Root nodule initiation in *Gymnostoma* (Casuarinaceae) and *Shepherdia* (Elaeagnaceae) induced by *Frankia* strain HFPGpI1

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Seedlings of *Gymnostoma papuanum* (Casuarinaceae) and *Shepherdia argentea* (Elaeagnaceae) were grown in water culture and inoculated with *Frankia* strain HFPGpI1. Root nodule initiation and early nodule development were examined using light microscopy. *Gymnostoma papuanum* was infected by penetration of *Frankia* into deformed root hairs, followed by development of a prenodule region and one to several nodule lobe primordia in the root cortex. *Frankia* hyphae grew directly through cell walls from cell to cell, colonizing cells of the prenodule prior to invading nodule lobe cells. *Shepherdia argentea* roots were infected by *Frankia* via intercellular penetration of the root epidermis and cortex with direct infection of cells of the nodule lobe primordia. No prenodule region was formed. Thus far, the mode of infection appears to be characteristic for each of the plant families. Subsequent to nodule initiation, plants were assayed at 4-week intervals (up to 12 or 16 weeks) for acetylene reduction activity. Low and variable activity was observed. The presence of symbiotic vesicles in *G. papuanum* nodules is reported as the first instance of vesicles seen in nodules taken from a member of the family Casuarinaceae.

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Des semis du *Gymnostoma papuanum* (Casuarinaceae) et du *Shepherdia argentea* (Elaeagnaceae) ont été cultivés en culture hydroponique et inoculés avec la souche HFPCpI1 de *Frankia*. L'initiation des nodosités radiculaires et les premiers stades de leur développement ont été suivis au microscope photonique. L'infection du *G. papuanum* s'est faite par la pénétration du *Frankia* dans les poils absorbants déformés et le développement subséquent d'une région prénodulaire et de un à plusieurs primordiums de lobules nodulaires dans le cortex de la racine. Les hyphes de *Frankia* passent directement de cellule à cellule à travers les parois, colonisant les cellules des prénodosités avant d'envahir les cellules des lobules nodulaires. L'infection des racines du *S. argentea* par le *Frankia* s'est faite via la pénétration intercellulaire de l'épiderme et du cortex de la racine accompagnée de l'infection directe des cellules des primordiums des lobules nodulaires. Il n'y a pas eu de formation de région prénodulaire. Jusqu'à présent, le mode d'infection semble être particulier à chacune des familles de plantes. À la suite de l'initiation des nodosités, les plantes ont été testées pour l'activité de réduction d'acétylène à intervalles de 4 semaines (pour une durée de 12 ou 16 semaines). Une activité faible et variable a été notée. La présence de vésicules symbiotiques chez les nodules du *G. papuanum* est signalée comme une première mention de vésicules vues dans les nodosités provenant d'un membre de la famille des Casuarinacées.

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Introduction

Effective nodulation of actinorhizal plants by *Frankia* requires that numerous steps be completed successfully. These steps include initial plant and bacteria recognition, invasion of the root by *Frankia* hyphae, development of nodule lobe primordia, colonization of nodule cortical cells, development of oxygen protection mechanisms, and fixation of dinitrogen by nitrogenase. Although little is known about some steps, others have been studied in great detail. Early root infection by *Frankia* has been examined in some detail for several species. Evidence that initial penetration occurs through root-hair infection, in association with root-hair deformation, was first presented for *Alnus glutinosa* (L.)Gaert. in the 1950s (cf. Callaham et al. 1979). Callaham and Torrey (1977) showed root-hair infection in the nodulation of *Comptonia peregrina* (L.)Coult., and it was also confirmed for two species of *Myrica* and one species of *Casuarina* (Callaham et al. 1979).

Evidence presented up until the mid-1980s indicated that *Frankia* normally infected plants via entry of deformed root hairs. Then Miller and Baker (1985) demonstrated that *Frankia* infects *Elaeagnus angustifolia* L. via intercellular penetration of the epidermis. They also showed that certain *Frankia* isolates are capable of infecting different actinorhizal species by different methods (Miller and Baker 1986). It has never been observed that a single plant species can be infected by both routes, indicating that the mode of infection is probably determined by the plant.

