

Angiosperm *Gymnostoma* trees produce root nodules colonized by arbuscular mycorrhizal fungi related to *Glomus*

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Summary

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Received: 25 May 2000
Accepted: 10 August 2000

- Structure and fungal composition is presented here for 'mycorrhizal' nodules of two angiosperms of the genus *Gymnostoma* (Casuarinaceae), *G. deplancheanum* and *G. nodiflorum*. These species are endemic to New Caledonia, where they grow on ultramafic soils. The mycorrhizal nodules, which are modified lateral roots invaded by an arbuscular mycorrhizal fungus, occur in addition to N₂-fixing nodules.
- Techniques included PCR amplification of extracted DNA, for species identification, and histological studies to compare the developmental pathway of *Gymnostoma* mycorrhizal nodules with that of actinorhizal nodules.
- The fungal DNA suggested that the strain belongs to the genus *Glomus* (Glomales). The endophytic mycelium also contained typical *Glomus* arbuscules and hyphal coils. Structurally, *Gymnostoma* mycorrhizal nodules are similar to those described in some Coniferales and in Caesalpinioideae trees of French Guyana.
- The mycorrhizal nodules of *G. deplancheanum* and *G. nodiflorum* contain a fungus belonging to the Glomales. The role of the nodules might be linked to the ecological situation of the host plants, which are pioneers in exposed and rocky habitats.

Key words: arbuscular mycorrhiza, Casuarinaceae, *Gymnostoma*, morphogenesis, mycorrhizal nodule.

© *New Phytologist* (2001) **149**: 115–125

Introduction

Small non-N₂ fixing protuberances often designated as nodules or mycorrhizal nodules have been known for more than one century to be formed on the roots of some Gymnosperms (Bond, 1963). Members of the Podocarpaceae, Araucariaceae and Phyllocladaceae families have been reported to form such nodules (McLuckie, 1923; Saxton, 1930; Schaede, 1943; Bayliss *et al.*, 1963; Bergersen & Costin, 1964; Morrison & English, 1967). The nodules are generally closely and regularly spaced along the root in 2–4 longitudinal rows. This alignment, together with their uniform size, distinguishes them from N₂-fixing root nodules of actinorhizal plants and legumes. Since most Podocarpaceae, Araucariaceae and Phyllocladaceae nodules contain a nonseptate filamentous fungus, they are called mycorrhizal nodules, even though the characterization and role of the fungus have not been clearly shown.

In Angiosperms, Capellano *et al.* (1987) and Sequerra *et al.* (1994), described so-called myconodules in *Alnus glutinosa* and *A. incana* where the endophyte, *Penicillium*, is a parasitic fungus that induces a nodule-like structure through root hair infection. The occurrence of polymorphic short roots harbouring endomycorrhizae and resembling myconodules has also been reported in some Caesalpinioideae trees (Béreau & Garbaye, 1994).

We report here that, in addition to N₂-fixing nodules, the *Gymnostoma* root systems may bear numerous small root protuberances designated as mycorrhizal nodules since they are colonized by a mycorrhizal fungus. A study on *G. deplancheanum* mycorrhizal nodules has previously been carried out by Huguénin (1969).

The Casuarinaceae family is a large group of perennial Angiosperms composed of four genera: *Allocasuarina*, *Casuarina*, *Gymnostoma* and *Ceuthostoma* (Diem & Dommergues, 1990).

These plants have the ability to thrive under a range of environmental stresses and on poor soils (National Research Council, 1984). This outstanding ability is due partly to their symbiosis with an actinomycete, *Frankia*, which induces the formation of N₂-fixing actinorhizal nodules, also known as actinorhizae. The presence of ecto- and endomycorrhizae has also been reported for a number of Casuarinaceae (Diem & Dommergues, 1990). Endomycorrhizal fungi, the Glomales (Zygomycetes) are obligate symbionts of 80% of all land plants (Perotto & Bonfante, 1997). In arbuscular endomycorrhiza (AM), the fungal mycelium penetrates and colonizes the cortex to produce typical intracellular structures such as coils and arbuscules. AM infection can affect the activity of the root meristem, inducing a slight alteration of the root system architecture, with increased branching reported for several species (Schellenbaum *et al.*, 1991; Hooker & Atkinson, 1996). Furthermore, AM infections may reinforce a dichotomous pattern of root development (Fitter, 1986).

This paper describes the anatomy and structure of both *Gymnostoma* N₂-fixing actinorhizal nodules and mycorrhizal nodules with emphasis on the latter structures. Two species of *Gymnostoma* were studied: *G. nodiflorum* and *G. deplancheanum*. Both species belong to the Casuarinaceae family and are endemic to New Caledonia. The fungus infecting mycorrhizal nodules of *Gymnostoma* was identified as a member of the Glomales. In addition, the structure of mycorrhizal nodules from *Gymnostoma* and Coniferales was compared. The mechanisms that may be involved in mycorrhizal nodule morphogenesis are discussed.

Materials and methods

Ecology of the studied plant species

G. deplancheanum is a gregarious species which occurs in shrub maquis and paraforest maquis formations (Jaffré, 1980). It is an important component of the ultramafic maquis vegetation found in the southern massif of New Caledonia at altitudes between 200 and 1000 m. *G. deplancheanum* is often associated with ferric soils characterized by low concentrations of N, P and K, toxic levels of Ni, Co and Cr, and an iron crust surface which contains >70% ferromagnetic oxides (Latham *et al.*, 1978).

G. nodiflorum occurs mainly on nonultramafic substrates, and is often encountered in riparian formations along the east coast of New Caledonia.

The climate is oceanic subtropical with air temperatures in the range of 20–30°C. Rainfall ranges from 1200 to 4000 mm yr⁻¹ occurring mostly during the wet season, from December to March (McCoy *et al.*, 1997).

Sampling of nodulated roots

The site selected for study and sampling was 'Les chutes de la Madeleine' (lat 22°14'S, long 166°51'E, elevation 250 m).

