977; Berg, 1983; Berg and McDowell, 1987; Laplaze *et al.*, 1997). *Frankia* grown in culture in nitrogen-free media usually exhibits three morphological forms: hyphae, vesicles and multilocular sporangia (Benson and Silvester, 1993; Akkermans and Hirsch, 2000). In actinorhizal nodules, vesicles are demonstrated site of nitrogen fixation and have been reported in mature nodule cortical cells in almost all actinorhizal plants (Huss-Danell and Bergman, 1990; Berg, 1999). *Casuarina* nodules differ from other actinorhizal nodules thus far described in that *Frankia* does not form vesicles within the nodules (Tayson and Silver, 1979; Berg, 1983). Instead of vesicles the micro-symbiont forms atypical hyphae in mature infected cells (Berg and

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McDowell, 1987). It has been suggested that the lack of vesicle formation in *Casuarina* is due to lowered oxygen concentration in cells where the wall may be an effective gas diffusion barrier (Berg and McDowell, 1987). However, Gardner (1976), Torrey (1976) and Becking (1977) described vesicle-like structure in *Casuarina* nodules. In this paper histochemical methods were used to describe the structure of *Casuarina* nodules and to elucidate the nature of the micro-symbiont within the nodules.

## MATERIALS AND METHODS

### Collection of plant material

Roots of *C. equisetifolia* trees growing for 4-6 years at Karachi University campus were excavated and examined for nodulation. The soil at the collection site was alkaline (pH 8.2-8.6) loamy sand with organic carbon ranging from 0.7 to 1.9%. Nodules were separated from roots and thoroughly washed under running tap water to clear the soil particles. They were then fixed in FAA (formalin : acetic acid : alcohol = 5: 5: 90) for 24 hours and transferred to 70% ethanol for storage.

### Preparation of material for microscopic examination

Nodules were prepared for microscopic examination following histological methods described by O'Brien and McCully (1981). The excised nodules were dehydrated with tertiary butyl alcohol series and embedded in paraffin wax (melting point 56-58 °C). Serial sections, 10-15  $\mu\text{m}$  thick, were cut on a rotary microtome. The ribbons of sections were floated on 4% formaldehyde solution on glass slides coated with Haupt's adhesive and dried overnight in formaldehyde vapors at 40 °C. Dried sections were passed through xylol-alcohol series and stained by different staining technique. The staining combinations used were fuchsin-methyl green, gram stain, safranin-light green stain, safranin-Harri's hematoxylin and Heidentain's hematoxylin. Staining with fuchsin-methyl green (0.15 g methyl green, 0.01 g fuchsin, 100 mL water) was found most satisfactory because it stained vascular system, infection hyphae and the infected tissues with equal clarity. The sections were rinsed with absolute alcohol, put in xylol-alcohol for one minute and finally in alcohol. If necessary they were brought back into xylol-alcohol for further differentiation. The stained sections were dehydrated with alcohol series, cleared in xylol and finally mounted in Canada balsam. The slides of nodule sections were observed under light, phase contrast and polarized light microscopes.

### Histochemical tests

Fresh hand-cut or FAA fixed microtome sections were tested for the presence of starch by using the IKI-H<sub>2</sub>SO<sub>4</sub> method (Jensen, 1962), tannins by ferric sulfate reactions (Reeve, 1959) and lignin by phloroglucinol in 20% HCl (Jensen, 1962). Presence of bacteria was tested by gram staining (Somasegaran and Hoben, 1994) and mycorrhizal hyphae by trypan blue in lactophenol (Philips and Hayman, 1970).

## RESULTS AND DISCUSSION

### Structure of the nodule

The roots of *C. equisetifolia* were abundantly nodulated (Fig. 1). Young nodules were ovate in shape which as result of repeated branching developed into spherical coralloid mass,

