The Biology and Management of
Red Alder

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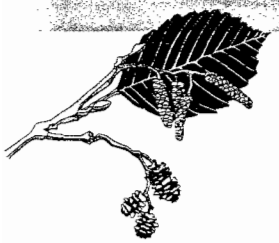

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Root Symbioses of Red Alder: Technological Opportunities for Enhanced Regeneration and Soil Improvement

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Red alder is unique among forest trees of the Pacific Northwest because it forms a tripartite symbiosis among roots, nitrogen-fixing actinomycetes in root nodules (actinorhizae), and mycorrhizal fungi. The amount of nitrogen fixed by the actinorhizal associations, the positive impact of this fixed nitrogen on growth of red alder, and the contribution made to soil fertility are well appreciated by foresters as reviewed in Binkley et al. (Chapter 4).

Past reviews of alder symbioses (Tarrant and Trappe 1971; Hall et al. 1979; Trappe 1979) called for intensive research on the biology of these root symbionts so that their benefits could be optimized through biotechnology. Consequently, over the last decade, characterization of the biology and ecology of the symbioses, especially the actinorhizal symbioses, has progressed considerably. The isolation of symbiotic actinomycetes in the late 1970s provided the biological framework for intensive study of their physiology and ecology; nearly 1000 papers have been published and several international symposia conducted over the last decade. Schwintzer and Tjepkema (1990) produced a comprehensive review on their systematics, host relations, and strain variations.

Mycorrhizal symbioses of alder have received less attention than actinorhizae, but identities of fungal symbionts, degree of host specificity, effects of mycorrhizae on seedling growth, and abundance of fungal symbionts in forest soils have been studied (Molina 1979, 1981; Rose 1980; Koo 1989; Miller et al. 1991, 1992). Most importantly, interactions between the two symbioses have been examined, resulting in a holistic appreciation of the tripartite symbiosis. Three general concepts are evident: each symbiosis provides unique benefits to host nutrition, the two symbioses interact synergistically to improve host nutrition; and the root endophytes are widespread in west-side forests of the Pacific Northwest.

In this chapter we describe the root symbionts, discuss their ecologies and interactions, and address current efforts to artificially inoculate red alder seedlings with selected isolates of root symbionts. Our overall objective is to emphasize how strongly red alder depends on the root symbionts and to develop strategies for using these symbioses in future management applications.

Actinorhizal (*Frankia*) Associations of Red Alder

Frankia species are sporulating actinomycetes capable of fixing nitrogen. They form symbiotic root nodules on several dicotyledonous plant genera, including alders. This symbiosis between *Frankia* and plant roots was termed "actinorhiza" by analogy to mycorrhizal associations (Tjepkema and Torrey 1979). Several review articles (Silvester 1977; Baker and Seling 1984; Baker 1988, 1989), proceedings of international conferences (Table 1), and a book (Schwintzer and Tjepkema 1990) on *Frankia* and actinorhizal plants are available for additional background.

Historical Perspective

Nonleguminous root nodules have been the object of observation and study for more than 100 years. Quispel (1990) recounted the history of actinorhizal studies before the successful isolation of *Frankia* in 1978. Although these earlier studies were mainly observational, they yielded important landmarks: Brunchorst (1886-88) concluded that root nodules are associated with a microorganism

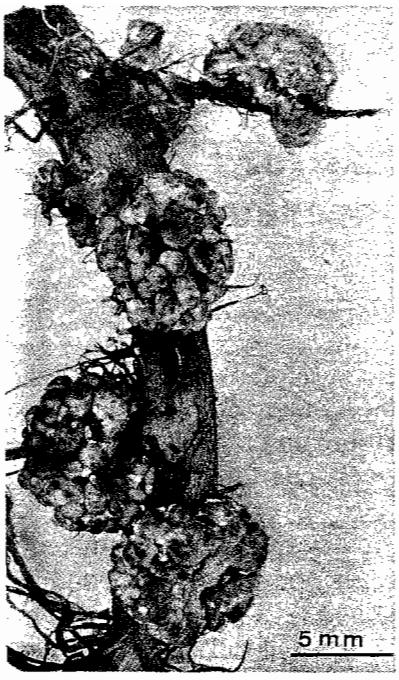


Figure 1. Alder root nodules. Photo courtesy of K. Huss-Danell, University of Umeå.

and suggested that these organisms be assigned to a new genus, *Frankia* (after B. Frank, his major professor); Hiltner (1895) demonstrated that nodulated *Alnus glutinosa* can grow in nitrogen-free soil and thus presumably fixed nitrogen; and Schaede (1933) confirmed the actinomycetous nature of the microsymbiont. In the 1950s, work on actinorhizae intensified and became increasingly quantitative. Nitrogen fixation was measured with $^{15}N_2$ for the first time in *Alnus* (Virtanen et al. 1954) and other actinorhizal genera (Bond et al. 1954). The first successful isolation of the microsymbiont was accomplished about this time by Pommer (1959). Unfortunately, the culture was lost, and it was not until 1978 that Callaham et al. (1978) once again isolated *Frankia* in pure culture. Since 1978, research on *Frankia* has increased rapidly, as evidenced by more than 900 citations in the bibliography published by the Program in Forest Microbiology (1988).

Root Nodules and Nodulation

Alnus species almost universally harbor root nodules. Even tiny alder seedlings quickly become nodulated in nature. Exceptions to rapid nodulation occur on sites not previously inhabited by actinorhizal plants (e.g., mine wastes and peat bogs) (Huss-Danell and Frey 1986).

Root nodule initiation. Nodulation of alder roots begins by entry of *Frankia* through a root hair. Only deformed root hairs, those which have curled, presumably in response to *Frankia* or other rhizosphere bacteria, are colonized. *Frankia* grows within the root hair and is encapsulated by host tissue. Although *Frankia* may penetrate plant cell walls, it does not penetrate the plant cell membrane. Proliferation of *Frankia* within the root coincides with increased production of cortical cells, which give rise to a macroscopic swelling on the root known as a prenodule. Nodule development continues with the formation of nodule lobe primordia. Nodule lobes are modified lateral

Table 1. List of international conferences on Frankia and actinorhizal plants, and resulting proceedings.

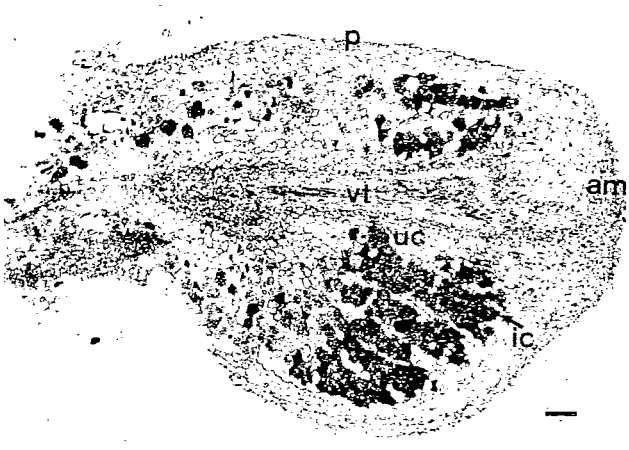
Year	Meeting site	Proceedings reference
1987	Harvard Forest	*Botanical Gazette* 140(S)
1979	Oregon State University, Corvallis	*Symbiotic Nitrogen Fixation in the Management of Temperate Forests* (J. C. Gordon, C. T. Wheeler, and D. A. Perry, eds.), 1979
1982	University of Wisconsin, Madison	*Canadian Journal of Botany* 61(11), 1983
1983	Landbouwhogeschool Wageningen, The Netherlands	*Plant and Soil* 78(1/2), 1984
1984	Laval University, Ste-Foy, Quebec, Canada	*Plant and Soil* 87(1), 1985
1986	University of Umeå, Umeå, Sweden	*Physiologia Plantarum* 70(2), 1987
1988	University of Connecticut, Stoors	*Plant and Soil* 118(1/2), 1989
1991	University of Lyon, Lyon, France	*Acta Oecologia*
1993	University of Waikato, Hamilton, New Zealand	*Soil Biology and Biochemistry*

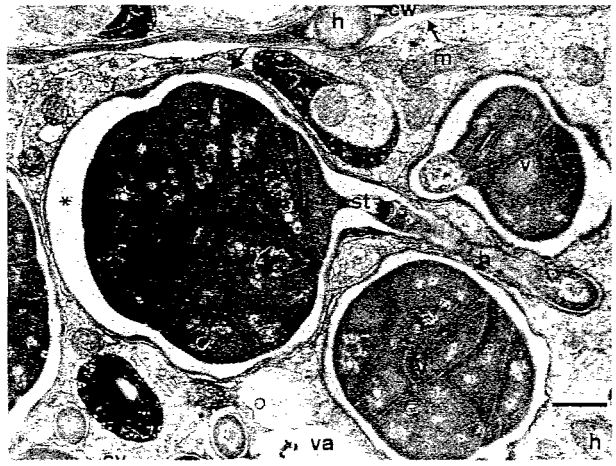
roots. Details of morphogenesis of actinorhizal nodules are reviewed by Newcomb and Wood (1987) and Berry and Sunnell (1990).