The mechanism of infection has actually been described for only a few plant species. Root-hair penetration has been demonstrated in four genera, with two of these genera (*Myrica* and *Comptonia*) being from the same family, the Myricaceae. Intercellular penetration has been demonstrated in only a single genus, *Elaeagnus*.

Many actinorhizal genera have not been examined with regard to their mode of infection. The two species studied in this paper, *Gymnostoma papuanum* (S. Moore) L. Johnson and *Shepherdia argentea* Nutt., represent two such genera. As a member of the family Casuarinaceae, *G. papuanum* is a close relative of several tropical trees with worldwide importance. *Gymnostoma papuanum* itself is an important fuel-wood tree in its native country of Papua New Guinea. *Shepherdia argentea* is a temperate tree, native to North America, and is a member of the family Elaeagnaceae. Based on light microscopy, the following account describes the contrasting routes of infection and nodule development for these plants following inoculation with a single, pure, cultured strain of *Frankia*.

Materials and methods

Seeds of *S. argentea* were collected at the Arnold Arboretum of

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Harvard University and subsequently stored at room temperature. Germination required a 24-h presoak in 500 ppm gibberellic acid (Sigma Chemical Co., St. Louis, MO) prior to sowing. Seeds were sown in flats of washed sand with top and bottom layers of vermiculite and placed in a growth chamber with 16 h light : 8 h dark at 26:19°C (light:dark). Seedlings were transferred to water culture 8 weeks after sowing.

Seeds of *G. papuanum* were collected by the Department of Forests at Papua New Guinea (Frangipani Street, P.O. Box 5055, Boroko, Papua New Guinea) and stored in a refrigerator after being received at Harvard Forest. No pretreatment was required for gemination. Seeds were sown as described above and germinated in a growth chamber with 16 h light : 8 h dark at 33:28°C (light:dark). Root growth of *G. papuanum* was slower than for *S. argentea*, so transfer to water culture was delayed until 12 weeks after sowing.

Seedlings were transferred from flats to foil-wrapped glass jars (three per jar) containing ¼-strength Hoagland's nutrient solution lacking nitrogen (Hoagland and Arnon 1950). Solution pH was adjusted to 6.0 for *S. argentea* and 7.0 for *G. papuanum*. The jars were then placed in a temperate greenhouse (approximately 24:18°C, light:dark) for observations on the mode of infection, or in the appropriate growth chamber (same conditions as for seed germination) for observation of nodule development.

Plants were inoculated with inoculum consisting of *Frankia* isolate HFPGpI1, catalogue No. HFP021801 (cf. Racette and Torrey 1989), one to several days after transfer to water culture, grown in modified BAP medium (Murry et al. 1984) for 3–4 weeks, washed twice with distilled water, homogenized, and resuspended in distilled water. A volume of 0.005–0.01 mL of packed cell volume per plant was applied by pipetting the *Frankia* suspension directly onto the plant roots.

Microscopic methods

Plants were observed daily, and at the first appearance of root swellings (indicative of nodule initiation) nodulated segments of root 2–3 mm long were harvested. These segments were fixed immediately for 2–3 h at room temperature in 2.5% glutaraldehyde fixative in 25 mM sodium phosphate buffer at pH 6.8. Fixed material was then rinsed and stored in 25 mM sodium phosphate buffer at 4°C. Nodules were likewise harvested at 4, 8 (*S. argentea* only), 12, and 16 weeks (*G. papuanum* only) after nodule initiation.

When searching for the route of infection, fixed material was trimmed to 1- to 2-mm lengths, dehydrated with a graded acetone series, and embedded in Spurr's low viscosity resin (Spurr 1969). Ten primordial nodules from each plant species were sectioned transversely with respect to the root axis, using a Porter–Blum ultramicrotome. Sections approximately 1 μm thick were transferred to water drops on slides, dried on a slide warmer set at 58°C, and stained with 0.05% toluidine blue O in 1% sodium borate for 1–2 min while on the slide warmer. Slides were then rinsed with distilled water and allowed to air dry before mounting cover slips with Permount.