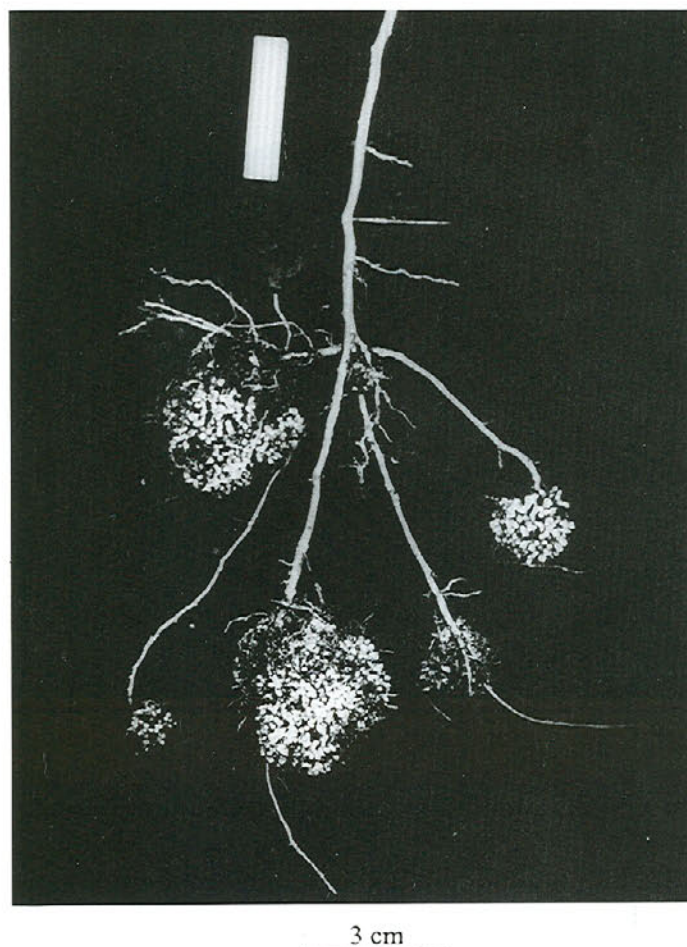


Fig. 1. Root system of *Casuarina equisetifolia* showing distribution of coralloid nodules. Bar = 3 cm.

5 cm or more in diameter, composed of close packed radiating nodule lobes. Another external feature of *Casuarina* nodules was that the apex of each nodule lobe gave rise to root (nodule-root) which showed upward growth (Fig. 1). Most nodules were found on the young roots in the upper 10-15 cm of the soil. Nodules were brown in color and existed throughout the year.

*Casuarina* nodules were similar to a root in general anatomical structure (Fig. 2). The nodules consisted of a distinct periderm enclosing the cortex. The cortex showed patches of infected cells interspersed among the uninfected ones (Fig. 3). Inside the cortex there was a stele bounded by an endodermis, which contained tannins (Fig. 3). A poorly defined meristem was also observed at the tip of each nodule lobe (Fig. 3). The infected cells were scattered through most of the cortex and were fairly large. These cells were conspicuous because of their dark stained contents and by their large size (Fig. 4). Starch was not found in any of the nodule tissue. The endodermis consisted of single layer of cells, heavily laden with tannin. The phloem-xylem relationship was amphicribal (Fig. 3). Apart from simple nodules, dichotomously branched and coralloid nodules were also observed (Fig. 4). The stele in simple and branched nodules reached up to the tip of the nodule.

The general structural of *Casuarina* nodules is essentially similar to that of other actinorhizal nodules as described by various workers (Gardner, 1976; Torrey, 1976; Callahan and Torrey, 1977; Kant and Narayana, 1977; Newcomb *et al.*, 1978; Berg *et al.*, 1999; Laplaze *et al.*, 2000; Wall, 2000). The structure particularly resembles with *Comptonia*

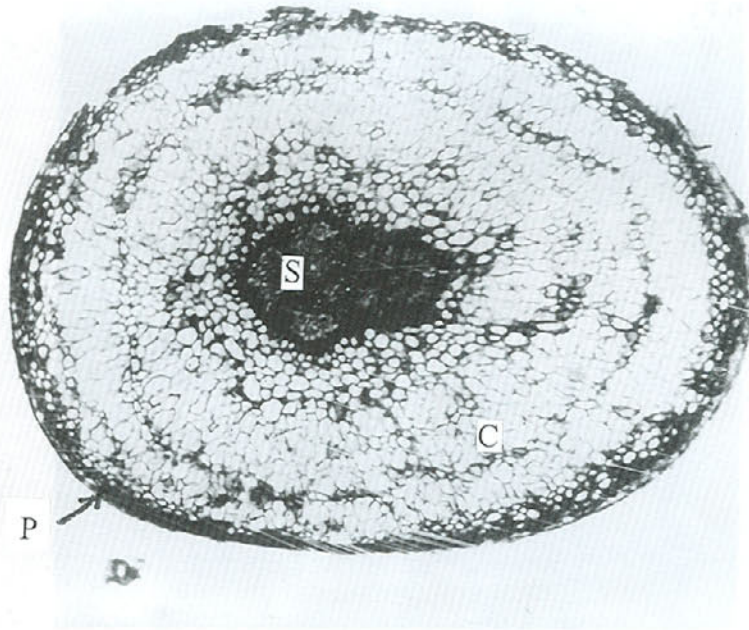


Fig. 2. Transverse section of a nodule lobe of *Casuarina equisetifolia* showing central stele (S) enclosed by cortex (C) and periderm (P). 130 x.