Root nodule structure. Actinorhizal root nodules are perennial structures with yearly cycles of growth and senescence. The youngest, most active tissue is located on the periphery of nodule lobes. Nodules come in a variety of shapes and sizes; some have discrete, branched lobes, others branch profusely and develop into compact clusters. Alder nodules are typically compact and become quite large, with diameters of several centimeters (Fig. 1). Nodule morphology is probably under host plant control but may reflect environmental conditions (e.g., presence of rocks, aeration of soil).

The anatomy of alder nodules resembles that of lateral roots: a central stele is surrounded by the endodermis, layers of cortical cells, and a periderm (Fig. 2). The periderm of alder nodules contains numerous lenticels, presumably to facilitate gas transport. Cells colonized by *Frankia* abound throughout the cortical region of the nodule. Unlike actinorhizal nodules of other plant genera, alder nodules contain relatively low concentrations of hemoglobin (Silvester et al. 1990).

Within alder nodules, *Frankia* can display all three morphological structures observed in pure culture: hyphae, vesicles, and sporangia (Fig. 3). Hyphae differentiate to form spherical, septate vesicles (Newcomb and Wood 1987). Nitrogen fixation occurs within the vesicles. The thick-walled vesicles serve as a diffusion barrier to oxygen and protect the nitrogenase enzyme from inactivation (Silvester et al. 1990). The formation of vesicles and





Figures 2 and 3. Anatomy and ultrastructure of alder nodules.

Figure 2 (left). Longitudinal photomicrograph of an alder nodule lobe surrounded by periderm (p), with vascular tissue (vt) in the center. Dark cortex cells are infected (ic) with Frankia. *Many cortex cells are uninfected (uc). The apical meristem (am) gives rise to new nodule cells. Bar = 100 μm. Photo courtesy of K. Huss-Danell.*

Figure 3 (right). Transmission electron micrograph of symbiotic Frankia *within an alder root nodule. Symbiotic vesicles (v) are compartmentalized by septa (s) and attached to the hyphae (h) by a stalk (st) located at the constricted basal portion of the vesicle. Vesicles are surrounded by a so-called void space (*), which most likely results from extraction of the lipid containing vesicle envelope during sample preparation. Hyphae are shown in both transverse and longitudinal sections. The large arrow points to the capsule, which is a barrier contiguous with the plant cell wall (cw) and consists of pectin and some fibrils. Other plant cell features include: mitochondrion (m), vacuoles (va), plastids (pl), which are largely devoid of starch, cytoplasm (cy), and cell membrane (small arrows). Bar = 1 μm. Photo courtesy of P.-Å. Vikman, P.-O. Lundquist, and K. Huss-Danell; published previously by Gallon (1992).*

their senescence follows a seasonal pattern of growth within the perennial nodules.

Ineffective nodules that do not fix nitrogen occasionally occur. They are typically small, contain relatively little *Frankia* (Berry and Sunnell 1990), and normally do not contain vesicles (Mian et al. 1976; Hahn et al. 1988).

Although virtually all *Frankia* strains can be induced to form sporangia in pure culture, this is not necessarily the case in nodules (Schwintzer 1990). *Frankia* strains observed within nodules form either many sporangia (sp^+) or none to very few sporangia (sp^-). Sporangia form as the *Frankia* strain ages, generally developing after vesicles have matured. The spores held within the sporangia presumably serve as a resting stage, thereby allowing *Frankia* to survive nodule senescence and ultimately to germinate after nodule decay. Whether nodules are sp^+ or sp^- seems to be a function of the *Frankia* genotype, although the host can influence whether sporulation is expressed (Schwintzer 1990). No sp^+ *Frankia* strains exist in pure culture, which complicates research in this area.

Nodule physiology. Carbon, nitrogen, and hydrogen metabolism are important aspects of nodule physiology (Tjepkema et al. 1986; Huss-Danell 1990). Alder root nodules and consequently symbiotic *Frankia* obtain their carbon from photosynthate. The plant metabolites used by *Frankia* in nodules are not known with certainty, but both simple sugars and carboxylic acids have been shown to stimulate respiration of symbiotic *Frankia* (Huss-Danell 1990).

Nitrogen metabolism in nodules includes both nitrogen fixation and assimilation of fixed nitrogen (Huss-Danell 1990). *Frankia* fixes nitrogen in its vesicles, which contain the common FeMo nitrogenase. Nitrogenase activity in alders follows seasonal changes in photosynthesis, suggesting the close coupling of the two processes. Once fixed as NH_3, nitrogen is assimilated by the plant via the glutamine synthase-glutamine oxo-glutarate aminotransferase system. In alder, subsequent transamination reactions result in the production of citrulline, the major nitrogenous compound transported in the xylem.

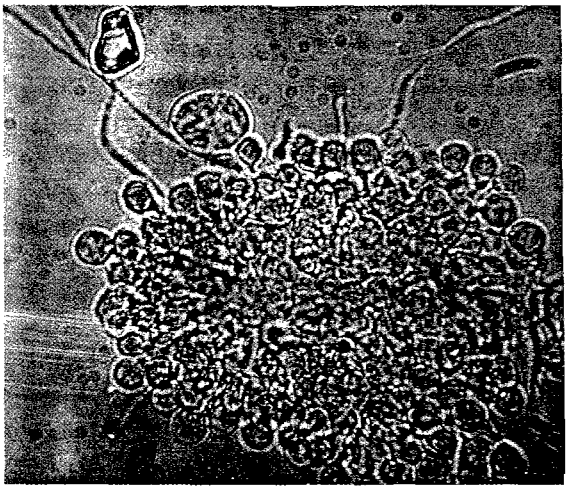
Hydrogenase acts as a potential energy conservation mechanism within nodules; nodules with active hydrogenase are more efficient at fixing nitrogen than are nodules without this enzyme (Huss-Danell 1990). Hydrogenase activity is universally present in *Frankia* and actinorhizal nodules, with the one exception of a "local source" of *Frankia* from Sweden that is infective on alders (Sellstedt and Huss-Danell 1984; Sellstedt 1989).

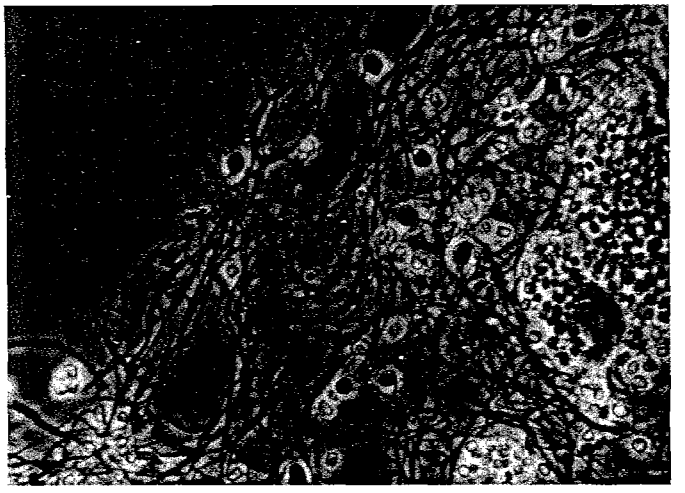
Isolation, Identification, and Physiology of Frankia Isolates

Since the isolation of *Frankia* by Callaham et al. (1978), numerous strains have been isolated from various actinorhizal plants and cultured for physiological, biochemical, inoculation, and genetic studies. *Frankia* associated with hosts in the families Rosaceae and Rhamnaceae have not yet been reported to be cultured. *Frankia* is unique among actinomycetes because it forms irregular shaped sporangia in liquid culture. Sporangia can be intercalary or terminal. The aseptate hyphae, 0.5 to 2.0 μm wide, are poorly branched. Aerial mycelium does not form in solid medium. Cells may be colorless or pigmented, and produce various soluble pigments depending on growth medium. Vesicles, site of nitrogen fixation under ambient oxygen concentrations (Tjepkema et al. 1980; Murry et al. 1984; Noridge and Benson 1986), form in the absence of combined nitrogen. Presence of combined nitrogen *in vitro* inhibits vesicle formation and nitrogen fixation.

Isolation of *Frankia*. The slow growth of *Frankia* and presence of contaminating microorganisms on the nodule surface impeded early progress in isolating *Frankia*. Several methods are now available to successfully isolate *Frankia* from actinorhizal plants: serial dilution (Berry and Torrey 1979), micro-dissection (Diem and Dommergues 1988), selective incubation (Quispel and Burggraaf 1981), filtration (Benson 1982), and sucrose density gradient centrifugation (Baker and O'Keefe 1984). Li (unpub.) has modified Benson's (1982) filtration techniques to routinely isolate *Frankia* from root nodules of Alnus species as follows:

Isolation medium. A defined, modified liquid BAP medium (Murry et al. 1984) in test tubes consists of the following in mM in distilled water: KH_2PO_4 $7H_2O$, 3.4; sodium propionate, 5; $MgSO_4$, 0.1; $CaCl_2$, 0.07; FeNaEDTA, 10 mg/l; biotin, 450 μg/l; and trace





Figures 4 and 5. Vesicles and hyphae of Frankia.