It was characterized by natural populations of *G. deplancheanum* usually associated with ferralitic ferritic soils. Mycorrhizal infections of *G. nodiflorum* seedlings were obtained by growing the seedlings in a nonsterile soil taken from that site and maintaining them under natural light and temperature in a shelter at Noumea I.R.D. (Institut de Recherche pour le Développement) Center.

In the shelter, mycorrhizal nodules did not form when seedlings were grown under sterile conditions but only in pots filled with vermiculite inoculated with a nonsterile soil taken from underneath a stand of mature trees. Roots exhibited intra- and intercellular mycelia only when they were 8-month-old and mycorrhizal nodules appeared a few months later. Histological studies were performed on samples collected in mature stands.

Microscopical analyses

N₂-fixing nodules and mycorrhizal nodules harvested in the field were fixed in 4% paraformaldehyde, 0.25 glutaraldehyde, 10% dimethyl sulfoxide (DMSO), 70 mM ethyleneglycol-bis (α-aminoethyl-ether)-N,N₄-tetraacetic acid (EGTA) and 100 mM phosphate buffer, pH 7.2, for 5 h and embedded in resin (Histo Technik 7100; Templemars, France). Two to 3 μm thick sections were cut with a microtome (Jun GRM 2055 Leica Microsystems, Wetzlar, Germany) and stained with 0.05% (w/v) toluidine blue. The fungus was localized using fluorescent wheat germ agglutinin, AlexaTM 488 conjugate 250 μg ml⁻¹ in PBS. Two filter sets were used: a UV filter set with a 340 to 380 nm excitation and a 425 nm barrier filter, and a blue filter set with a 515–560 nm excitation filter and a 590 nm barrier filter.

The confocal microscope used was a Bio-Rad 1024 CLSM system. The beam scanning system used a Nikon Optiphot II upright microscope and an Argon-Krypton ion laser (15 mW). Series of optical sections were collected and projected onto a single image plane in the laser sharp 1024 software and processing system.

After staining, sections were mounted in the staining reagent or in glycerine plus water (15%, v/v) and examined with a light microscope (model DMRB, Leica).

Analysis of the fungal endophyte Whole genomic DNA was extracted from mycorrhizal nodules of five different plants of *G. nodiflorum*. Each sample contained *c.* 20 mycorrhizal nodules. DNA isolation was carried out on carefully washed roots and mycorrhizal nodules using a Dneasy plant mini kit (Qiagen, Courtaboeuf, France). A fungal DNA fragment of the nuclear small ribosomal subunit (SSU) was amplified by polymerase chain reaction (PCR) using the universal eukaryotic primer NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3'; Simon *et al.* (1992)) and a general fungal primer AM1 (5'-GTTTCCCCTAAGGCGCCGAA-3'; Helgason *et al.* (1999)). Positive controls were DNA from a *Glomus* isolate (Glo2, ys2.4; gift from T. Helgason, University of York, UK).

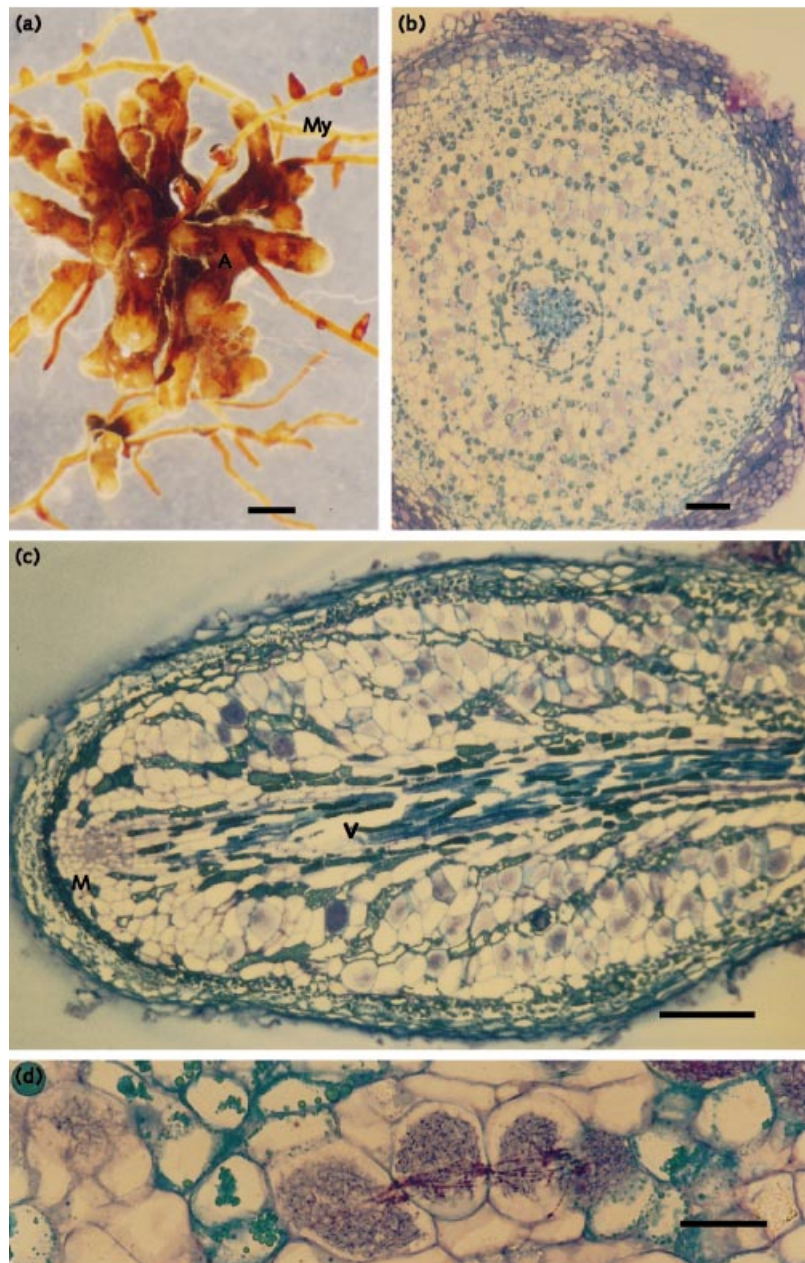


Fig. 1 Actinorhizal nodules (actinorrhizae) of *Gymnostoma nodiflorum*. (a) Root system bearing actinorhizal nodules (A) and mycorrhizal nodules (My) (bar, 0.1 cm). (b) Transversal section of actinorhizal nodule. Cortical purple cells infected by *Frankia* are localized between concentric files of cells accumulating phenolic compounds (green vesicles) (bar, 100 μ m). (c) Longitudinal section of a nodule lobe showing a central vascular bundle (V) and a meristem (M) at the apex. Purple cells are *Frankia*-infected cells (bar, 100 μ m). (d) Close-up of infected cells developing in nodule cortex (bar, 40 μ m).