For each species and within 1 day prior to each sampling of nodules, plants were tested for nitrogenase activity using the acetylene reduction method (Burris 1974). Plants were incubated with 10% acetylene for approximately 1 h, and then ethylene was measured with a Carle 9500 flame-ionization gas chromatograph. Whenever possible, plants with moderate to high acetylene reduction rates were selected for sampling nodules for structural study. At each sampling time, lobes from several nodules from each species were embedded and sectioned according to the procedures described above.

Results

Infection and nodulation of G. papuanum

Early nodule development was not visible on *G. papuanum* until 21 days after inoculation. *Gymnostoma papuanum* was always infected via root-hair penetration. Infections occurred in regions of root-hair deformation (Fig. 1), and infected hairs were themselves deformed. After penetrating the root hair, *Frankia* filaments grew down the hair cell, across the cell walls, and into cortical cells subjacent to the epidermis (Figs. 1, 2). The infection of two or more root hairs at a single site of nodule development was quite common under the conditions of inoculation used (Figs. 3, 4). Over 50% of early nodules examined showed multiple infected hairs. In most cases, it could be determined that *Frankia* from each of the infected hairs had infected prenodule cells as well. At this early stage of development, it could not be determined whether each infection would have proceeded to infect different nodule lobes or if different infections could infect the same nodule lobe.

Early nodule development was similar to that observed in *Comptonia* and *Myrica gale* (Callaham and Torrey 1977; Torrey and Callaham 1979). Cells of the cortex near the infected hair showed both cell division and cell hypertrophy (Fig. 1), resulting in the initial swelling of the root (the prenodule). One to several nodule lobe primordia developed at each infection site (Fig. 2). Hyphal growth proceeded from the infected hair into cells of the prenodule, with penetration of the hyphae from cell to cell directly through the cell wall.

At 4 weeks, the nodules on inoculated roots of *G. papuanum* were small, with one to several lobes that were generally ≤1 mm in diameter. Most nodule lobes had a single nodule root. In general, the nodule roots were short (<2 cm) and spikey in appearance, with no apparent directional orientation in their growth (unlike nodule roots of *M. gale* or *Casuarina* spp. that usually have nodule roots that grow in an upward direction). Numerous cells within the nodule cortex were colonized by *Frankia*.

Nodule growth beyond 4 weeks varied considerably from plant to plant. As nodules grew larger, most of them became multilobed and developed an aerenchymatous surface layer, which gave them a white, rough appearance. While some nodule lobes formed nodule roots of normal length, others showed nodule roots of reduced length (1–2 mm) or failed to form them at all.

FIGS. 1–4. Transverse sections through roots of *Gymnostoma papuanum* at sites of early nodule development harvested 21 days after inoculation in water culture with *Frankia* isolate HFPGpI1. Bar = 30 μm. Fig. 1. Prenodule formed by cells of the root cortex that have undergone cell division and cell hypertrophy in response to *Frankia* infection. Initial infection is via a deformed root hair (small arrowheads), with subsequent growth of *Frankia* into subjacent cells and cells of the prenodule (large arrowheads). Note asymmetry caused by prenodule formation. Fig. 2. The prenodule (*pn*) region and two nodule lobe primordia (*nlp*). *Frankia* has infected the cells within the prenodule (arrowheads). Figs. 3 and 4. Two sections taken from a single region of early nodule development showing two infected root hairs. Figure 3 shows the base of one infected hair (*ih*1) and *Frankia* within the root (arrowheads) near the region of the second infected hair. Figure 4 shows the first infected hair (*ih*1) and the base of the second infected hair (*ih*2). Although not shown in either figure, *Frankia* had also penetrated the cortex from the first infected hair. FIGS. 5 and 6. Sections through nodules of *G. papuanum* at 4 weeks after the initiation of nodule development on roots of plants inoculated with *Frankia* isolate HFPGpI1, and cortical cells of nodules of *G. papuanum* showing the presence of symbiotic vesicles (large arrowheads) in infected cells. Secondary thickening of the cell walls (small arrowheads) of infected cells is particularly evident in Fig. 5. Bar = 15 μm.