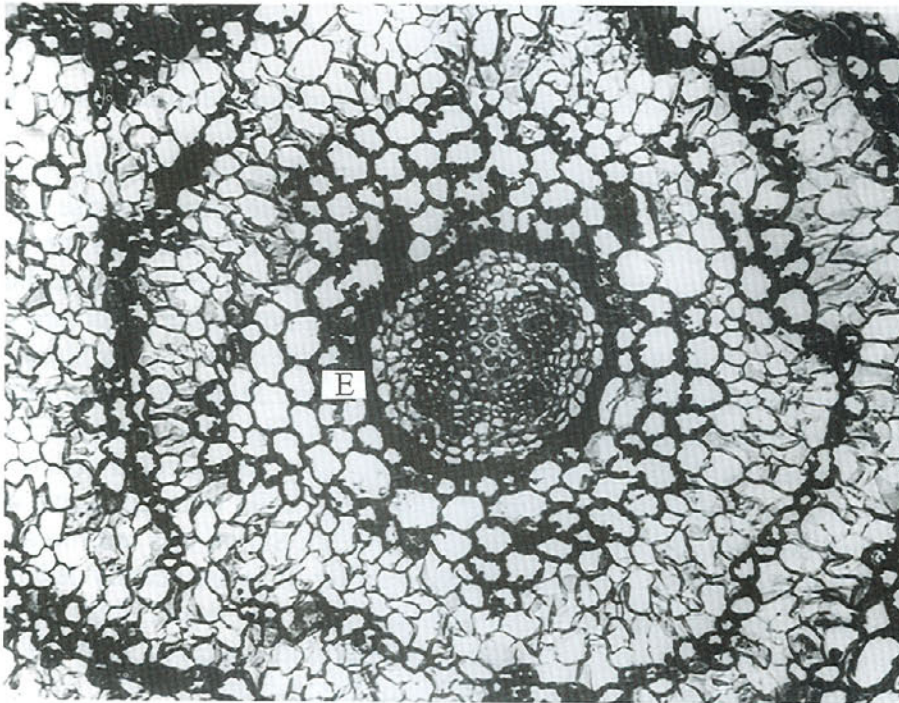


Fig. 3. Transverse section of a nodule lobe of *Casuarina equisetifolia* nodule showing amphicribal vascular bundle. The infected cells can be seen scattered in the cortical region (dark stained). The endodermis (E) is very distinct. 320 x.



Fig. 4. Longitudinal section of a *Casuarina equisetifolia* nodule. Note the coralloid branching and a poorly defined apical meristem (M). 80 x.

*peregrina* nodules studied by Newcomb *et al.* (1978). Though the arrangement of tissues within the *Casuarina* nodules is comparable with roots in many aspects, yet there are some obvious differences. Each nodule lobe contains a meristematic region located near the tip or distal end of the nodule. This meristem gives rise to nodule cortex and the endodermis. Micro-symbiont is found in the large cortical cells scattered among smaller uninfected cells. Points of difference between nodule and lateral root are that there is no root cap, root hairs are absent and there is a superficial cork layer enclosing the nodule.

The infected cells in *Casuarina* do not form any particular zone but were scattered throughout the cortex (Fig. 5). This resembles with *Myrica* (Silver, 1964). However, this is in contrast to the infected regions of other actinorhizae. In *Alnus* the infected cells form concentric zones, old nodules show several of them but young nodules contain only one (Stewart, 1966). In *Ceanothus*, the infection is confined to the middle or the outer cortex (Strand and Laetsch, 1977). In *Comptonia* infection is confined only to the middle cortex. The cortical cells adjacent to the endodermis and epidermis remain free of infection (Callaham and Torrey, 1977; Newcomb *et al.*, 1978).

#### Cytology of the micro-symbiont

Only the hyphal form of the micro-symbiont was observed. Cell walls of infected cells stained brilliant red with phloroglucinol indicating presence of lignin. The hyphae are the