and DNA extracted from leek roots colonized by *Glomus intraradices*. Negative controls were DNA extracted from noninfected roots of *Gymnostoma* seedlings and distilled water. Each 50 μ L reaction mixture contained 1.5 mM MgCl₂, 200 μ M each dATP, dCTP, dGTP and dTTP, 15 pmoles of each primers NS31 and AM1, 100 ng of DNA, 5 μ L of 10 \times concentrated reaction buffer and 0.5 U of Taq DNA polymerase (Promega, Charbonnière, France). Amplifications were performed with a DNA thermal cycler (PTC100, MJResearch, USA) programmed as follows: 1 cycle for 4 min at 95°C followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 1 min. One cycle for 15 min at 72°C was conducted afterwards. After amplification, 7 μ L of the PCR product

was used for cloning in the pGEMT vector following the manufacturer's recommendations (Promega). The insert was sequenced using the Applied Biosystems 373 A (Foster City, CA, USA) automatic sequencing system. The DNA sequence was analysed using the BLASTN 2.0.11 search tool (Altschul *et al.*, 1997) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

Results

Gymnostoma nodiflorum and *G. deplancheanum* usually bear N₂-fixing root nodules (actinorrhizae) and mycorrhizal nodules on the same root system (Fig. 1a).

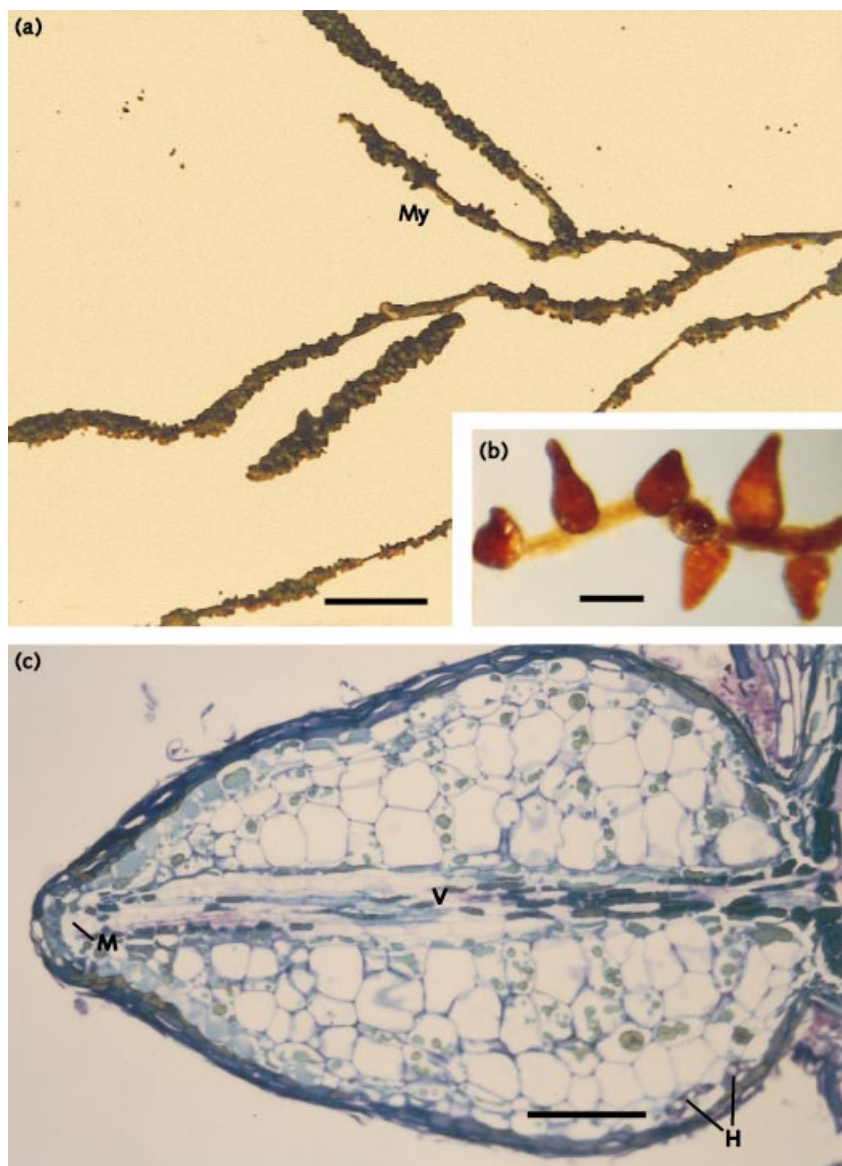


Fig. 2 Mycorrhizal nodules of *G. nodiflorum*. (a) General view of root system with mycorrhizal nodules (My) (bar, 500 μm). (b) Mycorrhizal nodules are arranged on three longitudinal rows (bar, 1 mm). (c) Longitudinal section showing a central vascular bundle (V) and a few meristematic cells at the apex (M). The swelling of the basal part of the mycorrhizal nodule is due to hypertrophy and cellular divisions of cortical cells. In this section the fungus is represented only by few hyphae (H) (bar, 100 μm). Toluidine blue staining.

N_2 -fixing root nodules of *Gymnostoma* spp.

Actinorhizal nodules of *G. nodiflorum* are composed of closely packed nodule lobes (Fig. 1a). Each nodule lobe arises as a lateral root from the pericycle and contains cortical cells infected by the endophyte *Frankia* (Torrey, 1976; Racette & Torrey, 1989). Lobes of 3–4-month-old N_2 -fixing root nodules are several mm wide.