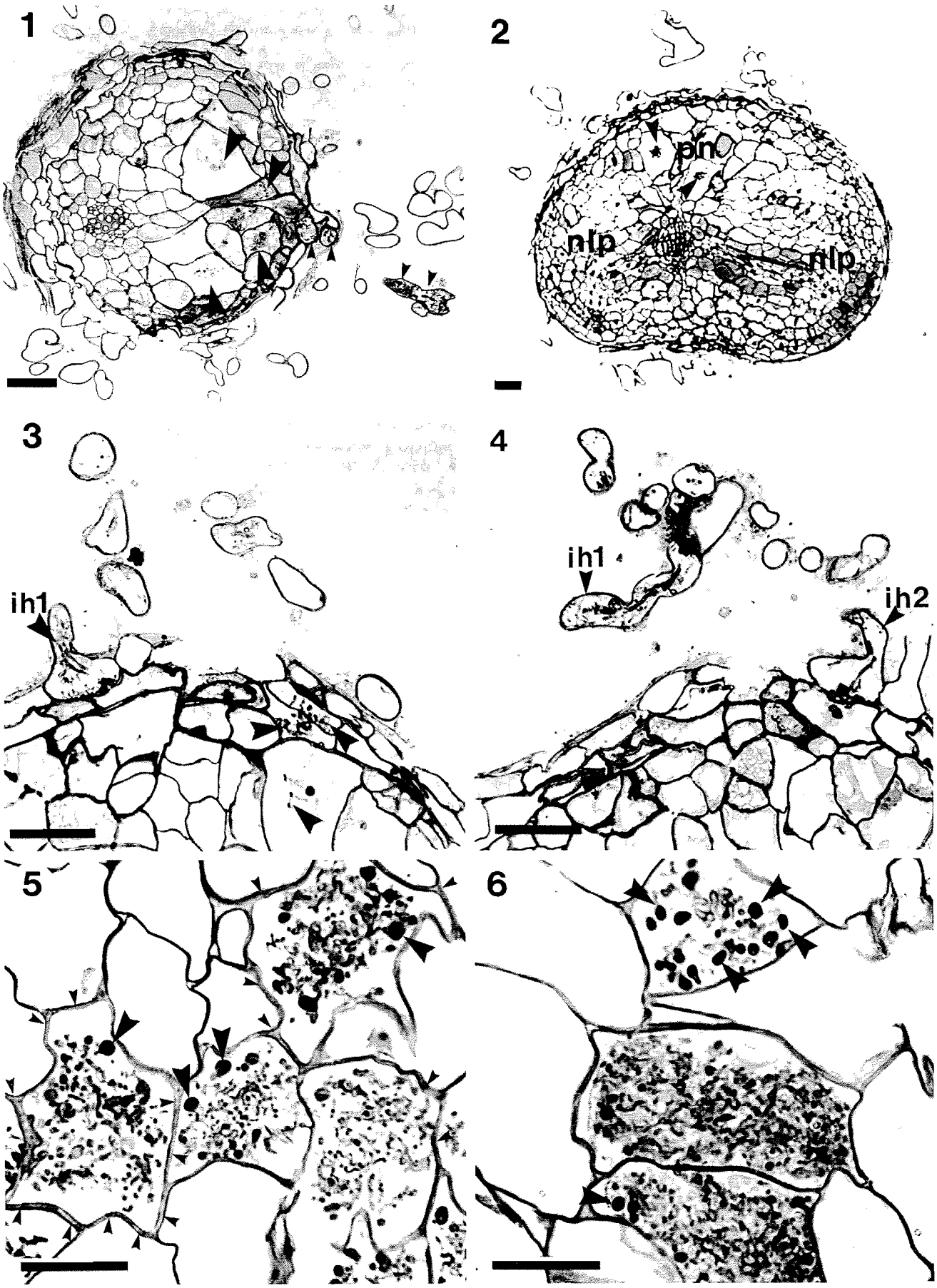


TABLE 1. Acetylene reduction activity in whole seedlings of *Gymnostoma papuanum* and *Shepherdia argentea* at 4, 8, 12, and 16 weeks after nodulation with *Frankia* strain HFPGpI1