Figure 4 (left). Vesicle clusters of Frankia *isolated from root nodules red alder (1100x).*

Figure 5 (right). Vesicles formed on hyphae in liquid culture when Frankia *is grown without a nitrogen source (1100x).*

elements and vitamins according to Tjepkema et al. (1981). The pH of the medium is adjusted to 6.7.

Nodule preparation. Wash root nodules in a stream of water to remove loose soil. Separate nodule lobes and place them in a beaker of 2.5-percent sodium hypochlorite solution plus one drop of Tween 20. The surface is sterilized by continuous agitation with a magnetic stir in the solution for 20 min. Then rinse the lobes thoroughly in sterile distilled water and homogenize them in 5 ml sterile distilled water in a tissue grinder to release the vesicle clusters. Filter the homogenate through autoclaved nylon screens with mesh openings of 50 and 20 µm. Then thoroughly wash vesicle clusters (Fig. 4) retained on the 20-µm screen with sterile distilled water. Collect vesicle clusters with a Pasteur pipette, place in tubes of modified BAP medium, and incubate at 30°C with occasional gentle shaking. *Frankia* filament mats appear within two to three weeks and can be transferred to new tubes of BAP containing 5 mM NH_4Cl to enhance growth. *Frankia* has not been isolated from soil under alder, although Baker and O'Keefe (1984) isolated a strain from the rhizosphere of *Cercocarpus montanus.*

Confirmation of *Frankia* isolation. Confirmation of *Frankia* isolates is based on presence of characteristic sporangia and vesicles, branching hyphae, lack of aerial mycelium, and ability to colonize and form nodules with a host plant. Some isolates, however, lack the ability to colonize host plants even though they can fix nitrogen in culture (Diem et al. 1982; Torrey 1990). Acetylene is not reduced without vesicle formation (Tjepkema et al. 1980, 1981). Vesicles are surrounded by multi-laminate envelopes of lipid that limit diffusion of oxygen into the vesicle cytoplasm, thereby protecting nitrogenase (Parsons et al. 1987; Lamont et al. 1988; Berry et al. 1991; Harriott et al. 1991). Vesicles can differentiate into hyphae for vegetative growth (Schultz and Benson 1989). Some *Frankia* strains sporulate in nodules of host plants after inoculation (Racette and Torrey 1991).

***In vitro* growth of *Frankia* isolates.** Most physiological studies of *Frankia* use homogenized cultures so that numerous hyphal fragments are available to initiate active growth. Regrowth from hyphal fragments is optimized when homogenization is performed during the exponential stage of growth. Thus, only healthy cultures that have been homogenized are transferred to fresh medium for physiological studies (Benson and Schultz 1990).

Carbon use. Propionate and acetate are universal carbon sources for growing *Frankia* (Stowers 1987). Some *Frankia* strains use sugars such as glucose, fructose, mannitol, trehalose, maltose, and sucrose (Burggraaf and Shipton 1983; Lechevalier et al. 1983; Tsai et al. 1983; Lopez and Torrey 1985).

Other strains, particularly from *Alnus, Comptonia,* or *Myrica* root nodules, prefer short-chain fatty acids or organic acids, such as succinate, fumarate, malate, and pyruvate (Shipton and Burggraaf 1982; Lopez and Torrey 1985).

Nitrogen use. Ammonium chloride is most widely used as a nitrogen source. Some isolates can use amino acids, such as aspartate and glutamate, and inorganic sources, such as ammonium nitrate and potassium nitrate (Stowers 1987). *Frankia* also grows in the absence of combined nitrogen and under these conditions forms vesicles (Fig. 5).

Growth determination. Frankia grows slowly and is typically measured by correlating biomass to total protein (Burggraaf and Shipton 1983). To determine total protein, the *Frankia* culture is harvested by centrifugation and washed with 0.01 M potassium phosphate buffer, pH 6.8. The washed culture is homogenized in a tissue grinder and digested with 0.5 ml of 1.0 M NaOH in a boiling water bath for 10 minutes. The amount of protein released is determined by the methods of Markwell et al. (1978).

Soil Ecology of Frankia

Much of what is known or surmised about the ecology of *Frankia* in soil has been learned indirectly by studying the ecology of the symbiosis. Many studies have focused on either the nitrogen-fixing ability of particular host-strain combinations or on the sp^+/sp^- nature of the nodules. The outcome of such studies will be discussed first, followed by a review of studies that have followed populations of *Frankia* in soil.

Studies based on symbiotic characteristics. Ineffective nodules often occur because of intergeneric or interspecific incompatibilities (i.e., *Frankia* strains isolated from one host genus may form only ineffective nodules when tested on a different actinorhizal host genus) (Weber et al. 1987; van Dijk et al. 1988). Some *Frankia* isolates form ineffective nodules even when inoculated on the same host species from which they were isolated (Hahn et al. 1988).

The nitrogen-fixing ability of various *Frankia* strain-plant combinations often differs significantly in laboratory studies (Carpenter et al. 1984; Hooker and Wheeler 1987; Domenach et al. 1988; Sheppard et al. 1988; Weber et al. 1989; Kurdali et al. 1990). For example, sp^- and sp^+ nodules can differ in both absolute and relative nitrogenase efficiency (Normand and LaLonde 1982; Wheeler et al. 1986). Presumably the differences in effectiveness among *Frankia* strains influence their competitiveness and fitness in nature, but this has not been studied.

The spore type of *Alnus* nodules displays interesting and complex ecological relations (Schwintzer 1990). Reasons for these ecological patterns include the following: degree of host selection (e.g., sp^+ strains form effective nodules on *A. incana* subsp. *incana* but not on *A. glutinosa*) (Domenach et al. 1988; van Dijk et al. 1988; Kurdali et al. 1990); sp^- strains often dominate on newly disturbed sites and may be more saprophytically competent than sp^+ strains, which often are found in established actinorhizal stands and may be maintained primarily through nodule turnover (van Dijk 1984; Weber 1986; Holman and Schwintzer 1987; Smolander and Sundman 1987); and low soil pH tends to favor sp^+ strains (Holman and Schwintzer 1987; Smolander et al. 1988). *Alnus rubra* forms both sp^+ and sp^- nodules, but their ecological significance has not been studied.

Soil *Frankia* populations. Nearly all the ecological information about *Frankia* populations in soil has been collected through a plant bioassay system (Myrold 1993). Surveys of forest sites in Finland (Smolander and Sundman 1987; van Dijk et al. 1988; Smolander 1990) and the Pacific Northwest (Hilger and Myrold, unpub. data) have shown *Frankia* populations to range from 0 to 4600 infective units g^{-1} soil. An infective unit is the amount of *Frankia* necessary to form one nodule. This variation in *Frankia* populations is caused partly by differences in plant species present and by soil conditions. Often higher numbers are found under nonactinorhizal plants (e.g., birch) than actinorhizal plants (Smolander and Sundman 1987; van Dijk et al. 1988; Smolander 1990).

In a greenhouse bioassay, Miller et al. (1992) determined the nodulation potential of red alder seedlings when grown in soils of six forest sites in the Oregon Coast Range: young alder, old alder, conifer clearcut, conifer plantation, rotation-age conifer, and old-growth conifer. They reported that nodules formed on red alder grown in all soils, but highest nodule numbers and rates of nodule for-

mation occurred on seedlings grown in the alder and conifer plantation soils. Because they found that levels of nitrate and mineralizable nitrogen in the bioassayed soils were highest in the alder and conifer plantation sites, they suggested that a nitrogen-priming effect might be involved in the nodulation rates observed in these soils.

Laboratory studies of soil and rhizosphere effects on *Frankia* populations mostly have confirmed field observations. *Frankia* populations are inversely correlated with soil pH (Smolander and Sundman 1987). Survival of added *Frankia* was greater in limed than in unlimed soil (Hilger and Myrold, unpub. data; Smolander et al. 1988; Smolander and Sarsa 1990); however, rhizosphere effects are quite variable (Hilger and Myrold, unpub. data; Smolander and Sarsa 1990). Van Dijk and Sluimer-Stolk (1990) found populations of ineffective *Frankia* present at much higher levels than effective *Frankia* in sand dunes soils planted with alders.

Hilger and Myrold (unpub. data) have developed a method of estimating *Frankia* biomass in soil based on *Frankia*-specific DNA sequences. Their method determines the proportion of *Frankia* biomass to total soil biomass, not just those *Frankia* that are infective under the conditions of a seedling bioassay. This technique provides a useful tool for studying *Frankia* populations in soil and their response to environmental conditions and forest management practices.