Each nodule lobe consists of an outer epidermis and a cortex with an endodermis that surrounds the central vascular cylinder; the nodule meristem is situated at the distal end of the vascular cylinder (Fig. 1c). Cells infected with *Frankia* (Fig. 1b,c) are restricted to the cortex. As already reported in *Casuarina glauca* (Laplaze *et al.*, 1999), histological studies revealed a cell-specific accumulation of

phenolics causing a compartmentalization in the nodule cortex (Fig. 1b,c). On transversal sections cortical cells that accumulate phenolics are organized in concentric layers (Fig. 1b). *Frankia* grows acropetally inside layers of cortical cells limited by strands of cells containing phenolic compounds (Fig. 1d).

Mycorrhizal nodules

In both plant species, small (<500 μm long) and numerous spherical or pyriform mycorrhizal nodules were arranged in two or three close rows along the side of the roots (Figs 1a, 2a,b). On roots of *G. nodiflorum* (Fig. 2) as well as *G. deplancheanum*, the number of mycorrhizal nodules increases with age. A 300- μm wide root of *G. deplancheanum*

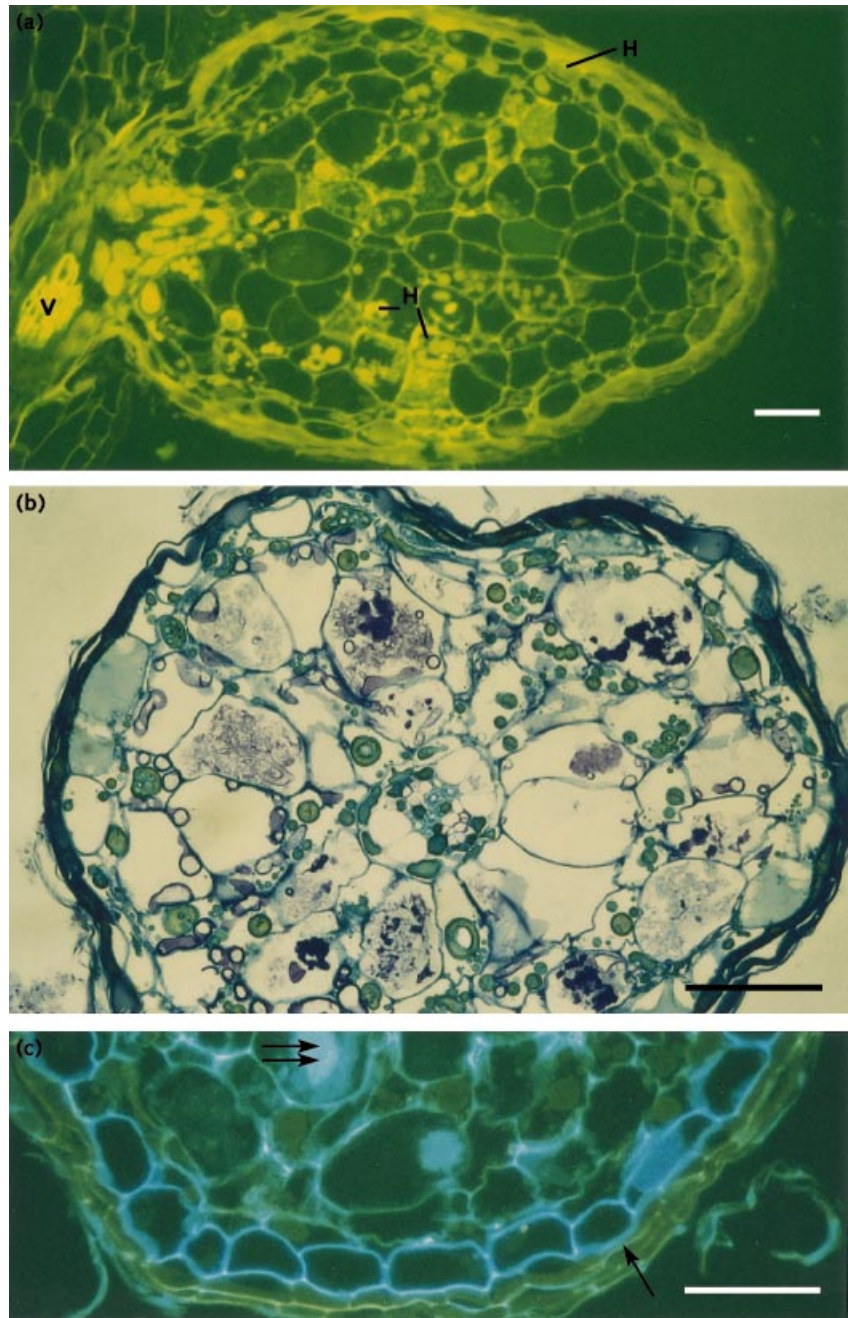


Fig. 3 Structure of mycorrhizal nodules in *G. nodiflorum*. (a) Longitudinal section of mycorrhizal nodule attached to the adjacent root. Intense staining of hyphae (H) in swollen basis and lignin from vascular bundle V; fluorescence view WGA-Alexa 488 (bar, 35 μm). (b) Transversal section of mycorrhizal nodule; toluidine blue staining. Extensive development of fungal hyphae in hypertrophied cortical cells. Various structures of fungi localized in cortical cells, some of them adjacent to endodermis (bar, 25 μm). (c) Transversal section of mycorrhizal nodule, autofluorescence view. Outer cortical cells of mycorrhizal nodule with dead cells (arrow) and exodermis (E). Hypertrophied cells with degenerate fungus (double arrows) (bar, 25 μm).

can bear up to 120 mycorrhizal nodules cm^{-1} . Young mycorrhizal nodules are white, whereas mature ones are brown and surrounded with tannins; the epidermis of mycorrhizal nodules never form root hairs. In *G. deplancheanum*, mycorrhizal nodules die within 3–4 months after their full development and become detached after secondary thickening of the root. Dead mycorrhizal nodules are so numerous that the surface of the soil under mature trees may be covered with a layer of mycorrhizal nodules (data not shown).

