AN ABSTRACT OF THE THESIS OF

Sharon Lee Rose (name) for the degree of Doctorate of Philosophy

in Soil Science presented on December 5, 1979

Title: Root Symbionts and Soil Microorganisms Associated With Actinorrhizal Plants

Redacted for Privacy

Abstract approved: (Major professor)

Symbiotic associations are established between non-leguminous (actinorrhizal) nitrogen-fixing flowering plants and two categories of microorganisms: mycorrhizal fungi and a filamentous actinomycete. The actinomycete induces nodule formation and produces nitrogenase, the enzyme responsible for the reduction of atmospheric nitrogen to a form available to higher plants. The mycorrhizal fungus is found both inter- and intracellularly in the root system, and may be found within the nodules. The two major nutrients, nitrogen and phosphorus, can be supplied to the host plant by means of these two symbiotic microorganisms.

Twenty-five species of flowering plants that fix atmospheric nitrogen in actinomycete-induced nodules were sampled for mycorrhizal associates. Both mycorrhizae and nodules were present on: (1) four species of Alnus; (2) two species of Casuarina; (3) eight species of Ceanothus; (4) four species of Myrica; (5) and one species

each of Shepherdia, Hippophae, Cercocarpus, Dryas, Purshia, Comptonia, and Datisca. Soil sieving revealed species of the following genera of vesicular-arbuscular (VA) mycorrhizal fungi; Gigaspora, Glomus, Acaulospora, and Entrophospora. The VA mycorrhizal fungi exhibited distinct distributional patterns when associated with actinorrhizal hosts in different habitats.

Mountain mahogany (Cercocarpus ledifolius Nutt.) is an actinorrhizal shrub native to Oregon and California. Nodulated seedlings along a roadbed in central Oregon were colonized by VA mycorrhizal fungi. Greenhouse seedlings inoculated with soil from this central Oregon site became nodulated and mycorrhizal within six months.

Snowbrush (Ceanothus velutinus Dougl.), an actinorrhizal shrub species native to the Pacific Northwest, is able to establish, grow, and improve infertile soil. The root system of snowbrush can be dually colonized. The possibility of a direct interaction between the endophytes in the symbiosis was investigated. Dually infected plants showed greater increases in total dry weight, number of nodules, nodule dry weight, increases in nitrogenase activity as measured by acetylene reduction, as well as higher levels of tissue nitrogen, phosphorus, and calcium than nodulated plants without mycorrhizae.

In assessing mycorrhizal associations of actinorrhizal plants, soil was sampled for Endogonaceae by wet sieving and decanting. Four new species of Glomus were isolated from under actinorrhizal shrubs in central Oregon and England: Glomus gerdemannii, G. halonatus, G. lacteus, and G. scintillans are described herein.

An actinomycete was isolated from the rhizoplane of nitrogen-fixing nodules of Ceanothus velutinus and was identified as an isolate of Streptomyces griseoloalbus. This isolate is a strong antagonist to three destructive root-rot pathogens: Phellinus weirii, Fomes annosus, and Phytophthora cinnamomi. This organism may confer protection to the nodule by presenting an antimicrobial barrier at the nodule-soil interface. The stability and longevity of the antimicrobial substance, its consistent effect on the pathogens on all substrates examined, and its ability to colonize wood suggest biological control possibilities for this organism in the Pacific Northwest.

Root Symbionts and Soil Microorganisms Associated With Actinorrhizal Plants

by

Sharon Lee Rose

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1980

APPROVED:

Redacted for Privacy

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Date thesis is presented December 5, 1979

Typed by: Mary Ann Airth

ACKNOWLEDGEMENTS

I wish to express my appreciation and extend a very sincere thank you to my friends and associates who participated in bringing this study to a conclusion: especially Dr. C.T. Youngberg who directed me to this area of investigation; Dr. J.M. Trappe who helped me with fungal taxonomy and encouraged me to pursue this aspect of my research; Drs. Chilcote, Hagedorn, Bottomly, and Vinson for cooperating and serving on my graduate committee; and Dr. D. Knutson and Mrs. Anita Hutchins for providing laboratory space at the Forestry Science Laboratory and constant support throughout my stay and study at Oregon State University.

I would also like to express my gratitude to the entire clerical staff at the Department of Soil Science for their help during my graduate studies.

The financial support for this project, made possible by the Agricultural Experiment Station, is gratefully acknowledged.

