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OBSERVATIONS ON THE FINE STRUCTURE OF THE ENDOPHYTE IN ROOT NODULES OF *ALNUS NITIDA* ENDL.

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Abstract

The ultrastructure of root nodules of the Asian alder, *Alnus nitida*, reveals that the developmental stages of the endophyte (*Frankia* sp.) inside the nodules resemble with other actinorhizae of *Alnus* spp., and with pure cultures of *Frankia* strains isolated from the same plant. The young stage of the endophyte shows hyphae which penetrate the cell wall of the cortical cells and establish in the centre of the cell as ramified mass. The ends of the hyphae near the periphery of the host cell are swollen to form spherical vesicles. The mature vesicles are septate, each compartment having its own nuclear material. Void areas are present around the vesicles. An upper layer and a basal loose layer of the vesicle cell wall have been found inside the void area. The plant nucleus is characterized by a double porous membrane at the periphery. Concentrations of mitochondria are present near the vesicles, suggesting a close association between the endophyte and the host. The possible function of the capsular material surrounding the vesicles for the protection of nitorgenase against oxygen is discussed.

A. nitida nodules, in contrast to pure cultures of *Frankia* isolates from the nodules, are spore-negative, suggesting that spore formation is affected by the host plant.

Introduction

Alnus nitida Endl., a small tree with a height of 20 m, is distributed in the Western Himalaya and Northern areas of Pakistan at elevation of 850-2550 m (Bhopal & Chaudhri 1977, Nasir, 1975). Nitrogen-fixing root nodules (also called actinorhizae) on this non leguminous plant have been found in all of the plants searched in these areas (Chaudhary *et al.*, 1981, Khan, 1971). The various aspects of the comparative morphology and physiology of *A. nitida* and *Datisca cannabina* have been investigated in previous reports (Hafeez *et al.*, 1984, 1984b). Recently we have described the isolation of two *Frankia* strains from root nodules of *A. nitida* (Hafeez *et al.* 1984a). Both of the isolates are infective and effective on *A. nitida* and *A. glutinosa* seedlings and have much similarities with other *Frankia* strains isolated from other actinorhizae. The ultrastructure of actinorhizae and its endophytes have well been described but largely have been restricted to relatively few plant species. The investigated species belong to *Alnus* (Lalonde 1979, Lalonde *et al.* 1976), *Ceanothus* (Strand & Gardner 1970; *Elaeagnus* (Henry 1979) *Comptonia* (Callaham *et al.*, 1979; Newcomb *et al.*, 1978) and *Datisca* spp. (Calvert *et al.* 1979; Akkermans *et al.*, 1984, Hafeez *et al.*, 1984, Hafeez *et al.*, 1984c). In most of these reports only one of the many species have been described. Since many genera of actinor

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hizal plants, particularly *Alnus, Myrica* and *Ceanothus* consist of more than one species, one has to be careful in drawing general conclusions.

In *Alnus* most structural studies are restricted to *A. glutinosa* (Becking *et al.*, 1964; Gardner, 1965; Gourret, 1975; Lalonde, 1979; Dijk & Merkus, 1976) and *A. crispa* (Lalonde & Knowles, 1975a; 1975b; Lalonde *et al.*, 1976; Lalonde & Quispel 1977). the alder species from Western Europe and North America. Since information is lacking about *Alnus* species which are native to Asia, a study of actinorhyzae of *A. nitida* from Pakistan has been made. In the present paper a general description of the ultrastructure of these nodules and its endophyte inside the nodules has been presented.