Histological studies of mycorrhizal nodules

Mycorrhizal nodule primordia are formed in the root pericycle in front of a xylem pole like lateral root primordia (data not shown). A vascular strand enters the mycorrhizal nodule and connects with the vascular tissue of the main root (Figs 2c, 3a). Mycorrhizal nodules may attain 300–450 μm in diameter. They represent radially symmetrical pointed lobes with a swollen base (Figs 2c, 5). Histological observations of longitudinal sections (Fig. 2c) showed that

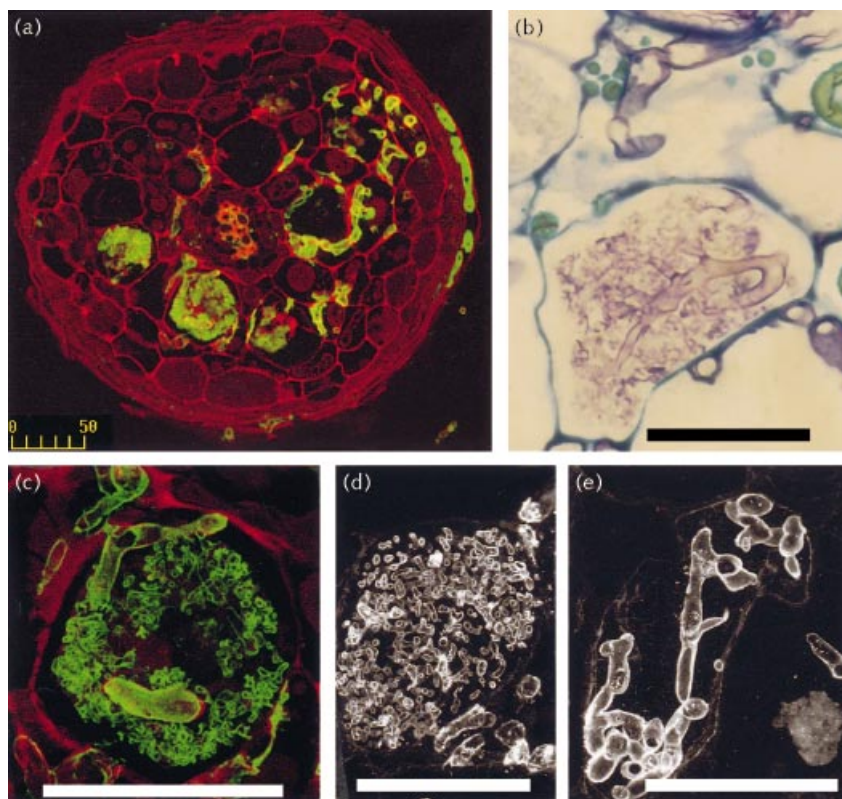


Fig. 4 Endomycorrhizal infection in *G. nodiflorum* mycorrhizal nodule. (a,b,c,d,e) laser confocal microscopy, WGA-Alexa 488 (bars, 50 μ m). (a) General view of a transversal section of a mycorrhizal nodule. Fungal hyphae (green colour) are localized in cortical cells of mycorrhizal nodule. At the periphery some hyphae between dead cells appear outside the exodermis. (b) Laser confocal microscopy of mycorrhizal nodule cortical cell colonized by fungus. (c) Intracellular arbuscule structure showing how the hyphal branches fill the cell volume. (d) Stage of arbuscule disintegration. (e) Early stage of peloton formation, and collapsed hyphal mass (arrow) in an adjacent cell.

the infected cortical cells enlarge considerably so that a swelling develops in the basal part of the mycorrhizal nodule. Furthermore, cortical cells proliferate during infection. The apical structure of the mycorrhizal nodule is that of a modified lateral root (i.e. there is no root cap and the meristematic area is reduced to a few cells near the tip (Fig. 2c)). The apical meristem of a mycorrhizal nodule is an arrested lateral root meristem which retains the potential to reactivate (data not shown), supposedly depending upon internal stimuli or inhibitors provided by the endophyte. In contrast to nodule roots that grow out of actinorhizal nodule lobes from other Casuarinaceae species (Torrey, 1976), these roots do not show agravitropic growth. Fluorescence microscopy of transversal sections shows two or three peripheral layers of dead cells containing heavy polyphenol deposits (Fig. 3c). Autofluorescence emission showed well differentiated xylem vessels with lignified cell walls, an endodermis and a complete exodermis (Figs 3c, 4a). Transversal sections of *G. nodiflorum* mycorrhizal nodules (Figs 3b, 4a) showed a small central cylinder and an expanded cortical parenchyma.

In mature mycorrhizal nodules, hyphae colonize the cortical tissue of the swollen base of the protuberance where cells show both cell divisions and hypertrophy (Fig. 2c). With the exception of some cells filled with crystals of calcium oxalate

(Fig. 5), most cortical cells of the mycorrhizal nodules, including the cortical cells adjacent to the endodermis, become infected by the fungus (Fig. 3b). The fungus is never observed in the vascular tissues nor in the mycorrhizal nodule meristem.

After formation of the first mycorrhizal nodules, 9–10 months after inoculation under our experimental conditions, formation of mycorrhizal nodules proceeds while fungal mycelium is also present inter- and intracellularly in the cortex of the adjacent root (Fig. 3a) and on the root surface (Fig. 5). The presence of the fungus within the roots where mycorrhizal nodule formation is induced, and the presence of a continuous exodermis in mycorrhizal nodules suggest that infection may occur from mycelium present in adjacent cells of the young root cortex. A sequential study of the process of mycorrhizal nodule formation would be necessary to clarify this process.