Diversity of *Frankia* strains. Initially, *Frankia* strains were classified by their host, much as has been done with *Rhizobium.* It is now clear that the boundaries between strains do not fall strictly along the lines of host infectivity (Torrey 1990). Four host-specificity groups are recognized: strains that nodulate *Alnus* and *Myrica, Casuarina* and *Myrica, Elaeagnus* and *Myrica,* and only members of the Elaeagnaceae (Baker 1987). Some *Frankia* strains, however, cross these host-specificity boundaries, and there also is variation within *Frankia* strains that nodulate different *Alnus* species. For example, an effective strain isolated from one *Alnus* species may form only ineffective nodules on another species of *Alnus* (Weber et al. 1987). Different combinations of *Frankia* strain by *Alnus* species also can yield differences in tree growth and rate of nitrogen fixation (Wheeler et al. 1986).

The diversity of *Frankia* in nature typically is determined by isolating *Frankia* strains from nodules. Isolates obtained from nodules are differentiated by several means. For example, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis first was used to differentiate *Frankia* strains by Benson and Hanna (1983); they separated 43 isolates from one stand of *Alnus* into six groups, with one group being dominant and containing 35 of the isolates. They also found evidence for dual occupancy within a nodule. Another method used isozyme patterns to compare the diversity of strains isolated from a single *A. rubra* (Faure-Raynaud et al. 1990) and from individual nodules of *A. glutinosa* (Faure-Raynaud et al. 1991). Thirteen *Frankia* strains were found on a single red alder root system, but individual nodules harbored only one strain. These studies indicate a wide diversity of *Frankia* strains capable of colonizing alder.

Methods using differences in DNA or RNA sequences to differentiate *Frankia* strains eliminate the need for isolating *Frankia* from nodules. For example, Simonet et al. (1988) used a radio-labeled *Frankia* plasmid to probe nodule DNA obtained from stands of *Alnus*. This allowed them to detect a single strain in those stands. Chromosomal genes also can be used. Oligonucleotide probes based on sequence differences in the variable region of the 16S rRNA gene were used to detect a particular *Frankia* strain in root nodules of *Alnus glutinosa* (Hahn et al. 1990b), and Simonet et al. (1990) used oligonucleotide probes based on sequence differences in the *nifH* gene to detect and differentiate between two *Frankia* strains in *A. glutinosa* and *A. incana* nodules. Such techniques and others currently being developed (e.g., the use of polymerase chain reaction with *Frankia* specific primers) will provide means for performing sophisticated autecological studies of *Frankia.*

Interactions between soil organisms and *Frankia*. Nodule initiation by *Frankia* can be depressed by low pH, combined nitrogen, or by lack of other rhizosphere microorganisms. Knowlton et al. (1979, 1980) and Knowlton and Dawson (1983) demonstrated that coinoculation of *Frankia* with certain bacteria could enhance *Frankia* colonization and nodule development of red alder; inoculation of *Frankia* strains under aseptic conditions resulted

in few nodules. When plants were inoculated with the bacteria plus *Frankia,* nodulation increased even at low pH (pH 4.0 to 6.0). These bacteria grew rapidly in low pH conditions and raised the rhizosphere pH to levels conducive to *Frankia* growth and infection (Knowlton and Dawson 1983). Bacteria other than *Frankia* also can cause massive root-hair deformation, thereby promoting nodulation by *Frankia.* Studies by Mansour and Torrey (1991) found that *Frankia* spores applied in nonsterile conditions caused root-hair deformation, which led to nodule formation by germinating spores; under axenic conditions, the incidence of root-hair deformation was low and nodulation did not occur with germinating spores. Research by Perinet and LaLonde (1983) and Myrold (unpub.), however, has shown that effective nodulation occurs under aseptic or axenic conditions. Further research is needed to address the role of "helper" bacteria on nodulation.

Dual inoculation of *Frankia* with root-associated nitrogen-fixing *Azospirillum* results in significantly larger seedlings than inoculation with *Frankia* alone in *Alnus rubra* and *Casuarina cunninghamiana* (Li 1987; Li et al. 1987, p. 247; Rodriguez-Barrueco et al. 1991). This dual inoculation also produces larger sizes and greater quantities of root nodules than inoculation with *Frankia* alone. *Azospirillum* may facilitate nodule formation by promoting *Frankia* infection. Simultaneous inoculation of effective and ineffective *Frankia* strains also enhances growth and nodule formation of *A. glutinosa* (Hahn et al. 1990a). Similarly, *Alnus* species inoculated with mixtures of *Frankia* strains grew better than *Alnus* inoculated with single strains, but the number of nodules formed remained the same for inoculations of both single and mixed strains (Prat 1989). Thus, several different strains of *Frankia* may normally be present in the nodules of actinorhizal plants, as demonstrated for Casuarinaceae by Reddell and Bowen (1985) and for *Alnus* by Simonet et al. (1985). Interactions between mixed strains likely take place in natural soils and are apparent in inoculation studies. Rojas et al. (1992) demonstrated that other actinomycetes on roots, nodules, and in soil of red alder do not affect *Frankia* infection and nodule development and can, in fact, reduce red alder growth by production of allelochemicals.

Soil and root chemistry effects on actinorhizae. Nodulation and endosymbiont nitrogenase activity are highly sensitive to excess inorganic nitrogen in some actinorhizal plants (Hughes et al. 1968; Righetti and Munns 1981; Granhall et al. 1983; Righetti et al. 1986). In contrast, nodulated alders provided with ammonium or nitrate nitrogen can produce greater nodule and plant biomass than those without added nitrogen (Ingestad 1980). Lipp (1987) found that 100 mg nitrogen/l enhances nodulation and acetylene reduction rates over low (1 or 10 mg) or high (1000 mg) nitrogen additions. Adequate phosphorus availability is also required for nodulation and nitrogen fixation (Benoit and Berry 1990). Potassium, magnesium, and calcium are needed by some alders for maximum growth and nitrogen fixation (Pregent and Camire 1985). Salinity in soils is not a major concern for growing actinorhizal host plants because growth of *Frankia* and nodule development are not greatly affected by high salt concentrations (Dawson and Gibson 1987; Ng 1987).

Root nodules of red alder contain phenolic compounds (Li et al. 1972). Some of these compounds also exist in soil and understory species in stands of red alder (Li et al. 1970; Li 1974). Compounds such as ferulic, *o*-coumaric, *p*-coumaric, and caffeic acids inhibit the growth of *Frankia* isolates. Some phenolic compounds cause increased hyphal ramification, and others induce *Frankia* to form numerous spherical structures while not affecting vesicle formation (Perradin et al. 1983; Vogel and Dawson 1986). Vogel and Dawson (1986) suggested that plant phenolics may affect growth and development of *Frankia* in soil and in actinorhizal hosts by acting as chemical mediators in the growth regulation of *Frankia* within the root tissues from the stage of initial infection through the latter stage of its development within the root nodule (Perradin et al. 1983). Root exudates also can facilitate nodulation by stimulating spore germination and the colonization process.

Mycorrhizal Symbioses of Red Alder

"Mycorrhiza" translates literally as "fungus root" and represents the symbiotic association between

plant roots and specialized soil fungi (mycorrhizal fungi). The fungi act as extensions of the root systems and improve host nutrition by their ability to extract nutrients and water from a volume of soil hundreds to thousands of times greater than the volume roots alone can explore. Other host benefits include protection of fine roots from pathogens, increased drought-resistance, and increased root development and longevity. In return, the mycorrhizal fungi depend on the host for carbon, primarily in the form of simple sugars, and some vitamins. The mycorrhizal symbiosis has strongly coevolved over the millennia such that each partner depends on the other for survival and fitness in natural ecosystems. Readers are referred to Allen (1992), Harley and Smith (1983), and Safir (1987) for greater detail on mycorrhizae.

Only two of the several types of mycorrhizae are relevant to alder: ectomycorrhizae (EM) and vesicular-arbuscular mycorrhizae (VAM). The former (EM) are characterized by the formation of a sheath or mantle of fungus mycelium that surrounds the fine, feeder roots and by the intercellular penetration of the fungus between epidermal or cortical cells to form a network of host-fungus contact called the Hartig net. The fungi are typically basidiomycetes or ascomycetes (occasionally zygomycetes). In the Pacific Northwest, ectomycorrhizal hosts occur in the families Betulaceae, Ericaceae (*Arbutus* and *Arctostaphylos*), Fagaceae, Pinaceae, Rosaceae, and Salicaceae.

Vesicular-arbuscular mycorrhizae are formed by zygomycetes in the order Glomales (Morton and Benny 1990). Unlike EM, VAM do not cause differentiation of the roots (swelling and branching); roots must be stained to reveal the internal structures of the fungus colonization. The VAM fungi ramify within the cortical tissue, both intercellularly and intracellularly, forming balloon-shaped vesicles (storage organs) and arbuscules (multibranched, intracellular structures that function in nutrient exchange with the host).