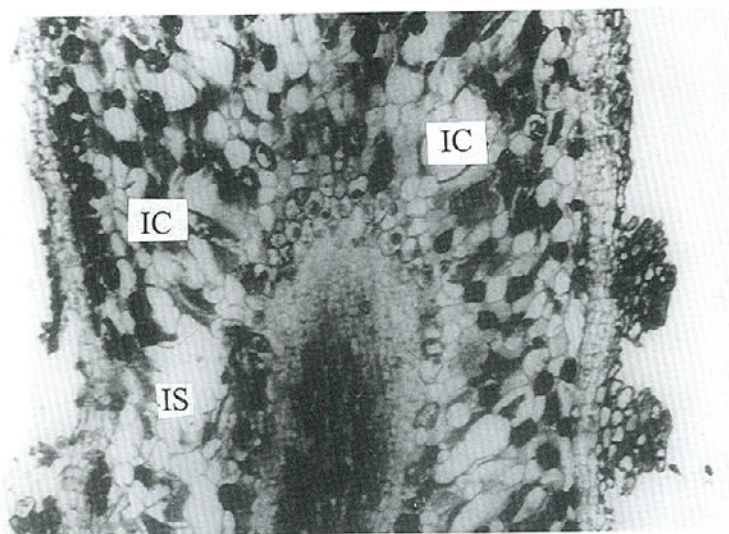


Fig. 5. Longitudinal section of a nodule lobe of *Casuarina equisetifolia*. Note the interstitial cells (IS) scattered among the infected cells (IC). 400 x.

primary form of the micro-symbiont. They were found only in the young nodules. In older nodules hyphae were present within cells immediately adjacent to the meristematic region. The hyphae were septate and branched measuring 0.5 to 1.0  $\mu\text{m}$  in diameter. They were gram positive. Hyphae did not stain with trypan blue and lactophenol indicating that they were not mycorrhizal in nature. In several cells, clusters of hyphae penetrated the cell wall intracellularly and could be seen at both sides of the neighboring cells (Fig. 6). The hyphae may penetrate through the host cell wall by hydrolyzing the cell wall components. Cellulolytic and pectinolytic enzymes synthesized by *Frankia* have been reported in wall degradation at the site of penetration (Safo-Sampah and Torrey, 1988; Seguin and Lalonde, 1989). Passage of hyphae from one cell to another through intact cell walls has also been described in other actinorhizal nodules (Torrey, 1976; Callaham and Torrey, 1977; Kant and Narayana, 1977; Newcomb *et al.*, 1978; Miller and Baker, 1985, 1988; Liu and Barry, 1991a, b; Sunell and Berry, 1992; Berg, 1999). The penetrated cells enlarged rapidly. Shortly after penetration in the cell, the hyphae formed clusters which filled major part of infected cells. Vesicle-like structures which appeared as spherical bodies, 3.0 to 4.0  $\mu\text{m}$  in diameter, were seen in the nodule sections (Fig.7). Spherical vesicle-like structures have been reported by Torrey (1976) in nodules of *C. cunninghamiana*. Gardner (1976), Newcomb *et al.* (1978) and Becking (1977) have reported club-shaped vesicles in *Casuarina* nodules. However, Tayson and Silver (1979), and Berg (1983) could not find vesicular structures in *Casuarina* nodules. The vesicle-like structures found in present study resemble in general outline with vesicle-like structures shown by Torrey (1976). However, the nature of these vesicle-like structures remains obscure since hyphae were not found in association with these structures. *Casuarina* strains do not form vesicles in symbiotic associations (Berg, 1983; Tayson and Silver, 1979) and since vesicles are generally thought to be major site of nitrogenase activity (Huss-Danell and Bergman, 1990; Berg, 1999), a pertinent question arises, whether under symbiotic associations the hyphae act as site for nitrogenase activity in *Casuarina* nodules?. This question needs to be elucidated by further physiological studies.

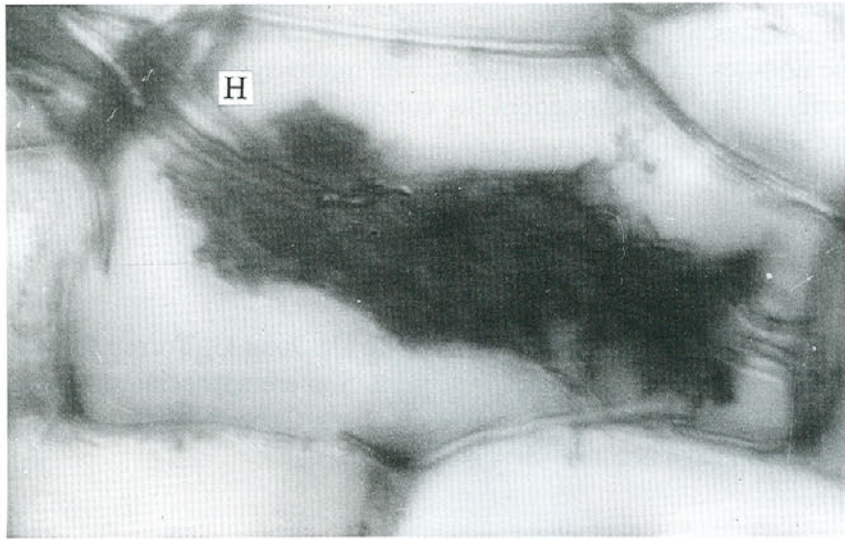


Fig. 6. Infected cortical cells of *Casuarina equisetifolia* root nodule showing clusters of hyphae (H) penetrating the cell wall observed under phase contrast microscope. 2400 x.