Materials and Methods

Alnus nitida seedlings were raised from seeds and inoculated with crushed nodules of *A. nitida,* collected from Swat, Pakistan. The plants were cultivated in liquid culture in a greenhouse at 20°C (Hafeez *et al.*, 1984a, b). Excised nodules were cut into small pieces and fixed in 3% glutaraldehyde in 0.05 *M* potassium phosphate buffer (pH 6.8) for 4 hours at 27°C. Fixed specimens were washed with phosphate buffer for overnight. and subsequently fixed in buffered OsO_4 for one hour After washing for 20 min in phosphate buffer, the fixed samples were passed through a dehydration series of ethanol in distilled water (10 to 100%; two times for 10 min each) followed by propylene oxide for 25 min. The samples were embedded in Epon 600 resin (a 1:1 mixture of propylene oxide and epon. for one hour; subsequently in a 1:4 mixture for 24 hours and finally in 100% epon for 4 days). The epoxy resin capsules were polymerized for 24 hours at 35°C, 48°C each. The blocks were cut at 500 A. Sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Philips EM 200).

Results

The ultra thin sections of *A. nitida* root nodule infected cortical cells reveal that the endophytic hyphae are located in the cell centre and the vesicles near the cell wall (Fig. 1). The hyphal form of the endophyte is highly septate and branched, diameter of the hyphae 0.5 – 1.0 μm (Figs. 1,2), hyphal septum continuous with the endophyte cell wall. The hyphal cytoplasm is rich in nuclear material and ribosomes. A nuclear membrane is lacking. The hyphae are surrounded by an electorn-dense capsule of variable thickness. A host membrane envelope separates the hyphal capsule from the host cytoplasm. The penetration of the hyphae in the cortical cells is through the host cell wall (Fig. 3). In the centre of the cortical cell, the hyphae form a thick mycelial mass. After intensive spreading in the living host cell, the ends of the hyphae become swollen to form vesicles near the host cell wall (Fig. 6). In young vesicles. the cytoplasm is continuous