Ectomycorrhizae of Red Alder

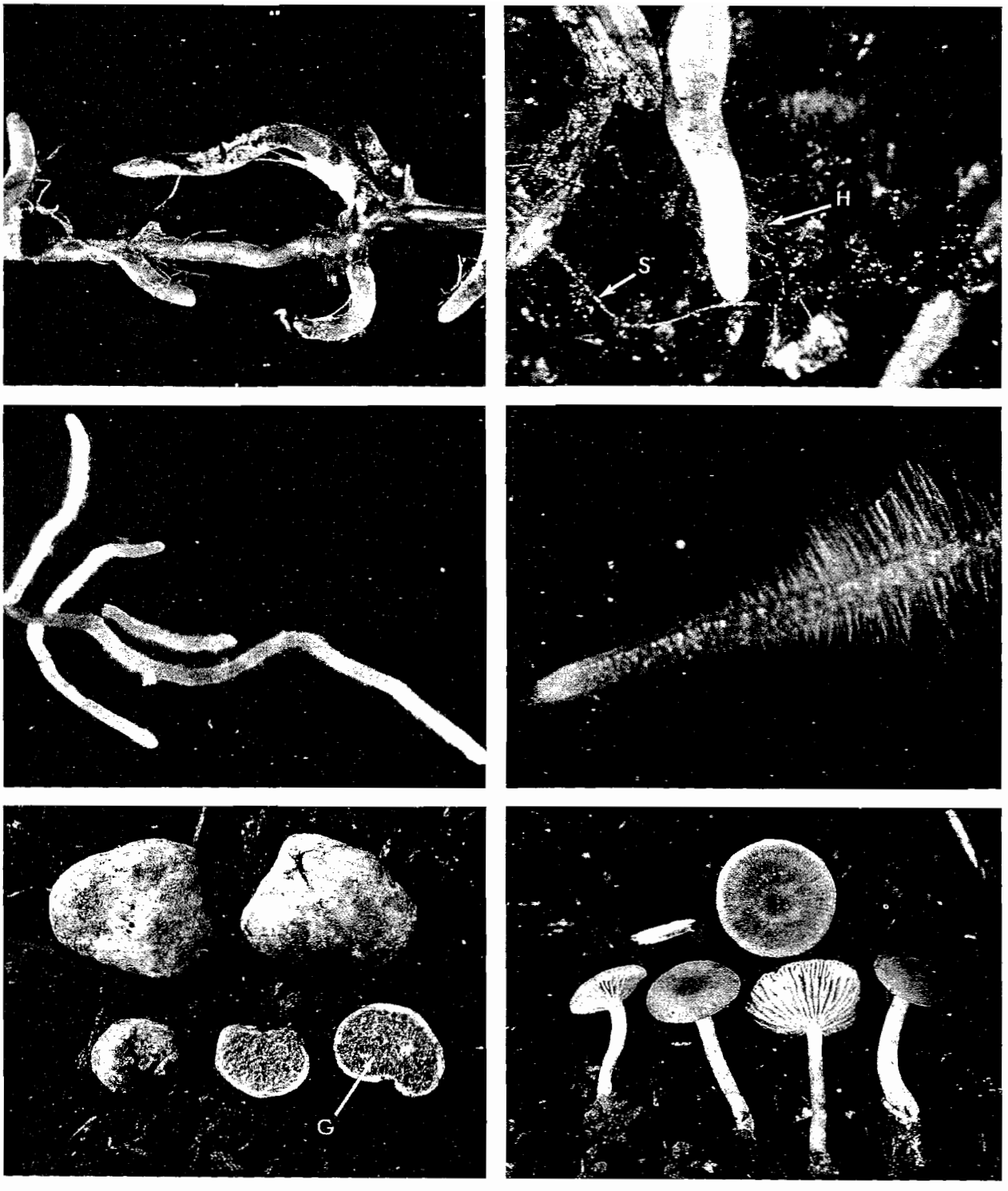
Ectomycorrhizae are by far the predominant type on red alder (Neal et al. 1968; Miller et al. 1991). Neal et al. (1968) described two EM types on red alder formed with unknown fungi; they referred to them as dark-brown clavate type and pale-brown glabrous type. Since that work, several EM fungi have been inoculated successfully onto red alder roots (Molina 1979; Miller et al. 1991) and additional suspected fungal associates have been noted from repeated occurrence of sporocarps (mushrooms and truffles) in association with red alder.

Miller et al. (1991) characterized many red alder EM from laboratory inoculations and field collected roots. They found two general morphological types: succulent (Figs. 6, 7) and flexuous (Fig. 8). The succulent EM type is short and thick, mostly determinate in growth with a rounded apex and thin or thick mantle; the Hartig net may penetrate only slightly or deeply and is well developed even near the root apex (Figs. 12, 13). The succulent type is typical of fungi host-specific to red alder. Flexuous EM typically are long and thin, seemingly indeterminate in growth, and usually have an acute root apex and thin mantle. The Hartig net commonly does not completely penetrate to the depth of one cell. Mantle and Hartig net are poorly developed at the apex. Flexuous EM are characteristic of fungi with broad host ranges.

Nonmycorrhizal short roots are rarely seen on red alders in nature. When red alder seedlings are grown in the laboratory or greenhouse, non-mycorrhizal short roots are densely covered with root hairs (Fig. 9). Red alder EM lack root hairs.

Miller et al. (1991) recognized 11 distinct EM types on red alder. The majority of fungal associates are basidiomycetes. Proven fungus associates include *Alpova diplophloeus* (Figs. 6, 7), *Lactarius obscuratus, Cortinarius bibulus, Thelephora terrestris* (Fig. 8), *Paxillus involutus,* and *Laccaria laccata.* Several species in the genera *Hebeloma, Inocybe, Naucoria,* and *Russula* are suspected EM fungus associates but have proven difficult to isolate and confirm in experimental mycorrhizal synthesis.

Of the fungi noted above, *Alpova diplophloeus* and *Lactarius obscuratus* are by far the most widespread. The former forms large, succulent types of EM on red alder that are pale brown to golden brown and darken with age (Figs. 6, 7); the tips show a distinct blue-bruising reaction (Miller et al. 1988). Miller et al. (1991) reported *A. diplophloeus* as "almost ubiquitous in the field" and particularly


H
S
G

Figures 6-11. Ectomycorrhizae and ectomycorrhizal fungi of red alder. Figures 7, 9, 10, and 11 are courtesy of Steven Miller, University of Wyoming.

Figure 6 (top left). Succulent ectomycorrhizae synthesized in pure culture between Alpova diplophloeus *and red alder. Hyphal strands emanate from the ectomycorrhizae on the left (7.5x).*
Figure 7 (top right). Ectomycorrhizal root tip of Alpova diplophloeus + *red alder on container-grown seedling. Hyphae (H) and hyphal strands (S) emanate from the ectomycorrhiza and ramify through the soil substrate (12x).*
Figure 8 (center left). Flexuous ectomycorrhiza formed by Thelephora terrestris + *red alder on container-grown seedling. The fuzzy appearance is due to the short, emergent hyphae on the mantle surface (3.5x).*
Figure 9 (center right). Nonmycorrhizal root tip of red alder showing abundant root hairs (10x).
Figure 10 (bottom left). Sporocarps of the truffle fungus Alpova diplophloeus. *The interior gleba (g) tissue bears abundant spores. Whole sporocarps can be macerated in water, and the resulting spore suspension used for seedling inoculation (1.5x).*
Figure 11 (bottom right). Sporocarps of mushroom fungus Lactarius obscuratus *(at natural size).*

abundant in mesic areas. The "dark-brown, clavate" red alder ectomycorrhiza described by Neal et al. (1968) likely was formed by *A. diplophloeus.*

Lactarius obscuratus also forms large, succulent EM, but they are pale yellowish orange to orange, with a smooth mantle surface typical of EM formed by other *Lactarius* species. *Lactarius obscuratus* EM also are abundant in the field. They correspond to the "pale, brown, glabrous" ectomycorrhizae described by Neal et al. (1968) and Froidevaux (1973).

The reader is referred to Miller et al. (1991) for detailed anatomical descriptions of these and other EM types of red alder; a key to field identification of red alder EM types also is provided. Massicotte et al. (1989a) provided additional detailed ontological characterization of red alder EM formed by *Alpova diplophloeus.* They emphasized that the EM morphology depends upon the stage of lateral root elongation at the time of fungal contact. Thus, some EM types may show a gradation between flexuous and succulent morphology. See Massicotte et al. (1989a, 1989b) for detailed light and electron microscopic descriptions of red alder + *Alpova diplophloeus.*

Host Specificity of Red Alder Ectomycorrhizal Fungi

Red alder, and alder species in general, form EM with comparatively few fungus species. For example, in contrast to Trappe's (1977) estimate of about 2000 EM fungi associated with *Pseudotsuga menziesii,* only 11 are known or suspected on red alder and less than 50 are known for the entire genus *Alnus* (Brunner et al. 1990; Miller et al. 1991). The reason is that most EM fungi of red alder are host specific to the genus. Molina (1979) attempted pure culture EM syntheses between 28 confirmed EM fungi and red alder; only four developed EM: *Alpova diplophloeus, Paxillus involutus, Scleroderma hypogeum,* and *Astraeus pteridis.* Several of the other 28 fungus species tested had typically broad host ranges when tested on eight species of Pinaceae (Molina and Trappe 1982a) yet were incompatible with red alder. In a related study, Molina (1981) found that four other *Alnus* species showed restricted fungus associates and hypothesized that the entire genus shows strong specialization regarding fungus associates.