Fungal structures in mycorrhizal nodules

The fungus colonizes the mycorrhizal nodule cortex as intercellular hyphae. Each infection unit develops longitudinally and to some extent radially in the cortex of the root. Hence the oldest arbuscules are located in the swollen basal part of the mycorrhizal nodules, close to the adjacent

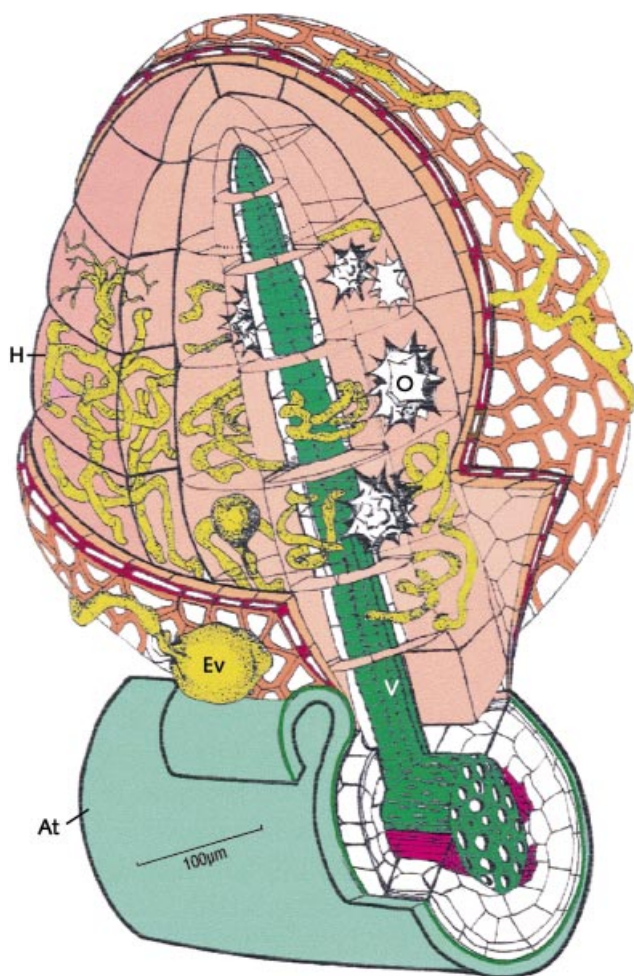


Fig. 5 Diagram illustrating anatomy and morphology of *G. deplancheanum* mycorrhizal nodule (Redrawn from Huguenin, 1969). At, adjacent root; Ev, extra-radical hyphae with vesicle; H, fungal hyphae; O, crystals of calcium oxalate; V, vascular bundle.

root, whereas immature arbuscules are found in the distal part of mycorrhizal nodules. The hyphal cell walls were labelled intensely by wheat germ agglutinin (Fig. 4a,b). This lectin has a strong affinity for oligomers and polymers of *N*-acetoglucosamine residues, especially chitin (Peters & Latka, 1986).

Short side-branches of the fungus penetrate the cortical cells and branch dichotomously to produce characteristic arbuscules with extensive ramifications (Fig. 4b,c). Finally, a large part of the volume of the host cell is occupied by fungal branches. As in mycorrhiza of other host plants, structural changes occur during arbuscule development as shown by variations in arbuscule morphology and patterns (Fig. 4b–e). Some intercellular hyphae located between peripheric cells outside the exodermis were observed by laser confocal microscopy (Fig. 4a). All these structures are easily recognizable

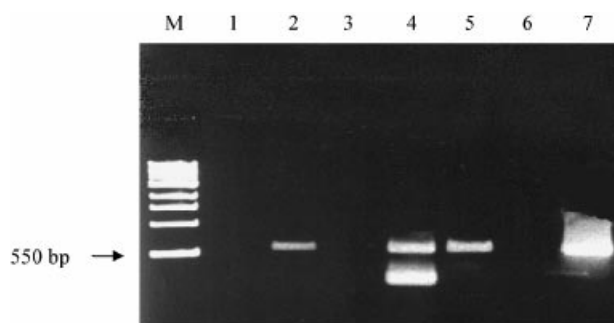


Fig. 6 Agarose gel-electrophoresis analysis of PCR products of the small subunit (SSU) rRNA fragment (size 550 bp) amplified with the universal NS31-AM1 fungal primers. PCR products were obtained from genomic DNA of *Gymnostoma nodiflorum* mycorrhizal nodules (lanes 2 and 5), and *Glomus* small subunit rRNA fragment as positive control (lane 7), leek roots infected with *G. intraradices* (lane 4), noninfected roots as seedlings (lane 3) and as mature plant (lane 6), control with water (lane 1). Lane M contains a size marker (1-kb ladder, Biolabs).

and clearly reminiscent of infection with an AM fungus (Smith & Read, 1997).

PCR amplification of fungal DNA extracted from mycorrhizal nodule

The general fungal primer AM1 and the universal eukaryotic primer NS31 were used to amplify a fungal-specific fragment from DNA extracted from mycorrhizal nodules. A PCR product of *c.* 550 bp was amplified from DNA of *G. nodiflorum* mycorrhizal nodules as well as in the positive controls (PCR products from *Glomus* DNA and DNA from *G. intraradices*-colonized roots; Fig. 6). Sequence analyses of the *G. nodiflorum* PCR product showed 93–99% identity with the corresponding sequences from several *Glomus* sp. isolates small subunit ribosomal RNA gene (Fig. 7), suggesting that the mycorrhizal nodule-colonizing fungus belongs to the genus *Glomus* in the family Glomales.

Discussion

Our observations suggest that *G. nodiflorum* and *G. deplancheanum* can develop mycorrhizal nodules, probably in response to colonization of plant roots by an endomycorrhizal fungus belonging to Glomales. Mycorrhizal nodules have also been found on root systems of *G. chamaecyparis*, *G. poissonianum*, and *G. leucodon* (Huguenin, 1969). All these species, which are particularly representative of New Caledonia, are trees or tall shrubs that grow on soils derived from ultramafic rocks enriched with peridotites. Since mycorrhizal nodules were also found in Caesalpinioideae species in French Guyana (Béreau & Garbaye, 1994) and in some Coniferales

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1  A G C A G C C G C G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A A g1o2
1  A G C A G C C G C G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A A myc

41  G T T G T T G C A G T T A A A A A G C T C G T A G T T G A A T T T C G G A A T T g1o2
41  G T T G T T G C A G T T A A A A A G C T C G T A G T T G A A T T T C G G A A T T myc

81  A G T A C G T T G G T C G T G C C T T A G G T A C G T A C T G G T G T T A C T G g1o2
81  A G T A C G T T G G T C G T G C C T T A G G T A C G T A C T G G T G T T A C T G myc