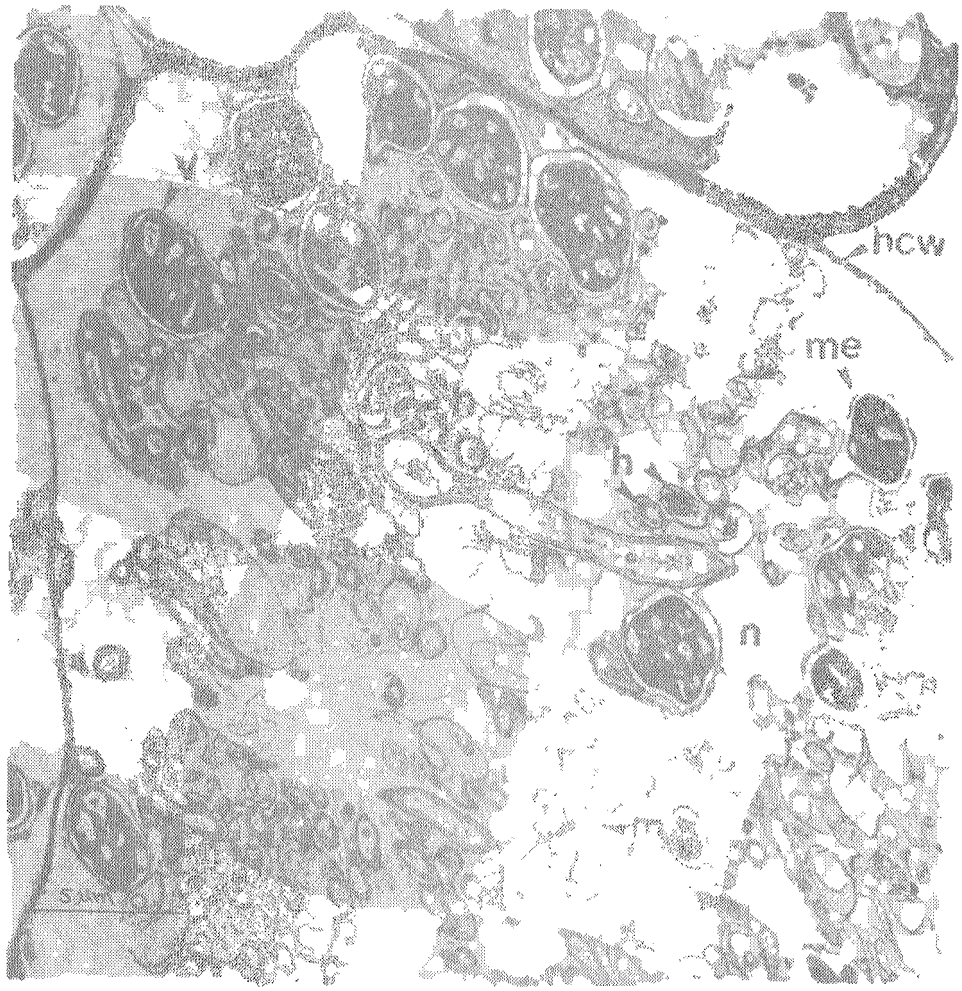

Fig. 1. TEM of root nodule of *Alnus nitida*, showing an infected cortical cell with the endophyte having branched and septate hyphae (h) in the centre and electron dense vesicles (v) near the host cell wall (hcw). Nucleus (n), mitochondria (m), host membrane envelope (me).

with the parental hyphal cytoplasm which contains a large amount of nuclear material. A septum between the parental hyphal and the young vesicle is formed. Finally the vesicle is compartmentalized by numerous septa (Figs. 3-6). The degree of compartmentalization of the endophyte cytoplasm by the formation of septa is a reflection of the vesicle maturity. The vesicle septum is a continuation of the endophyte cell wall and cytoplasmic membrane is seen on each side of the septum (Figs. 5,6). On a complete septum, side septa are added to produce further [illegible] compartmentalization of the vesicle. In each compartment of the vesicle the nuclear [illegible] and [illegible] ribosomes are distributed. Striated