It is important to note that both *Alpova diplophloeus* and *Lactarius obscuratus* are host specific to *Alnus.* Sporocarps of each species (Figs. 10, 11) occur exclusively with *Alnus.* Molina and Trappe (1982a, 1982b) and Massicotte and Molina (unpub. data) could not synthesize EM between *A. diplophloeus* and 10 species of Pinaceae and Ericaceae. Such close specialization of mycorrhizal association and predominance on root systems indicates a strong coevolutionary development and dependence between these symbionts (Molina et al. 1992).

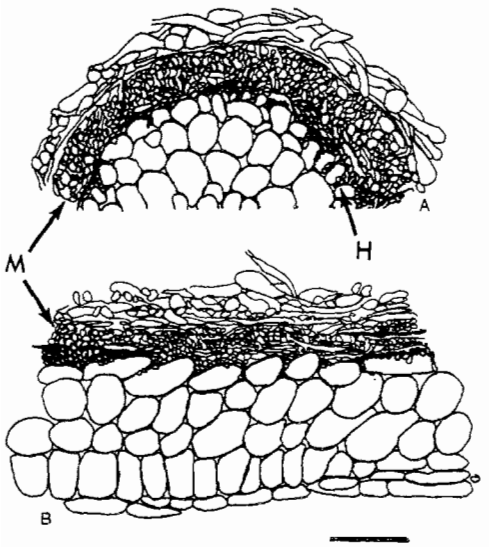


Figure 12. Cross section (A) and longitudinal section (B) of Alpova diplophloeus *+ red alder. Bar = 100 μM; M = mantle; H = Hartig net. Drawing courtesy of Steven Miller; previously published by Miller et al. (1991).*

Ecology and Distribution of Red Alder Ectomycorrhizae

The EM fungi of red alder are widespread in Oregon coastal forests and likely in other Pacific Northwest forest habitats wherever red alder is common on the landscape (Miller et al. 1992). Most of these fungi form mushrooms whose spores are dispersed by wind over great distances. The alder-specific *Alpova diplophloeus,* however, develops hypogeous (subterranean) sporocarps (truffles, Fig. 10) that are dispersed by small mammals. These mammals seek out the truffles by smell, dig them from the soil, consume them, and disperse the still viable spores in their fecal pellets (Maser et al. 1978). The Pacific Northwest is rich in truffle fungi (many hundreds of species), so this dispersal mechanism is common and highly successful. For example, Miller et al. (1992) noted that red alder seedlings develop abundant *A. diplophloeus* EM when grown in clearcut conifer forest soil; because *A. diplophloeus* is specific to red alder and therefore would not have been resident on previous conifer roots, the spores most likely were dispersed into the clearcut by mammals.

Miller et al. (1992) conducted a seedling greenhouse bioassay for EM fungal propagules using the same six forest soils noted previously for nodulation potential: young alder, old alder, conifer clearcut, conifer plantation, rotation-age conifer, and old-growth conifer. Red alder seedlings developed EM in all soils, with highest levels of colonization in young alder, old alder, and conifer clearcut soils; light to moderate levels of EM colonization occurred in plantation, rotation-age, and old-growth conifer soils. Development of EM also was delayed in the conifer soils, thereby indicating low initial inoculum potential. Five EM types developed during the course of the seedling bioassays. The highest diversity of EM types occurred in the clearcut conifer soil followed by the rotation-age conifer, young alder, and old alder soils; lowest EM type diversity occurred in the conifer plantation and old-growth conifer soils. *Alpova diplophloeus* was the most common and abundant type from all soils and also increased in abundance during the bioassay period, dominating the root system and displacing other EM types. This dominance and abundance by *A. diplophloeus,* especially in clearcut conifer soils, and competitive interactions with other EM fungi clearly emphasize the ecological importance of this host-specific fungus of red alder in the Pacific Northwest.

Vesicular-Arbuscular Mycorrhizae of Red Alder

The prevalence and ecological importance of VAM on red alder in Pacific Northwest forests is unclear. Rose (1980) reported VAM development on red alder growing in coastal Oregon and northern California forest habitats: VAM colonization ranged from 50 to 90 percent in the fine roots. Miller et al. (1992), however, found no VAM on red alder seedlings grown in soils from the six coastal forest soils noted above. They suggested that their soil bioassay may have negatively affected the VAM fungus inoculum or that the VAM fungi were

competitively excluded by EM fungi. Indeed, Rose (1980) was unable to develop greenhouse pot cultures of VAM fungi on red alder if EM fungi colonized first. She hypothesized that the EM present a physical barrier to VAM colonization. Development of VAM also has been reported on *Alnus incana* (Rose 1980; Arveby 1988, p. 173; Chatarpaul et al. 1989), *A. accuminata* (Russo 1989), *A. firma* (Lee 1988), *A. glutinosa* (Rose 1980; Fraga-Beddiar and Le Tacon 1990), and *A. sinuata* (Rose 1980); inoculation with *Glomus* spp. has increased growth and nutrient acquisition for some alders (see section on mycorrhiza-actinorhiza interactions).

Arveby (1988, p. 173) reported that VAM were present only during the first year of natural seedling establishment of *Alnus incana* in Sweden and that EM were dominant thereafter. Similar patterns of VAM to EM succession have been reported for other angiosperms that form both VAM and EM (Molina et al. 1992). The ecological advantage of this situation is that if either VAM or EM fungi are absent from the site, one can substitute for the other. The VAM fungi are widespread throughout Pacific Northwest forests, so they may sometimes be important in the early establishment of red alder seedlings. Thus, the natural development of VAM on red alder seedlings and mature trees, interactions with EM, and effects of VAM on plant nutrition need further study.

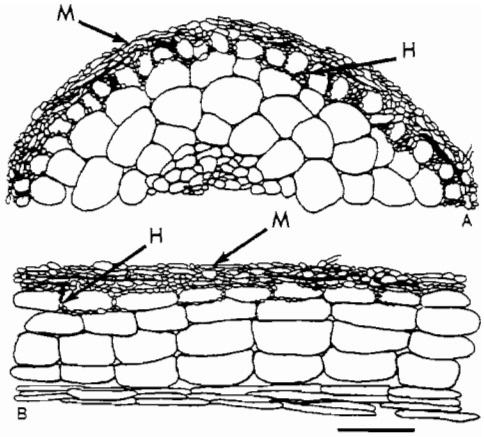


Figure 13. Cross section (A) and longitudinal section (B) of Lactarius obscuratus *+ red alder. Bar = 100 μM; M = mantle; H = Hartig net. Drawing courtesy of Steven Miller; previously published by Miller et al. (1991).*

Physiology and Host Benefits of Mycorrhizae

Mycorrhizae are most well known for improving phosphorus nutrition of host plants (Harley and Smith 1983). Mejstrik and Benecke (1969) found that EM of *Alnus viridis* absorb phosphorus five times as rapidly as non-mycorrhizal roots. Koo (1989) explored effects of light intensity, drought stress, and nitrogen and phosphorus fertilization on EM development of red alder by *Alpova diplophloeus* and subsequent effects on seedling growth and nutrition. Water stress decreased EM development and *Alpova diplophloeus* only enhanced red alder growth under well-watered conditions. Development of EM was greatest under strong light. Fertilization with nitrogen tended to enhance EM development and substitute for similar stimulating effects of nodulation (nitrogen fixation). Fertilization with phosphorus did not affect EM development. Overall, Koo (1989) found that EM formation by *Alpova diplophloeus* increased seedling growth and improved phosphorus status of the red alder seedlings. These mycorrhizal benefits were strongly dependent on the interaction with nodulation, however, and so are discussed in greater detail in the section on actinorhiza-mycorrhiza interactions.

The above studies by Miller et al. (1992) and Koo (1989) provide only a glimpse of the ecological and physiological contribution of mycorrhizae to the establishment and growth of red alder. Because mycorrhizae are essential to phosphorus acquisition for most plants, and because nitrogen fixation demands high phosphorus input, Trappe (1979) hypothesized that mycorrhizae of alder and other actinorhizal

plants are essential to the healthy functioning of these tripartite symbioses. Koo (1989) suggested that the function of mycorrhizae for red alder may become increasingly important as stands develop and soil phosphorus levels decline. Field studies are urgently needed to address these matters.