Species	Rate of acetylene reduction activity (μmol ethylene/(h · plant))			
	4	8	12	16
G. papuanum	0 (5)	0.015±0.015 (5)	0.023±0.010 (10)	0.053±0.046 (8)
S. argentea	0.026±0.010 (9)	0.125±0.035 (9)	0.135±0.068 (18)	

NOTE: Values are the mean ±SE, and the number of plants sampled for each period is given in parentheses.

At 12 and 16 weeks, for the nodules that were apparently healthy and growing, large numbers of cortical cells were infected. Near the tip of nodule lobes, *Frankia* was seen in the early stages of new cell infection with only a small portion of the cell occupied by hyphae. *Frankia* hyphae frequently were seen penetrating from cell to cell directly through cell walls. Further along the length of the nodule lobes, *Frankia* occupied the majority of the cell volume. It was not uncommon to see secondary wall thickening in these cells (Fig. 5), distinguishable also by differential, blue–green staining with toluidine blue O. Closer to the base of the nodule, the *Frankia* hyphae were quite senescent.

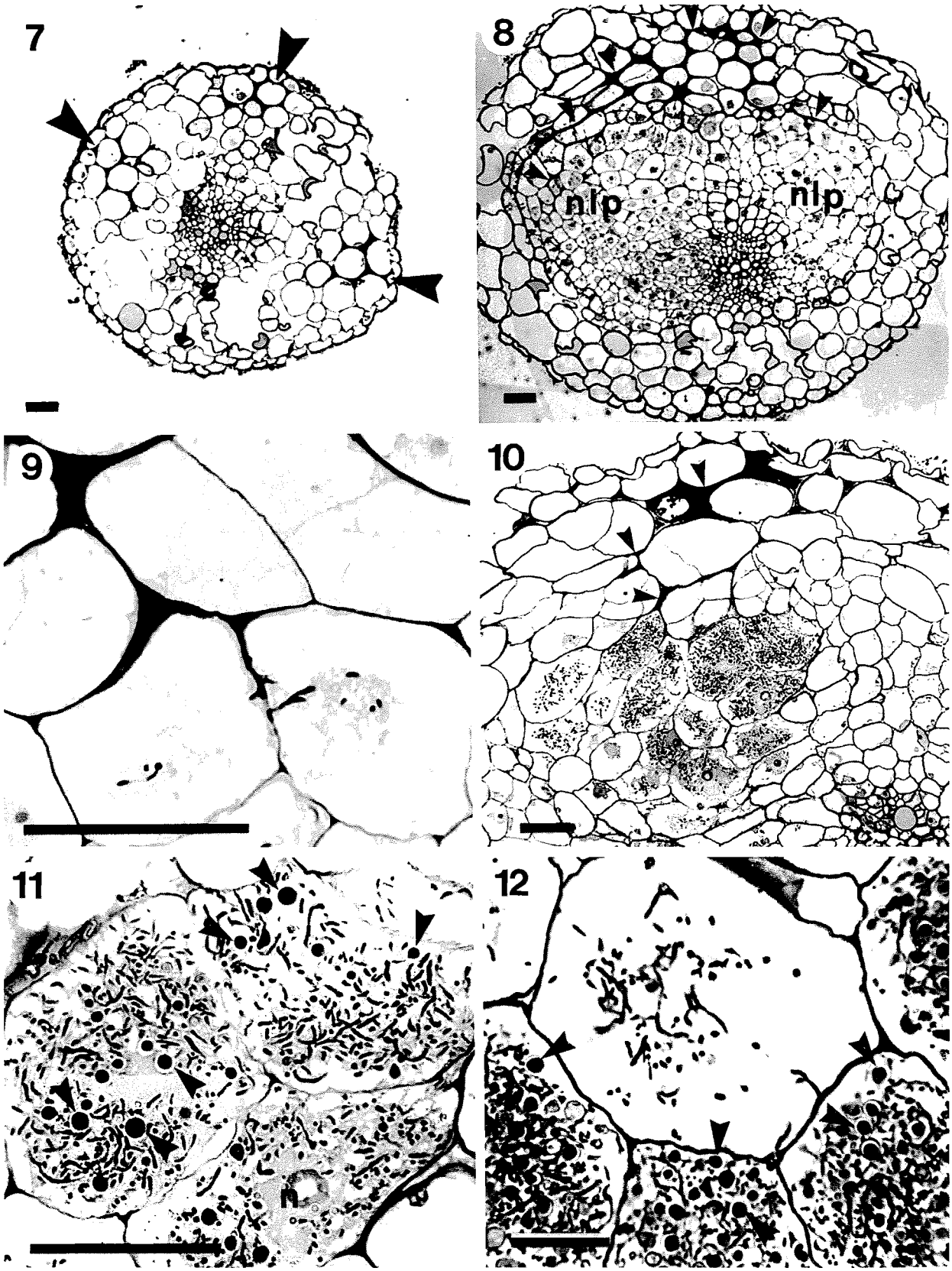
The first observation of the occurrence of symbiotic vesicles in the Casuarinaceae (cf. Torrey 1985) is illustrated in Figs. 5 and 6, which show the presence of symbiotic vesicles of *Frankia* within the cells of *G. papuanum*. The vesicles are mostly located at the periphery of infected cells and are more or less spherical in shape. Unlike the mature vesicles of *S. argentea* (Fig. 12), there are no pronounced void spaces seen around these vesicles. Vesicles were observed most frequently in nodules harvested at 4 weeks (when no acetylene reduction could be detected) but were also seen in nodules harvested at 12 and 16 weeks. At no time were their numbers any greater than shown in Figs. 5 and 6, and quite frequently many cells in any single 1-μm thick section lacked vesicles. On rare occasions, vesiclelike structures were also seen in cells of the prenodule.