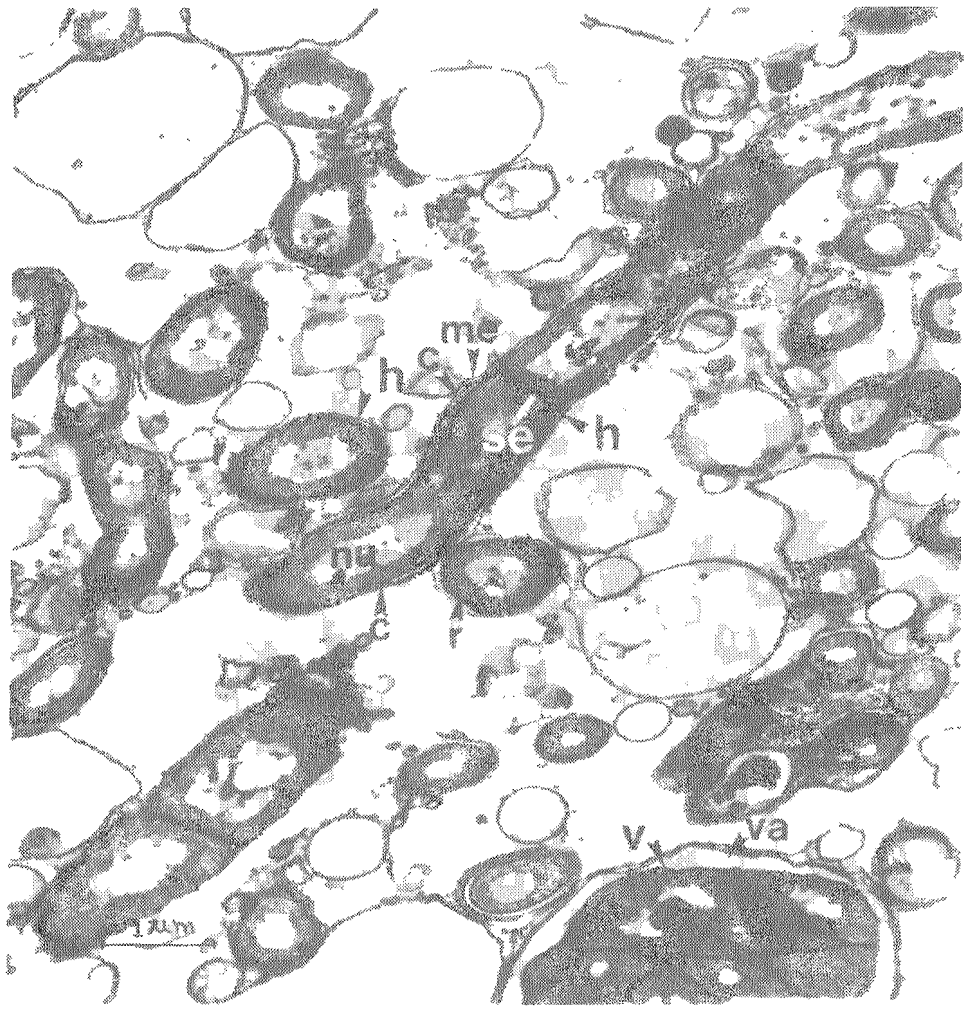

Fig 2. TEM of septate (se) hyphae (h) in the centre of the cortical cell. The endophyte is surrounded by electron dense capsular material (c) and a membrane envelope (me). Ribosomes (r) and nuclear material (nu) are visible in the hyphal cytoplasm. Septate vesicle (v), void area (va).

bodies and mesosomes are occasionally observed in the vesicles. Each vesicle is surrounded by a capsule and a host membrane envelope. The void area formed by the shrinkage of the vesicle cell wall from its encapsulation material is apparent. The upper loose layer and lower basal layer of the vesicle cell wall are seen in the void area (Fig. 5). The void area which surrounds the entire vesicle sometimes seems to extend along the parental hypha (Fig. 6). The nucleus in the host cortical cells is bounded by a porous nuclear membrane (Figs. 1,4). Groups of mitochondria are located in close vicinity of the vesicles (Figs 3,4,6).