Interactions Between Mycorrhizae and Actinorhizae

Several studies report the existence of synergistic interactions between legumes, *Rhizobium,* and VAM (see reviews by Barea et al. 1992; Bethlenfalvay 1992). In general, dual-inoculated legumes grow better than legumes inoculated with either VAM or *Rhizobium* alone. The VAM typically enhance phosphorus uptake and *Rhizobium* enhances nitrogen nutrition. Similar results are evident in actinorhizal-VAM interactions with nonleguminous plants (Cervantes and Rodríquez-Barrueco 1992). Dual inoculation of *Ceanothus velutinus* with VAM and *Frankia* (crushed nodules) increases total dry weight of shoots and roots, number and weight of nodules, and nitrogen fixation compared to *Frankia* inoculation alone (Rose and Youngberg 1981). *Causuarina equisetifolia* inoculated with *Glomus mosseae* (VAM) and *Frankia* contains twice as much nitrogen and grows better than plants inoculated with *Frankia* alone (Diem and Gauthier 1982). *Hippophae rhamoides* displays significantly better growth, uptake of phosphorus, and nitrogenase activity in dual-inoculated versus nodulated- or mycorrhizal-only plants (Gardner et al. 1984). Berliner and Torrey (1989), however, showed that actinorhizal *Comptonia* and *Myrica* species grow as well as those with both root symbioses under greenhouse conditions.

Similar results apply to interactions among actinorhiza, mycorrhiza, and *Alnus. Alnus* firma seedlings inoculated with *Glomus mosseae* or with *G. mosseae* + *Frankia* weighed 27 and 83 percent greater than noninoculated control seedlings, respectively (Lee 1988). At low phosphorus fertilization (10 ppm) *A. acuminata* seedlings inoculated with *Glomus* intraradices + *Frankia* displayed acetylene reduction rates 87 percent higher than *Frankia*-only inoculated seedlings, although seedling fresh weights did not differ; at 50 ppm phosphorus, *Glomus* + *Frankia* increased leaf and nodule weight over *Frankia*-only inoculated seedlings (Russo 1989). Inoculation with VAM of nodulated *A. glutinosa* increased dry weight of nodules tenfold, doubled nitrogenase activity, and increased seedling growth compared to seedlings inoculated only with *Frankia* plus supplemental (50 ppm) phosphorus fertilizer (Fraga-Beddiar and Le Tacon 1990). Chatarpaul et al. (1989) examined tetrapartite interactions of EM-VAM-*Frankia* on growth of *A. incana* and found that inoculation with all three symbionts produced larger seedlings than either fungus or *Frankia* inoculation alone; inoculation with all three symbionts increased total seedling dry weight over both *Frankia* + EM and *Frankia* + VAM treatments. Both EM and VAM colonization increased in the presence of actinorhizal development.

Koo (1989) found actinorhizae and EM (inoculation with spores of *Alpova diplophloeus*) interacted significantly in affecting red alder seedling growth, nutrient status, and nitrogen-fixation rates. *Alpova diplophloeus* EM only enhanced growth and phosphorus tissue concentration of red alder seedlings when *Frankia* also was present, and then increases were typically less than 20 percent compared to *Frankia* inoculation alone. Formation of EM by *A. diplophloeus* typically did not enhance nodulation or nitrogen-fixation rates. *Frankia* strongly influenced EM development, however; Koo (1989) reported that in some experiments *Frankia* enhances EM formation by as much as six times over nonnodulated seedlings grown with supplemental nitrogen fertilization. Nonnodulated, non-nitrogen-fertilized red alder seedlings remained stunted and with only a trace of *A. diplophloeus* EM development. Koo (1989) also notes that nodulation preceded EM development in the field; in greenhouse studies, early nodule formation promoted more rapid EM development than when nodulation was delayed. Koo's data emphasize the importance of actinorhizal development and function in influencing red alder growth and EM development; actinorhizae appear the dominant root symbiosis in the tripartite symbiosis during the seedling stage because of the seedling's high nitrogen demand. Development of EM and physiological function likely follow once these nitrogen demands are met.

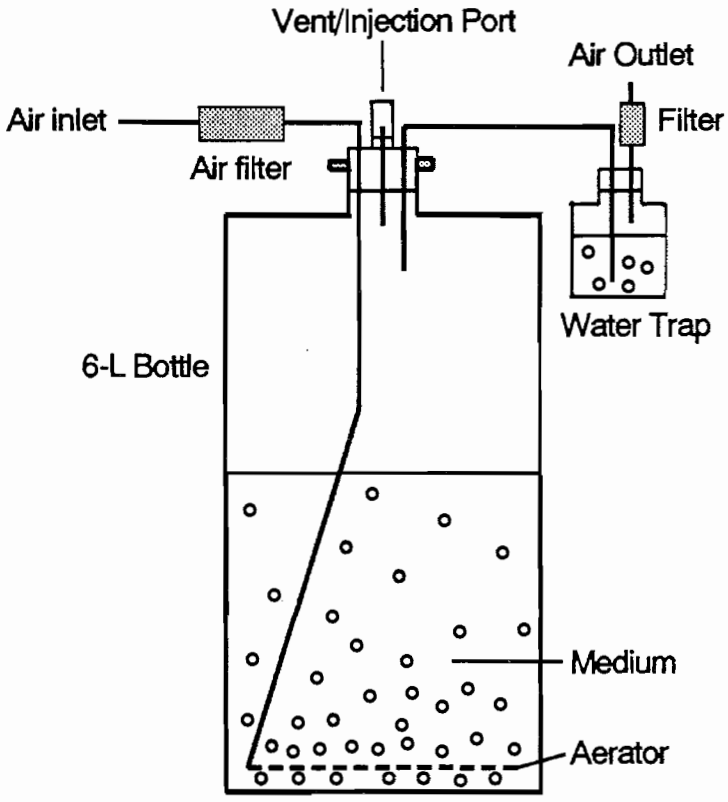


Figure 14. Batch culture apparatus for large scale production of Frankia, *suitable for production of inoculum for field use. The stopper in the 10-L bottle is held in place with two slotted steel plates, one below the bottle lip and one above the stopper, connected by four bolts and wing-nuts. The air inlet, air outlet, and vent are made from 1/8-inch stainless steel tubing and are inserted through the stopper. The air entering the bottle is passed through a filter consisting of foam plugs inside two 30-mL syringe barrels fitted onto a 20-mL syringe barrel and sealed with epoxy. This filter is autoclaved with capped needles at both ends to maintain sterility. The filter is attached to the air inlet via a Luer-lock fitting sealed to the end of the tubing. The other end of the air inlet tube is formed into an aerator by crimping the end and making a series of holes on one side with a triangular file and bending the tubing at a right angle. The air outlet is fitted with a small flask to catch condensate and an air filter to prevent contamination by back-flow. The top of the vent tube is inserted through the septum of a vacutainer tube so that it can be sealed after autoclaving. Under the vacutainer tube, a small length of glass tube, melted closed at one end, covers the end of the vent. This inner cap maintains sterility and allows for flame sterilization so that solutions may be injected through this port. BAP medium (6L) without phosphate buffer and propionate solutions are autoclaved separately in sealed serum bottles and later added through the vent.* Frankia *inoculum is also added through the vent, allowed to settle, and later siphoned from the media via the air inlet tube. The cells are concentrated from the remaining media by centrifugation.*

Management Implications

Nursery Inoculations with Frankia

Frankia inoculations have been developed primarily for experimental purposes, but with some modifications, these techniques can be used for nursery practices. What follows is practical advice on preparation of crushed nodule inoculum, an example of how production of pure culture *Frankia* can be scaled-up, guidelines for seedling inoculation, and a summary of the results of previous greenhouse and nursery inoculation trials.

Crushed nodule preparation. Healthy alder nodules are collected and used fresh, although storage at 4°C for a few weeks is acceptable; storage longer than a few weeks may result in nodule decay and growth of unwanted microorganisms. Nodules are cleansed of soil. Young nodule lobes are selected and homogenized in water in a blender (Martin et al. 1991; Wheeler et al. 1991), although more elaborate preparation procedures may be used to reduce possible adverse effects of released plant phenolics (Perinet et al. 1985). Inoculum amounts in the range of 1 to 6 g nodule material per 1000 seedlings produce good inoculation success in the greenhouse (Perinet et al. 1985) and field (Wheeler et al. 1991; Martin et al. 1992); amounts at the high end of this range are used for inoculating nursery beds. Crushed nodule inoculum can remain viable for at least one year when stored frozen at -20°C (Akkermans and Houwers 1979).

Large volume culturing methods. Scaling up from flasks to multiliter bottles is not particularly difficult and works well for producing large quantities of *Frankia.* Diem and Dommergues (1990) presented one such approach using a batch culture method. Diem et al. (1988) used a chemostat to develop mass inoculum.

Myrold et al. (unpub. data) used a batch culture approach similar to that of Diem and Dommergues (1990). Large batch culture of *Frankia* is achieved in a closed bottle with slow agitation by bubbles (Fig. 14). In this system, 1 liter of medium produces about 1 ml of packed cell volume (pcv—the volume of cells in a 1.5-ml microfuge tube that has been spun at 16,000 x *g* for 10 min.) in about one week and provides inoculum for 1000 to 10,000 seedlings.