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CHAPTER 1

INTRODUCTION

The symbiosis of a flowering plant, a mycorrhizal fungus, and a nitrogen-fixing microorganism has been the subject of interest since 1896 when Janse first described such a tripartite symbiotic association between the legum Pitchecolobium montanum, a bacterium, and a fungus (Janse, 1896). Jones (1924) examined 18 species of nodulated legumes and found that 15 were colonized by vesicular-arbuscular (VA) mycorrhizae. Asai (1944) first suggested that mycorrhizae were a necessary precondition for effective nodulation in legumes.

The symbiosis in actinomycete-induced nodulating (actinorrhizal) plants is less well known than that of the Rhizobium-legum symbiosis. Legumes are the primary means of adding fixed atmospheric nitrogen to agricultural soil, whereas non-legumes are the major means of adding nitrogen to forests, bogs, and deserts in temperate regions of the world. About 160 species in eight families of flowering plants have been reported to fix atmospheric nitrogen by actinomycete-induced nodules (Torrey, 1978). These diverse plants are characteristically woody perennials common to ealy successional stages in areas low in combined soil nitrogen. The ectomycorrhizal fungal associates of three genera, Alnus, Cercocarpus, and Dryas, have been reported, but only a few actinorrhizal plants have been examined for endomycorrhizae.

From summer 1976 to spring 1979, 25 species of actinorrhizal angiosperms, representing eleven genera, were examined for nodules and mycorrhizae. The plants were collected from a variety of natural habitats over a range of edaphic and geographic conditions. The vesicular-arbuscular mycorrhizal fungal component of the tripartite symbiotic association was characterized and identified when possible. The number of endomycorrhizal fungal spores in the soil samples from around each host was counted.

Cursh (1974) and Daft and El Giahmi (1974, 1976) found that the weight of nodules, amount of nodular tissue, nitrogen and phosphorus content of the plant, concentration of leghaemoglobin, and rates of acetylene reduction were greater in mycorrhizal nodulated legumes than in nonmycorrhizal nodulated plants. Gates (1974) and Mosse et al. (1976) found similar results with species of Trifolium, Stylosanthes, and Centrosems, and noted that only the mycorrhizal plants were able to nodulate in severely phosphorus deficient soils. Carling et al. (1978) demonstrated that nitrogen-fixing capabilities of soybeans increased in response to added increments of phosphorus and/or mycorrhizal infection.

Harley (1970) suggests that dual symbiotic associations are particularly successful as primary colonizers due to their ability to compensate for the infertility of the habitat. There are eight species of nodulated Ceanothus in Oregon, all early colonizers in edaphically or climatically stressed sites. These shrubs contribute to the nitrogen balance of the ecosystem through their association with the endophytic nitrogen-fixing organism. Delwiche et al. (1965)

reported that snowbrush (Ceanothus velutinus Dougl.) can improve depleted soil by adding 60 Kg/ha/yr of nitrogen to the shrub community. Youngberg and Wollum (1976) have shown that accretion of nitrogen in the 0-23 cm depth of soil in a snowbrush stand was 556 Kg/ha in a 10-year period. In xeric forest habitats, snowbrush is able to establish, grow, and improve infertile soil.

It has been reported that the phosphorus and copper concentration in plant tissue influences the effectivity of nodulation and the rate of nitrogen fixation in the field (Hewitt, 1958). The intensity of mycorrhizal infection has been shown to positively influence the development of nodules and favors an effective symbiosis (Crush, 1974). The possibility of improved nutrition, enhancement of growth, and nitrogen fixation in response to dual infection of snowbrush was investigated in a greenhouse experiment.

In assessing the mycorrhizal associations of nonleguminous nitrogen-fixing plants, the soil was sampled for spores of vesicular-arbuscular (VA) mycorrhizal fungi. Four new species of VA mycorrhizal fungi were isolated from soil associated with the nitrogen-fixing shrubs. These spores were characterized and described and subsequently used as inoculum for mycorrhizal colonization of their actinorrhizal hosts.

In attempts to isolate the nitrogen-fixing endophyte, an orange pigmented actinomycete was repeatedly isolated from the rhizoplane of snowbrush nodules. As this isolate did not grow in a nitrogen-limiting medium, could not reduce acetylene when incubated in an atmosphere of the gas, and did not induce the formation of nodules of snowbrush

seedlings, it was concluded that this organism was not the nodule endophyte. However, this isolate did produce an antimicrobial substance inhibitory to three root-rot pathogens. Antibiotics are thought to be restricted to the rhizophere and rhizoplane where there is a higher concentration of roots and organic matter (Soulides, 1969). The occurrence of this actinomycete at the nodule rhizoplane could mean that the actinomycete is confering protection from pathogens at the nodule-soil interface.