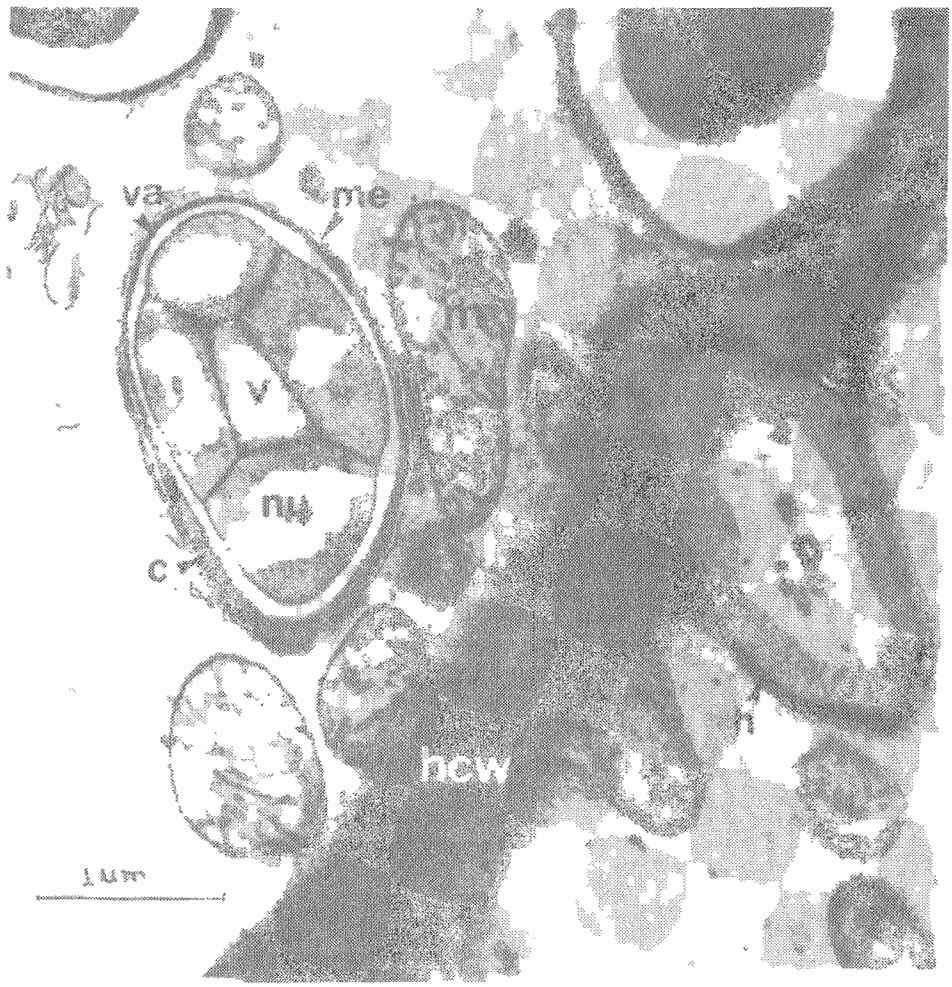

Fig. 3 TEM of *A. nitida* root nodule showing the penetration of the host cell wall (hcw) by hyphae (h) of the endophyte. Septate vesicles (v) with nuclear material (nu); mitochondria (m), electron dense capsule (c), membrane envelope (me) and void area (va).

The third stage of the endophyte i.e., sporangium formation, was not observed in the present study.

Discussion

The developmental stages of the endophyte within the root nodules are generally analogous to *Frankia* strains isolated from root nodules of *A. nitida* (Hafeez *et al.*, 1984a). The young form of the endophyte is hyphal and measured 0.5 – 1.0 µm in