Inoculation protocols. Although crushed nodules are effective inocula (Sheppard et al. 1988; Wheeler et al. 1991), many studies report pure culture inoculum to outperform crushed nodules (Perinet et al. 1985; Hooker and Wheeler 1987; Martin et al. 1991). Direct comparison between pure culture and crushed nodule inoculations is difficult, however, because of the difficulty in equating the two sources of inoculum. The selection of inoculum source must consider the relative availability of the two sources and the potential adverse affects of using crushed nodules. The latter may produce less uniform nodulation than pure cultures (Perinet et al. 1985) and also may be a source of pathogens (Stowers and Smith 1985).

Inoculum is best applied when seed is sown (Stowers and Smith 1985; Wheeler et al. 1991). Rates of 0.1 to 1.0 µl pcv per seedling prove adequate to nodulate seedlings in both the greenhouse (Berry and Torrey 1985; Stowers and Smith 1985) and field (Martin et al. 1991). Inoculum can be surface applied as a liquid suspension or injected into the soil. Although surface application has succeeded in nursery beds (Wheeler et al. 1991), Martin et al. (1991) found improved inoculation success when pure culture *Frankia* inoculum was mixed with peat moss. Using an inoculum stabilizer such as peat reduces the total amount of inoculum needed.

Effect of *Frankia* inoculation on seedling production. In all reported studies, inoculation improved seedling size and quality relative to noninoculated controls. This is likely always to be true in the greenhouse when sterilized potting mixes are used and in fumigated nursery beds. In nursery situations, a major benefit of inoculation is the early formation and functioning of nodules (Martin et al. 1991; Wheeler et al. 1991); noninoculated controls often become nodulated later in the growing season as inoculum invades from outside sources or as roots grow into soil not effectively fumigated. Inoculation of alders produces larger seedlings than noninoculated seedlings supplied with supplemental nitrogen (Hilger et al. 1991; Wheeler et al. 1991).

The performance of inoculated versus non-inoculated seedlings at outplanting has received little study. McNeill et al. (1989) measured better growth

of inoculated seedlings versus noninoculated seedlings over the first few seasons after outplanting, particularly on nutrient-poor soils. More studies of this sort are needed, especially to determine how long the apparent benefit of seedling inoculation lasts in nature where indigenous *Frankia* will begin to colonize the root system and interact with the introduced strain.

Nursery Inoculations with Mycorrhizal Fungi

Unlike the work cited above for *Frankia,* inoculation of red alder seedlings in nurseries with EM fungi has not received attention. Inoculation is possible, however, particularly with *Alpova diplophloeus.* Koo (1989) found that spores of this fungus were effective in inoculating red alder seedlings grown in containers in the greenhouse or growth chamber. He prepared slurry spore suspensions of homogenized sporocarps in water and added about 10 million spores in 5 ml water to each seedling; mycorrhizal colonization ranged from 35 to 90 percent of total short roots when inoculated concurrently with *Frankia.* Castellano and Molina (1989) reviewed the technology for operational mycorrhizal inoculation of container seedlings and reported high success of inoculating several species of Pinaceae with spores of EM fungi in the genus *Rhizopogon,* close truffle relatives of *A. diplophloeus.* In the most advanced inoculation system, spores of *Rhizopogon* are introduced through the irrigation mist system. It is likely that such a technology could work for introduction of *A. diplophloeus* spores onto red alder seedlings and could even be done in conjunction with introduction of *Frankia* cultures.

Much more research is needed to address the benefits of mycorrhizal fungi to red alder seedling nutrition, growth, and plantation performance before recommendations on the need for mycorrhizal inoculation can be made. Growth response of red alder seedlings to mycorrhizal inoculation is limited to data on only one symbiont (*Alpova diplophloeus*) and then only under growth chamber and greenhouse conditions in artificial soil mixes (Koo 1989). Trappe (1977) and Castellano and Molina (1989) outlined detailed research programs for selecting mycorrhizal fungi for inoculating seedlings based on nutritional benefits, ecological conditions of planting site, and ease of manipulating the candidate fungi. Although such research programs have been conducted for seedlings of Pinaceae in the United States and elsewhere, they are lacking for the most part on broad-leaf EM trees in North America. Given the positive benefits seen in EM inoculation programs elsewhere, it is likely that with adequate research, EM inoculation of red alder with selected, beneficial strains of EM fungi for use under prescribed ecological conditions could prove effective.

Two major considerations are important in deciding the need for mycorrhizal inoculation. First, does the fungus improve seedling growth or reduce cull percentage in the nursery? This can be answered only through a series of experimental inoculations in various nursery settings. Techniques that work in bare-root nurseries may not work in container nurseries. Secondly, does inoculation improve outplanting performance? Outplanting performance often is affected by the history of site disturbance and residual populations of mycorrhizal fungi. Because EM fungi are widespread for red alder (Miller et al. 1992), planted seedlings may quickly develop EM after out-planting. Thus, if adequate growth is obtained in the nursery with *Frankia* inoculation only, and seedlings quickly develop EM following outplanting, then inoculation with EM fungi may not be necessary. Inoculation with EM fungi may be necessary and beneficial, however, if the red alder seedlings are planted on severely disturbed sites or on sites far from natural red alder stands (e.g., plantations in agricultural settings) where natural populations of alder EM fungi are low or absent. Such need will require experimental validation of the low EM fungus inoculum potential of the sites and then development of the proper technology to inoculate seedlings with appropriate EM fungus plus *Frankia* symbionts.

Effects of Site Disturbance on Red Alder Root Symbioses

Few studies have examined direct effects of soil/forest disturbance on the presence and function of the root symbionts. The soil bioassay by Miller et al. (1992) clearly showed the widespread nature of both EM fungi and *Frankia* in a variety of forest

types in the Oregon Coast Range. They found that red alder forms as many nodules in a conifer plantation soil as in a young alder forest soil. Similarly, EM development was as great in conifer clearcut soil as in young alder soil as was the diversity of EM types. Development of EM and actinorhizae were delayed on seedlings grown in mature forest soil, but the symbioses did develop. The widespread dispersal characteristics of both root symbionts likely contribute to their quick development and physiological function for benefiting red alder establishment. Indeed, this pervasiveness likely contributes to the pioneering ability of red alder on disturbed sites.

Specific impacts of soil disturbances such as fire (e.g., hot, localized burns) or soil compaction have not been examined for effects on development and function of these symbioses. If such sites are planned for rehabilitation with red alder, then further study is warranted to decide the need for symbiont inoculation or amelioration of the disturbed site to allow the effective development and function of the symbioses.

Conclusions and Recommendations

Natural red alder seedlings typically develop both actinorhizae and mycorrhizae soon after seed germination in the field. Seedling studies indicate that red alder growth is highly responsive to nodule formation and subsequent nitrogen fixation. Mycorrhizal development likewise improves growth and phosphorus status, but during the first year of seedling growth, increases in growth due to ectomycorrhizal development are small compared to the actinorhizal benefit alone. It must be emphasized, however, that the benefits of ectomycorrhizae to red alder have barely been explored, and what is known is limited to experimental greenhouse studies with seedlings. The two symbioses also interact, the most notable result being the strong stimulation in mycorrhizal development as a result of early nodule formation.

Both fungal and actinomycete endophytes are widespread in forest soils throughout the Pacific Northwest wherever red alder is common on the landscape. This pervasiveness likely contributes to the pioneer colonizing ability of red alder. It also indicates the high survival and dispersal capabilities of the root symbionts.

Previous reviews of alder symbioses called for optimizing the use of root symbionts through development of technologies to inoculate seedlings with selected, beneficial strains. Such techniques are now available, particularly for inoculation with specific strains of *Frankia.* At this point, we recommend at least the inoculation of red alder seedlings in nurseries with *Frankia,* preferably with pure cultures of proven, effective isolates. Such an inoculation scheme will improve seedling growth in the nursery, and reduce both the percentage of culls and need for nitrogen fertilizer to compensate for the absence of *Frankia.* To optimize such a *Frankia* inoculation scheme, more work is needed on selecting strains based on ecological characteristics that will benefit seedling survival and growth on specific planting sites (i.e., matching *Frankia* strains to outplanting locations and environments).

The immediate need for inoculating with specific mycorrhizal fungi is less clear, primarily because of the strong effect of nodulation on first-year growth. Active research is needed similar to that conducted for conifers. Mycorrhizal fungi of red alder are widespread, even on recently cut over forest sites, so adequate mycorrhizal development likely will take place soon after seedling planting. Mycorrhizal inoculation may prove beneficial if seedlings are planted on sites distant from natural stands of red alder. Techniques to inoculate red alder with the alder-specific fungus *Alpova diplophloeus* are available.

The next logical step in developing these management tools is the installation of carefully designed nursery inoculation studies using a variety of *Frankia* and mycorrhizal fungus isolates and then outplanting these inoculated seedlings over a variety of planting sites.

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