121  G T T T C T A C C T T C T G A C G A A C C A T C A T G T C A T T A A T T T G G T g1o2
121  G T T C T A C T T T C T G A C G A A C C A T C A T G T C A T T A A T T T G G T myc

161  G T G G C G G G G A A T C A G G A C T G T T A C T T T G A A A A A A T T A G A G g1o2
161  G T G G C G G G G A A T C A G G A C T G T T A C T T T G A A A A A A T T A G A G myc

201  T G T T T A A A G C A G G C T C G C G C T T G A A T A C A T T A G C A T G G A A g1o2
201  T G T T T A A A G C A G G C T C G C G C T T G A A T A C A T T A G C A T G G A A myc

241  T A A T G A A A T A G G A C G T T G A C C C T A T T T T G T T G G T T T C T A G g1o2
241  T A A T G A A A T A G G A C G T T G A C C C T A T T T T G T T G G T T T C T A G myc

281  G A T T G A C G T A A T G A T T A A T A G G G A T A G T T G G G G C A T T A G g1o2
281  G A T T G A C G T A A T G A T T A A T A G G G A T A G T T G G G G C A T T A G myc

321  T A T T C A A T T G T C A G A G G T G A A A T T C T T G G A T T T A T T G A A G g1o2
321  T A T T C A A T T G T C A G A G G T G A A A T T C T T G G A T T T A T T G A A G myc

361  A C T A A C T A C T G C G A A A G C A T T T G C C A A G G A T G T T T T C A T T g1o2
361  A C T A A C T A C T G C G A A A G C A T T T G C C A A G G A T G T T T T C A T T myc

401  A A T C A A G A A C G A A A G T T A G G G G A T C G A A G A C G A T C A G A T A g1o2
401  A A T C A A G A A C G A A A G T T A G G G G A T C G A A G A C G A T C A G A T A myc

441  C C G T C G T A G T C T T A A C C A T A A A C T A T G C C G A C T A G G G A T C g1o2
441  C C G T C G T A G T C T T A A C C A T A A A C T A T G C C G A C T A G G G A T C myc

481  G G A T G A T G T T A T T T T T A A T G A C T C A g1o2
481  G G A T G A T G T T A T T T T T A A T G A C T C A myc

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Decoration 'Decoration #1': Box residues that differ from g1o2.

Fig. 7 Alignment of partial sequences of small subunit ribosomal RNA gene (SSU) amplified by primers AM1-NS31 for *Glomus* sp. Glo2 isolate Bd4.7 (glo2) and *G. nodiflorum* mycorrhizal nodules (myc) DNA. The nucleotides that differ from glo2 sequence are indicated in a box.

families, the occurrence of such organs might be much more common than previously expected in tropical ecosystems.

It is difficult to identify AM fungi *in planta* as they possess few informative characters. Moreover, the taxonomy of AM endosymbiont is hampered by the inability to cultivate the fungus. However, Podocarpaceae mycorrhizal nodules are considered to be infected by an endophytic mycelium belonging to a member of the genus *Endogone* (Morrison & English, 1967). Also, six different species of AM could be identified based on spore morphology in *Araucaria angustifolia* (Breuninger *et al.*, 2000). The identification of fungus belonging to Glomales appears to be well established in the present molecular study. Arbuscules, the physiological exchange structure of AM, were observed in *Gymnostoma* mycorrhizal nodules, stressing the endomycorrhizal status of this plant.

Comparison of mycorrhizal nodules with actinorhizal and Parasponia nodules

In Podocarpaceae, Araucariaceae, Phyllocladaceae and Casuarinaceae, mycorrhizal nodules are modified short lateral roots (< 1 mm long) without root caps where the central stele is connected with the vascular system of the parent root. The cortex is colonized by fungi but no infection

occurs in the endodermis. The cortex periphery is surrounded by a periderm consisting of several layers of compressed dead cells. Mycorrhizal nodules have determinate development, with the already mentioned exception of *G. nodiflorum*, where the apical meristem can be reactivated giving rise to an elongated root. The morphology of these mycorrhizal nodules is remarkably similar to that of nodule lobes in actinorhizae (Franché *et al.*, 1998). The actinorhizal nodule structure can also be observed in N₂-fixing root nodules of *Parasponia*, the only nonlegume that forms nodules with *Bradyrhizobium* (Trinick & Hadobas, 1988). Both mycorrhizal nodules and N₂-fixing actinorhizal nodules are lateral roots whose developmental pattern has been modified by the endosymbiont.

Comparison with proteoid roots

Like proteoid roots, mycorrhizal nodules form clusters along the root and have determinate development. In proteoid roots, the number of rows of rootlets in a cluster depends on the structure of the root vascular system, since a row develops from each xylem pole (Watt & Evans, 1999; Diem *et al.*, 2000). However, contrary to proteoid roots, mycorrhizal nodules belonging to a cluster are generally all at the same

stage of development suggesting that they developed simultaneously from the root. While all species with proteoid roots can grow in soils with poorly available nutrients, most of them do not form mycorrhizal symbioses (Skene, 1998). Typical proteoid roots chemically modify the surrounding soil by exuding compounds (carboxylic acids, acid phosphatases, etc.) to facilitate the mobilization of mineral nutrients from soils.

Role of mycorrhizal nodules: comparison with typical AM mycorrhiza

All mycorrhizal nodule-bearing species in this study represent stabilizing and pioneering species of considerable ecological significance in exposed rocky habitats. However, reports on the selective advantage conferred by mycorrhizal nodules found in Podocarpaceae and Araucariaceae are contradictory. Morrison & English (1967) showed that AM infection stimulated phosphate uptake by mycorrhizal nodules, whereas Bergersen & Costin (1964), Becking (1966), and Furman (1970) found that mycorrhizal nodules were able to fix small but significant quantities of atmospheric nitrogen which, however, would require a prokaryotic symbiont. In fact, the actual function of mycorrhizal nodules is still unknown.