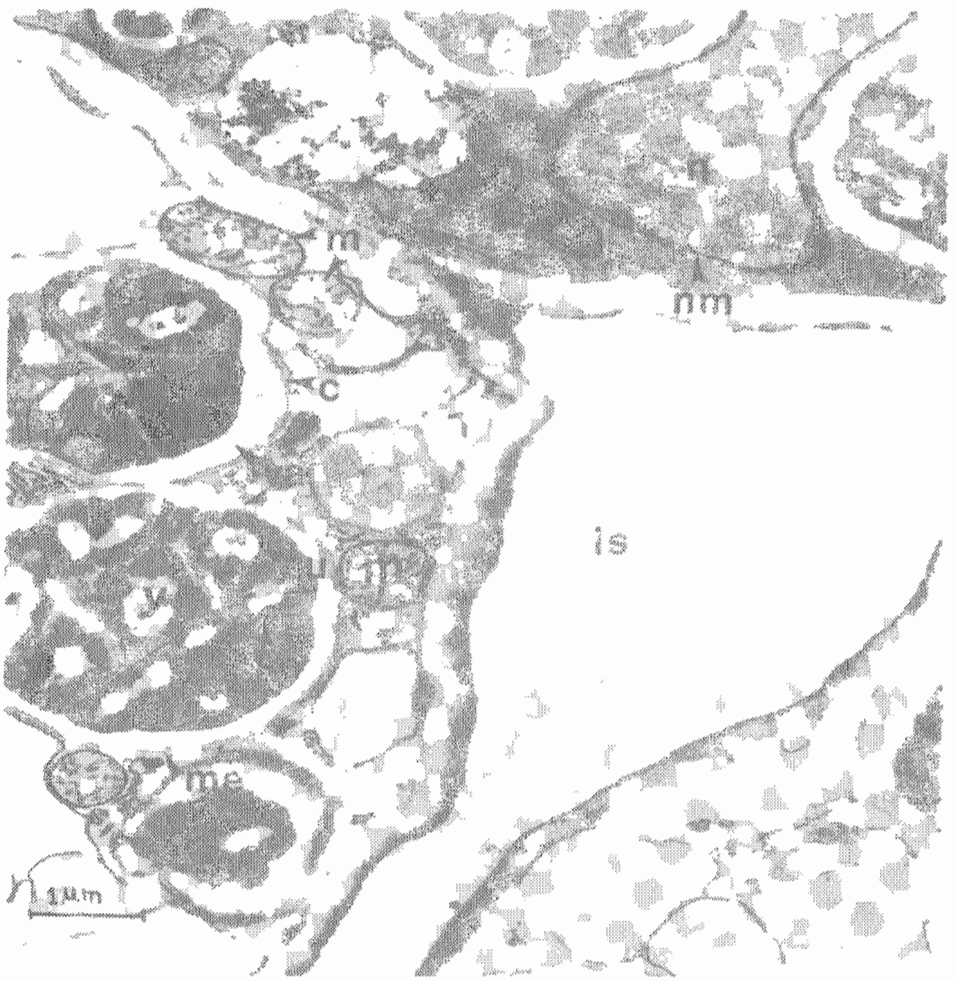

Fig. 4. TEM of three adjacent cortical cells with intercellular space (is). Cortical cells show a nucleus (n) surrounded by a porous nuclear membrane (nm). Note the mitochondria (m) in close connection with the vesicles (v). The vesicles are septate (se) and show nuclear material (nu) in each compartment. Void area (va), capsular material (c), host membrane envelope (me).

diameter. The hyphal form exhibits granular cytoplasm, branching, a non-membrane bound nuclear material and septa. The size and fine structure of the hyphae in *A. nitida* nodules are similar to the endophytes from other *Alnus* spp. (Becking *et al.*, 1964; Gardner, 1965; Lalonde & Knowles, 1975b; Lalonde & Quispel, 1977).

The cell to cell infection process occurs by penetration of hyphae across the host cell wall. The invasion of the host cell by the endophyte is a complex process. The phe-