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CHAPTER 2

MYCORRHIZAL ASSOCIATIONS OF SOME ACTINOMYCETE NODULATED NITROGEN-FIXING PLANTS [1/]

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ABSTRACT

Flowering plants that fix atmospheric nitrogen in actinomycete-induced nodules were sampled for mycorrhizal associates. Twenty-five species from 7 families (Betulaceae, Casuarinaceae, Myricaceae, Rhamnaceae, Rosaceae, Elaeagnaceae, and Datiscaceae) were examined. Samples included were from the United States, Japan, and England.

Both mycorrhizae and actinomycete-induced nitrogen-fixing nodules were present on: (1) four species of Alnus; (2) two species of Casuarina; (3) eight species of Ceanothus; (4) four species of Myrica; (5) and one species each of Shepherdia, Hippophae, Cercocarpus, Dryas, Purshia, Comptonia, and Datisca. Soil sieving revealed species of the following genera of Vesicular-Arbuscular mycorrhizal fungi:

1/ Technical Paper No. ____, Oregon Agricultural Experiment Station, Corvallis, OR.

Gigaspora, Glomus, Acaulospora, Entrophospora, and three undescribed taxa soon to be described. Spores of species in the first three genera of fungi were found most frequently from soil sievings. The VA mycorrhizal fungi exhibited distinct distributional patterns when associated with non-leguminous nitrogen-fixing hosts in different habitats. The ectomycorrhizae were not characterized.

INTRODUCTION

The symbiosis of a flowering plant, a mycorrhizal fungus, and a nitrogen-fixing microorganism has been the subject of interest since 1896 when Janse first described such a tripartite symbiotic association between the legume Pithecolobium montanum, a bacterium, and a fungus (Janse, 1896). Jones (1924) examined 18 species of nodulated legumes and found that 15 were colonized by vesicular-arbuscular mycorrhizal fungi. Asai (1944) first suggested that mycorrhizae were a necessary precondition for effective nodulation in many legumes. Crush (1974) and Daft and El Giahmi (1974; 1976) found that the weight of nodules, amount of nodular tissues, nitrogen and phosphorus content of the plant, concentration of leghaemoglobin, and rates of acetylene reduction were greater in mycorrhizal nodulated plants than in nonmycorrhizal nodulated plants. Gates (1974) and Mosse et al. (1976) found similar results with Trifolium, Stylosanthes, and Centrosem spp., and noted that only the mycorrhizal plants were able to nodulate in severely phosphorus deficient soils. Carling et al. (1978) demonstrated that the nitrogen fixing capability of soybeans increased in response to added increments of phosphorus fertilizer and/or mycorrhizal

infection, the mycorrhizal plants having five times more nodular dry weight than the nonmycorrhizal plants. It has been suggested that mycorrhizae aid in the uptake of phosphorus, sulfur, and the minor elements such as Zn, Co, and Cu (Mosse, 1973; Mosse et al., 1976; and Stark, 1971), and that enhanced nodulation and nitrogen fixation is a response to the improved nutrition of the host plant rather than a direct effect on the nitrogen uptake (Carling et al, 1978).

Nitrogen fixation by actinomycete-induced nodulating plants is less well known than that of the Rhizobium-legume symbiosis. Legumes are the primary means of adding fixed atmospheric nitrogen to agricultural soil, whereas non-legumes are the major means of adding nitrogen to forests, bogs, and arid areas in temperate regions of the world. Worldwide, about 160 species in 15 genera among 8 families of flowering plants have been reported to fix atmospheric nitrogen by actinomycete-induced nodules (Torrey, 1978). These diverse plants are characteristically woody perennials common to early successional stages in areas low in combined soil nitrogen. The actinomycete-nodulated plants are generally distributed in temperate regions on many soil types in varying ecosystems. Typically these plants are shrubs or small trees that invade disturbed areas and quickly establish pure stands.

The ectomycorrhizal associates of Alnus, Cercocarpus,, and Dryas spp., actinomycete-nodulated angiosperms, have been reported, but only a few actinomycete-induced nitrogen-fixing hosts have been examined for vesicular-arbuscular mycorrhizae (Truszkowska, 1953; Mejstrik, 1971; and Hall et al., 1979). Williams and Aldon (1976) reported vesicular-arbuscular (VA) mycorrhizal formations in Purshia

tridentata (Pursh) DC and Cercocarpus montanus Raff.; Kahn (1974) noted the presence of endomycorrhizal fungal spores in soil under Casuarina cunninghamiana Miq., although he did not find vesicular-arbuscular mycorrhizae.