Unlike typical endomycorrhizal infections, histological analysis supports the hypothesis that mycorrhizal nodules of *Gymnostoma* species are initially invaded by intraradical hyphae of an AM fungus. Mycorrhizal nodules form an exodermis, a barrier that might prevent fungal infection from outside. The cortex of adjacent roots of *Gymnostoma* mycorrhizal nodules contains hyphae displaying a structure similar to that found in actinorhizal nodules (data not shown). This suggests that the hyphae associated with roots are likely to be involved in the initial stages of infection.

Moreover, mycorrhizal nodule development has been described in sterile conditions in absence of the endophyte (Baylis *et al.*, 1963; Becking, 1966; Bond, 1967; Khan, 1967) suggesting that mycorrhizal nodule organogenesis is controlled by the host-plant. In legumes, root nodulation has also been described in absence of rhizobia (Truchet *et al.*, 1989). Our histological observations suggest that development takes place in three steps. First, cell divisions are triggered in numerous places in the pericycle that give rise to lateral root primordia. Second, these primordia are prompted to elongate resulting in the formation of root protuberances. Third, hyphae of AM fungi progress from adjacent roots to the apex of the rootlet. All these processes differ markedly from typical AM associations.

In AM associations, the fungus not only colonize plant cells but also develops a network of external hyphae which absorbs and translocates phosphate and other mineral nutrients from soil. In *G. nodiflorum* mycorrhizal nodules, extra-radical hyphae are absent since the homomorphic exodermis with suberin lamellae deposition constitutes a wall layer that would prevent penetration by the mycorrhizal fungus, in

contrast to the dimorphic exodermis of several mycorrhizal roots, where short cells lacking suberin resemble 'passage' cells (Bonfante-Fasolo & Vian, 1989; Matsubara *et al.*, 1999). Therefore, in mycorrhizal nodules, nutrients from the soil would not be able to reach the arbuscules. In AM, arbuscules are believed to be the highly differentiated hyphae of the fungus and the key site of interchange between root cells and fungi (Blee & Anderson, 1998). In *G. deplancheanum* as in AM roots, arbuscules are short-lived and subsequently collapse. After a few days arbuscules progressively degenerate to form clumps whilst the infected plant cells die, unlike typical AM, where cortical cells of the root remain alive when arbuscules degenerate. During the secondary thickening of the root, the dead mycorrhizal nodules detach and are deposited in the uppermost layer of the soil litter. Thus the period of activity of mycorrhizal nodules only lasts a few months. Subsequent degeneration of the fungus is accompanied by thickening of the cell walls and deposition of phenolics throughout the mycorrhizal nodule. The short-term association raises the question of the nature of symbiosis in mycorrhizal nodules.

Taken together, structural observations of *Gymnostoma* mycorrhizal nodules show that these organs differ markedly from both mycorrhizae or proteoid roots. Mycorrhizal nodules may, thus, be considered as an original structure that occurs in some Gymnosperm and Angiosperm species.

Evolutionary considerations

Phyllocladaceae, Podocarpaceae and Araucariaceae form a highly supported clade within Coniferales (Chaw *et al.*, 1997). All these families comprise very old genera that existed mostly in the Southern hemisphere at the very beginnings of Gondwanaland, even before Africa and India broke off and went their own way. Fossil roots with mycorrhizal nodules have been described within the lower cretaceous Otway Group (Australia) (Cantrill & Douglas, 1988). The most parsimonious explanation for the existence of mycorrhizal nodules in most species belonging to these three families would be the unique event of mycorrhizal nodule acquisition in Gymnosperms through a common ancestor of the three families. Since mycorrhizal nodules have now been found in Angiosperms including *Gymnostoma* and number of Caesalpinioideae, the hypothesis of a unique event of acquisition of the ability to form mycorrhizal nodules in Coniferales is unlikely. Another scenario would be that the ability to enter mycorrhizal nodule symbioses goes back to a common ancestor to Angiosperm and Gymnosperm and that most plants lost their ability to form mycorrhizal nodules.

Is the development of the different lateral root structures (actinorhizae, mycorrhizae, mycorrhizal nodules) governed by the same set of genes? This hypothesis could be tested by the study of the characterization and expression of genes involved in the development of actinorhizae and

mycorrhizal nodule in Casuarinaceae. The present study showed that within the Casuarinaceae family, the same genus *Gymnostoma* can be infected by both an actinomycete and by a member of the Glomale. The same modified lateral root structure can be induced by a variety of microorganisms like *Frankia* in actinorhizal plants, *Penicillium* in *Alnus*, Glomales in *Gymnostoma*, Podocarps and Araucarias, and also *Bradyrhizobium* in *Parasponia*. Whatever infection process is involved and whatever microorganism is present, mycorrhizal nodules and actinorhizal-type nodules share the same cellular morphogenetic events. A likely scenario is, thus, that *Frankia* in the rhizosphere of AM roots of *Gymnostoma* were progressively associated more tightly with roots, leading to the actinorhizal nodules. The plant predisposition to form nodules was used to direct a N₂-fixing nodule.

Molecular and genetic studies have shown that several common steps are involved in legumes that establish endo-mycorrhizal and rhizobial associations (Duc *et al.*, 1989; Gianinazzi-Pearson, 1996; Van Rhijn *et al.*, 1997). It is interesting that mycorrhizal nodules occur on root of Caesalpinieae species that also nodulate with rhizobia (Béreau & Garbaye, 1994). This raises the question of whether common host genes are involved in mycorrhizal, rhizobia and *Frankia* nodules formation.

In this article, structural details of *Gymnostoma* mycorrhizal nodules have been described. Whether and how mycorrhizal nodule morphogenesis is induced by the fungus or by soil mineral conditions is not yet established. Further research is needed to clarify this point. Because mycorrhizal nodule formation as well as N₂-fixing root nodules are developmentally regulated on the same root system, these structures provide a good experimental opportunity to study cellular events using molecular and cellular approaches.

Acknowledgements

We are grateful to T. Frutz for technical assistance and to Dr F. Guinel and Dr T. Helgason for the gifts of *Glomus intraradices* isolates, and Glo2 DNA, respectively. We thank Dr B. Vian, Dr K. Pawlowski, Dr M. A. Sélosse and, Dr L. Laplaze for critical reading of the manuscript.

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