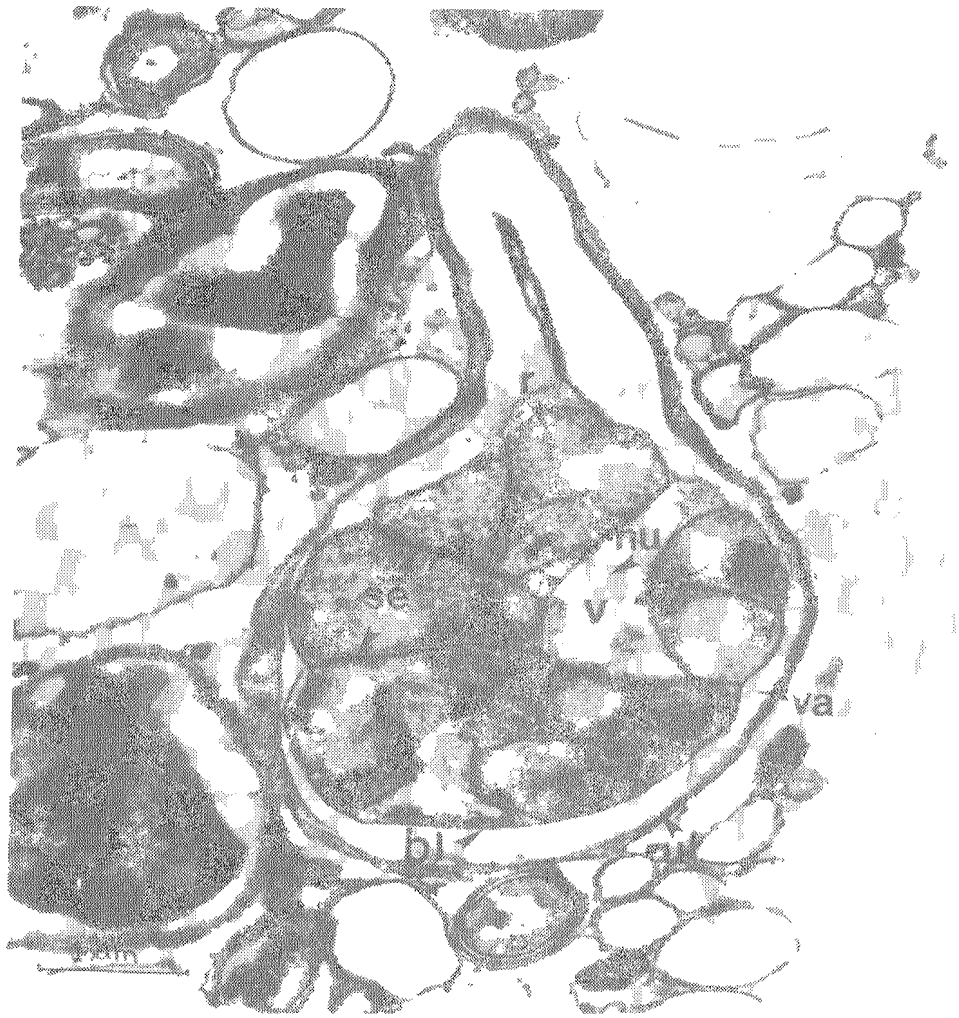

Fig. 5. TEM section of a mature vesicle. Note the upper loose layer (ul) of the vesicle cell wall in the void area (va). Septum (se), nuclear material (nu), ribosomes (r) and basal layer (bl) of the vesicle cell wall.

nomena has been noted by Gardner (1965) and Lalonde & Knowles (1975a). The hyphae branch from the central myceliar mass and form spherical vesicles near the periphery of the host cell. The endophyte is surrounded by a dense capsular material and a host plasma membrane. The capsular material around the endophyte may have arisen as an extension of the host cell wall material and is probably formed by the host in response to the invading microorganism (Benson & Eveleigh, 1979; Gardner, 1965; Lalonde & Knowles, 1975a). Since no polysaccharide capsule exists in cultured *Frankia* cells, the encap-