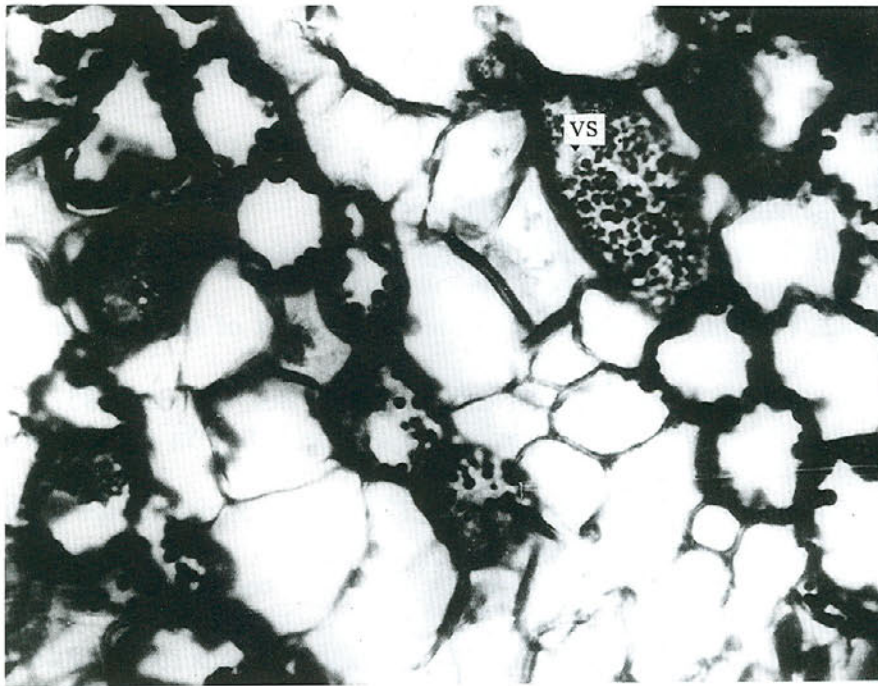


Fig. 7. Infected cortical cells of *Casuarina equisetifolia* root nodule showing vesicle-like structures (VS). 1280 x.

Vacuoles and starch grains are not observed in any of the nodule tissue. Transverse sections of *Casuarina* nodule show the micro-symbiont arbitrarily distributed in the cortical cells. The infected cells in *Casuarina* do not form any particular zone but are present scattered throughout the cortex. This resembles with *Myrica* where the micro-symbiont is found scattered throughout the cortex (Silver, 1964). However, this is in contrast to the infected regions of other actinorhizae.

The internal structure of *Casuarina* nodules is essentially the same as that of other actinorhizal nodules. It resembles with *Myrica*-type nodules. From the anatomical studies of the *Casuarina* nodules it is worth mentioning that micro-symbiont *Frankia* was found occurring in the nodules only in hyphal phase. The micro-symbiont did not form defined zone but was arbitrarily distributed throughout the cortex. These features have not been reported in any of the actinorhizal nodules.

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## 木麻黃放射菌根瘤的構造與其組織化學的研究

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## 摘 要

木麻黃是一種引進的放射菌根瘤的植物，在巴基斯坦它是路旁的景觀植物，也是種植於森林中的植物。它的根部被放射菌 *Frankia* 感染後即形成根瘤，使用組織化學的方法觀察根瘤內部構造，發現此放射菌根瘤與楊梅型根瘤相似。根瘤皮層外圍有一層明顯的外皮，皮層內部可看到感染的細胞散佈於未感染細胞之間。內皮層含有單寧，內部有中柱。韌皮部—木質部呈環生維管束，其中有一難以辨識的分生組織。根瘤除了簡單型外，尚有分叉(分枝)型及珊瑚型。簡單型及分枝型的根瘤，其中柱可延伸到根瘤的頂尖部位。根瘤組織內的共生菌成菌絲狀，用組織化學分析，發現根瘤中沒有液泡及澱粉粒，但含有單寧及木質素。

關鍵詞：木麻黃，放射菌 *Frankia*，放射菌根瘤，菌絲。

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