Gymnostoma papuanum seedlings were assayed for acetylene reduction activity at 4, 8, 12, and 16 weeks after the initiation of nodulation. At 4 weeks, there were no indications from plant appearance that nitrogen fixation was occurring, and testing for acetylene reduction activity confirmed this observation (Table 1). By 8 weeks, only one seedling showed significant greening, indicative of nitrogen fixation. This plant had a low rate of acetylene reduction activity, whereas other plants that were tested continued to have no measurable activity. By 12 weeks, a majority of nodulated plants appeared healthier than the uninoculated controls. Most plants tested for acetylene reduction activity still had little or no measurable activity. At 16 weeks, shoots of many plants were green, with some slow growth occurring, while the acetylene reduction assay continued to indicate little or no nitrogenase activity for all but one plant. The acetylene reduction assay may not be an accurate measure of overall nitrogenase activity in seedlings of *G. papuanum*. For instance, one plant continually tested negative for acetylene reduction activity, yet that plant was a healthy green color and showed a significant increase in size by 16 weeks. The results of the acetylene reduction assays do indicate that nitrogenase activity of GpI1 in nodules of *G. papuanum* was generally much lower than for the same isolate in *S. argentea*.

Infection and nodulation of S. argentea

Early nodule development was visible on *S. argentea* roots, 9 days after inoculation with GpI1. Infection of *S. argentea* by *Frankia* proceeded very differently from that of *G. papuanum*. Seedlings roots were essentially devoid of root hairs under the conditions in which they were grown, including the regions where nodules were initiated (Fig. 7). Infection occurred following intercellular invasion by *Frankia* through the epidermis and underlying cortical tissue of the root. The path of *Frankia* penetration into the root was followed easily in sections because of the intense staining reaction of material secreted into the intercellular spaces where *Frankia* grows (Figs. 7,8, and 9). Although *Frankia* hyphae could not be seen within this material at the light microscope level, the electron