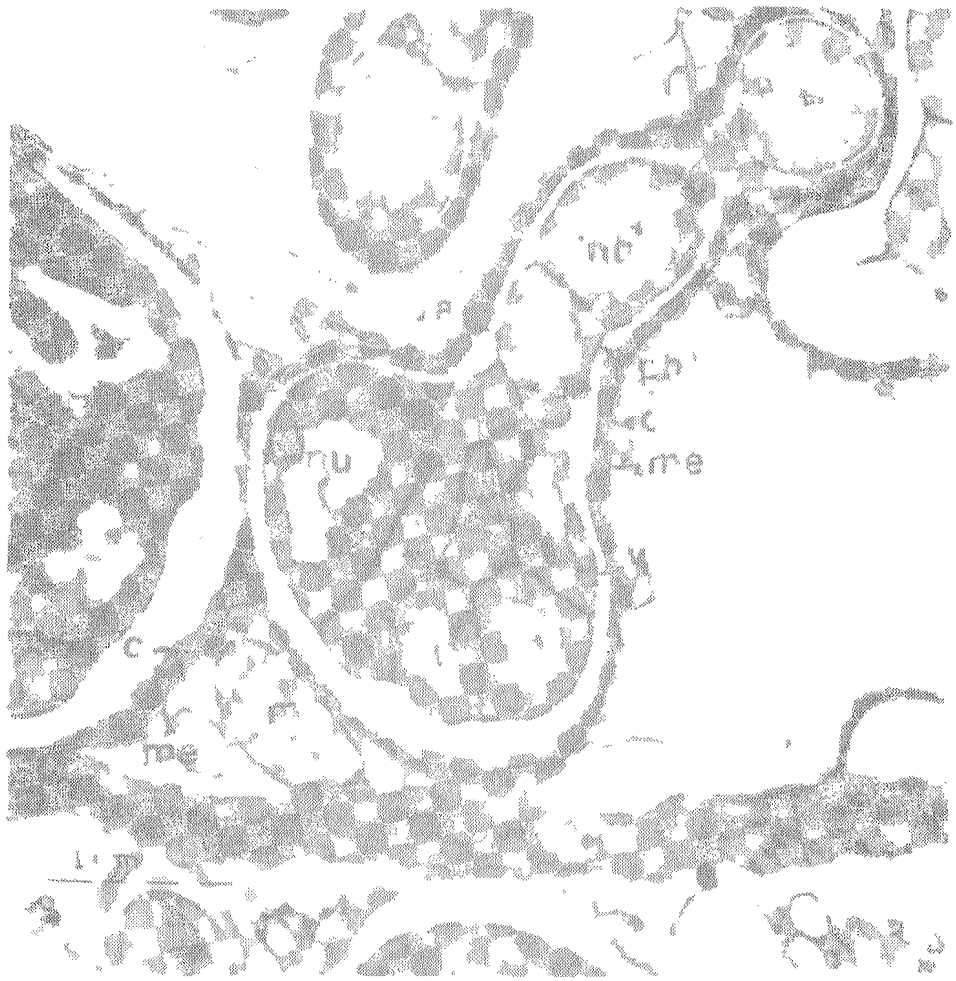

Fig. 6. TEM of [illegible] nodule of *[illegible]ria*. Adjacent to the host cell wall (hcw) are two septate vesicles (v) at the tip of a parental hyphae (ph). Note the parental septation (sp) and the continuity of the capsule (c) and membrane envelope (me) surrounding the parental hypha and its vesicle. Void area (va) surrounds the entire vesicle and extends along part of the parental hypha. Mitochondria (m), nuclear material (n).

sulation most probably is a product of the host cell and is only formed in the symbiotic state within the nodule (Lalonde & Knowles, 1975a).

A void area has been observed between the capsule and the endophytic cell wall in *A. gluinosa* (Becking *et al.*, 1969; Harper & Pankhurst, 1975; Lalonde & Quispel, 1977), *A. crispa* (Lalonde & Knowles, 1975a, 1977), and *A. mamnoides* (Gardner &

Gatner, 1973: Gatner & Gardner, 1970). Torrey & Callaham (1982) reported that the vesicles in the free-living *Frankia* spp. are surrounded by a multilaminar envelope which extends along the parental hyphae. They suggested that the void area around the vesicle is due to dissolution of the lipid laminae during transmission electorn microscopic methods (Lalonde *et al.*, 1976; Torrey & Callaham, 1982). Similar multilayered halo or envelope was also oberved around the vesicles of free-living cultures of *Frankia* spp., isolated from root nodules of *A. nitida* (Hafeez *et al.*, 1984a). No such void area has been observed around the visicles of the endophyte in root nodules of *Datisca cannabina* (Akkermans *et al.*, 1984; Hafeez *et al.*, 1984; Hafeez *et al.*, 1984c). Thus these void areas may not necessarily be an artifact of transmission electron microscopy. It is possible that the halo area around the vesicles in *Alnus*-type nodules other than *Datisca* and *Coriaria* spp. (Akkermans & Houwers, 1983; Hafeez *et al.*, 1984c), may be the part of the vesicle structure. Uptil now there is no successful cross inoculation reports on *Datisca* or *Coriaria* and *Alnus* spp., and they can be placed as two distinct and different groups in *Frankia*.

The presence of multilaminated layers around the vesicles in pure culture of *Frankia* have been compared with the inner most layer of heterocysts of *Anabaena* sp. (Torrey & Callaham, 1982). The envelope around the vesicle may protect the nitrogenase, which probably is present inside the vesicle, against oxygen. The absence of such structures in vesicles of *Datisca* and *Coriaria* spp., root nodules can be explained on the basis of their pattern of orientation within the host cell. The vesicles in *Datisca* and *Coriaria* spp., root nodules are directed towards the centre of the cortical cell and are compactly packed (Calvert *et al.*, 1979; Akkermans *et al.*, 1984; Hafeez *et al.*, 1984; Hafeez *et al.*, 1984c). The mitochonodria are present in between the hyphae and there may be low oxygen tension in the vicinity of the vesicles due to respiration of the host and the endophyte. Therefore it is likely that in *Datisca* and *Coriaria* no additional protection of nitrogenase against oxygen is needed (Hafeez *et al.*, 1984c). In *Alnus* root nodules, however, the visicles are loosely arranged near the periphery of the host cell and the mitochondria are in close connection with the vesicles. Thus the vesicles may need some kind of protection of the nitrogenase against oxygen.

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