FIGS. 7–11. Transverse sections through roots of *Shepherdia argentea* at sites of early nodule development harvested 11 days after inoculation with *Frankia* isolate HFPGpI1. Bar = 30 μm. Fig. 7. Very early infection stage, showing three areas of intercellular deposition of intensely stained material, indicative of intercellular invasion by *Frankia* (arrowheads). Fig. 8. Later stage of development, showing a large amount of intercellular deposition (arrowheads) in the root cortex and extending into the two nodule lobe primordia (*nlp*) that have developed. Fig. 9. Higher magnification of cells of a nodule lobe primordium where *Frankia* hyphae have penetrated into cells from the region of intercellular deposition. Fig. 10. A more advanced stage of nodule development, showing extensive infection of the nodule lobe primordium by *Frankia*. The pathway of intercellular invasion can still be seen (arrowheads). Fig. 11. Enlarged view of several cells in the prenodule seen in Fig. 10, showing the beginning of symbiotic vesicle formation (arrowheads) even at this early stage in development. Penetration of *Frankia* directly from cell to cell is not seen. *n*, nucleus. FIG. 12. Section through cortical cells of a nodule of *S. argentea* at 4 weeks after the initiation of nodule development on roots of plants inoculated with *Frankia* isolate HFPGpI1. Cells show early and mature stages of infection by *Frankia*. At the early stage of infection, *Frankia* proliferates in hyphal form (central cell). As the infection progresses, numerous symbiotic vesicles are produced throughout the infected cell (cells at bottom and right). Mature vesicles stain dark, with a pronounced void space around them (arrowheads). Bar = 15 μm.



micrographs of early infection in *E. angustifolia*, published by Miller and Baker (1985), clearly showed the presence of hyphae in this darkly stained matrix.

As was also the case in *E. angustifolia* (Miller and Baker 1985), no prenodule formed in the root cortex in *S. argentea* nodule formation. The initial penetration of *Frankia* occurred directly into cortical cells of the nodule lobe primordium (Fig. 9). Multiple nodule lobe primordia developed in close proximity to one another (Fig. 8), but whether each lobe initiated was due to a separate infection or whether a single infection resulted in multiple lobes being initiated could not be determined. Conversely, multiple sites of invasion were often seen within single 1-μm-thick sections (Fig. 7), so it is possible that a single lobe was infected through more than one invasion site.

Colonization of the nodule lobe primordium cells occurred rapidly, with the most advanced nodules harvested at 11 days showing numerous cells filled with hyphae and many symbiotic vesicles starting to differentiate (Fig. 10). Dark-staining material was seen between infected cells of the nodule lobe primordium, and no cases of direct cell to cell penetration were seen (Fig. 11).

Unlike *G. papuanum*, which initiated all nodules at approximately the same time, *S. argentea* continually initiated new nodules throughout the 12-week sampling period. By 12 weeks, even the oldest nodules were usually still single lobed, with the largest being approximately 2 mm in diameter and 3–4 mm in length. Many of these larger nodules grew in a curved shape. All nodules were coralloid, that is, they lacked nodule roots. Nodules generally had an aerenchymatous surface layer composed of loosely organized tissue, with misshapen cells showing a great deal of air space between them and infected cells bordering directly on that air space.

Frankia was never seen to cross directly from cell to cell through cell walls in nodules of *S. argentea*, as was observed in nodules of *G. papuanum*. Initial cell infections usually consisted of hyphal proliferation followed by the production of numerous vesicles (Fig. 12). By 8 weeks, older nodules had significant regions near the base of the nodule lobes in which the *Frankia* had undergone senescence. During the infection process, vesicles were also occasionally seen in the dark-staining intercellular material deposited in the root cortex.

Shepherdia argentea seedlings were assayed for acetylene reduction activity at 4, 8, and 12 weeks after the initiation of nodulation. At 4 weeks, acetylene reduction assay results indicated considerable variation in nitrogenase activity from plant to plant (Table 1). *Shepherdia argentea* plants at 8 weeks continued to show great variation in nitrogenase activity, reflected both in the acetylene reduction assay and in the overall growth of the plants . At 12 weeks, plants again showed considerable variation in both growth and acetylene reduction activity. Shoot height at 12 weeks varied from approximately 6.5 to over 28 cm.

Discussion

Frankia isolate HFPGpI1 is capable of infecting two types of plants that undergo very different processes of infection and nodule initiation. Thus, GpI1 is the type of *Frankia* that has been termed 'developmentally flexible' by Miller and Baker (1986). In this study, GpI1 infected *G. papuanum* by root-hair penetration, with development of a prenodule region, and *S. argentea* by intercellular invasion of the epidermis and cortex followed by direct infection of cells of the nodule lobe primordium, with no prenodule development. The differences between these two modes of infection are best seen by comparing Fig. 1 (*G. papuanum*), which shows an infected root hair and numerous infected cortical cells in the prenodule region, with Fig. 8 (*S. argentea*), which shows the invasion path leading to the nodule lobe primordium and no prenodule region.

As in all cases reported to date, the mode of infection and initial nodule development were found to be consistent for each given plant species. Observations on the modes of infection of these two genera lend support to the hypothesis that this trait is determined by the host plant and is characteristic of the family to which the plant species belongs. *Shepherdia argentea*, like *E. angustifolia* (Miller and Baker 1985), shows infection by direct epidermal invasion, and both are members of the family Elaeagnaceae. *Gymnostoma papuanum* and *Casuarina cunninghamiana* (Callaham et al. 1979), both members of the family Casuarinaceae, show infection by root-hair penetration.

Other striking differences in nodule formation were apparent between these two genera. Nodules developed more rapidly in *S. argentea* than in *G. papuanum* and continued to be initiated successively over time, unlike *G. papuanum* where nodule initiation occurred all at once. In *G. papuanum*, individual nodules frequently branched to become multilobed; in *S. argentea*, nodules usually remained single lobed. Nodule roots were formed in *G. papuanum*, whereas none developed in *S. argentea*.

Once *Frankia* entered root nodule cells of *G. papuanum*, invasion continued from cell to cell across nodule lobe cell walls. In contrast, each infected cell in *S. argentea* nodules resulted from invasion of bacteria from intercellular spaces. No growth of *Frankia* across cell walls was observed in *S. argentea*. Other intracellular differences were observed. *Frankia* differentiated rapidly in *S. argentea* nodules, developing abundant vesicles early in nodule development. The vesicles were spherical and showed clear zones in the position of the vesicle envelope, reminiscent of those seen in *Elaeagnus* (Newcomb et al. 1987). In *G. papuanum*, vesicle formation was sparse and erratic both in spatial distribution within the nodule and in nodules over time. Acetylene reduction activity reflected the differences in nodule initiation and development, with *S. argentea* plants showing a great increase in acetylene reduction activity between 4 and 8 weeks with increasing age and numbers of root nodules, whereas *G. papuanum* showed low levels of acetylene reduction throughout the 16-week period studied.

The formation of symbiotic vesicles in the nodules of *G. papuanum* is of particular interest, since they have never been reported in nodules from other members of the family Casuarinaceae. It cannot be stated with certainty whether vesicles have any role in nitrogen fixation in *G papuanum*, whether they persist in nodules beyond 16 weeks after nodule initiation, or whether their presence is specific to the isolate GpI1. Further studies on the occurrence and ultrastructure of vesicles in root nodules of *G. papuanum* are in progress.

Although only a single isolate was used as inoculum in the experiments reported here, the frequency of multiple root-hair infections in *G. papuanum* and multiple infection sites in *S. argentea* support the view that multiple *Frankia* strains in a single nodule are possible and even likely. Evidence that double infections do occur has been obtained by protein analysis of several isolates from the same alder nodule (Benson and

Hanna 1983). Multiple infections in the field would not be expected to be as frequent as suggested by the number seen here, since the density of infective particles in the soil would be much lower than that used for these experiments. Nevertheless, the potential for dual infections certainly exists, and this fact has important implications for isolations of *Frankia* from nodules collected in the field.

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