From summer 1976 to spring 1979, 25 species of actinomycete-nodulating angiosperms, representing eleven genera, were examined for nodules and mycorrhizae. The plants were collected from a variety of natural habitats over a range of edaphic and geographic conditions. The VA mycorrhizal fungal component of the tripartite symbiotic association was characterized and identified when possible. The number of vesicular-arbuscular mycorrhizal fungal spores in soil samples from around each host was counted. The distribution of these spores and their nitrogen-fixing hosts is herein reported. The presence of ectomycorrhizae was noted, but the fungi were not characterized or identified.

METHODS AND MATERIALS

Plant root and soil samples were collected extensively in six physiographic provinces of Oregon and northern California: 4 sites on the coast and in the Coast Range; 2 sites in the Blue Mountains; 2 sites in the High Cascades; 4 sites in the High Lava Plains; and 2 sites in the Basin and Range province (Table 1: Franklin and Dyrness, 1973). Root and soil samples were collected from 5 plants at each study site. Main roots were traced from the crown until the first nodulated lateral root was reached. Ten samples of thin lateral roots were excised from the main laterals, placed in

collecting bags, sealed and stored at 5° C until examined. Soil (200 to 1000 g) was collected from the soil surface to a depth of 15 cm around the lateral roots and included rhizosphere soil when possible. The soil was stored in sealed paper bags at 5° C until analyzed for mycorrhizal spores and hyphae. In addition, collaborators in Alaska, Florida, Massachusetts, California, England, and Japan sent sealed plant and soil samples of actinomycete-nodulated plants and their rhizospheric soil.

To prepare the roots for assessment of mycorrhizal colonization, 20 fine root segements 1 cm in length were excised from the specimens collected, washed, cleared, and differentially stained with 0.05% trypan blue in lactophenol following a modification of the procedure of Phillips and Hayman (1970): heavy pigmentation in the roots require that root segments be bleached in 10% KOH under steam for a period of 5 to 10 min depending on thickness of the sample. Staining was also improved by placing the roots in small glass petri dishes (50 mm dia), adding trypan blue in lactophenol to cover the specimens, and placing the petri dishes under steam for 5 min. The root segments were mounted on microscope slides in clear lactophenol and examined under magnification for endomycorrhizal root structures: vesicles, arbuscules, and hyphae. A root segment was considered infected when one or more of these structures was observed. The percentage of VA infection was estimated by the root slide technique and calculated thus (Read et al., 1976):

$$\%\text{VA infection} = \frac{\text{No. of infected segments}}{\text{Total no. of segments examined}} \times 100$$

Table 1. Principal great soil groups within the physiographic provinces of Oregon where actinomycete-nodulated plant communities were sampled.

Province	Great Soil Groups-1938 System	Great Soil Groups-1967 System
High lava plains	Brown Chestnut Lithosol Regosol (pumice)	Haplargids Camborthids Vitrandepts
Basin and Range	Brown Chestnut Lithosol Regosol (pumice) Western Brown Forest	Haplargids Durargids Vitrandepts
Coast Range	Reddish Brown Lateritic Sols Bruns Acides Regosol Lithosol	Haplumbrepts Haplohumults
Western Cascades	Brown Podzolic Regosol Reddish Brown Lateritic	Haplumbrepts Xerumbrepts
High Cascades	Regosol Brown Podzolic	Vitrandepts Cryorthods
Blue Mountains	Western Brown Forest Regosol Lithosol	Argixerolls Vitrandepts

Thin sections of roots were prepared and stained for examination under light and scanning electron microscope to facilitate determinations and degree of mycorrhizal infection.

Spores of vesicular-arbuscular mycorrhizal fungi were recovered from 100 g soil subsamples from each collection by wet-sieving and decanting (Gerdemann and Nicolson, 1963) and by modified wet-sieve methods (Smith and Skipper, 1979). Spore populations were calculated from 100 g soil subsamples. Total counts from the sievings were made by picking out the spores from the soil while examining the sample under a dissecting microscope at 20x magnification. Illumination was by both transmitted and incident light as described by Mosse and Bowen (1968). Members of the Endogonaceae were determined from spore characteristics as detailed by Gerdemann and Trappe (1974), Hall and Fish (1979), and Nicolson and Schenck (1979).

RESULTS AND DISCUSSION

All of the 25 species of actinomycete-nodulated angiosperms were mycorrhizal (Table 2). Two species, Dryas drummondii Richardson from Alaska and Comptonia peregrina (L.) Coult. from Massachusetts were exclusively ectomycorrhizal. Of the 23 remaining species, 6 were infected by both ecto-and VA mycorrhizal fungi and 17 by VA mycorrhizal fungi only. Plants that were exclusively endomycorrhizal and the 6 species with both types of mycorrhizae had 50 to 90% or more of their fine roots colonized by the VA mycorrhizal fungus. Hyphae of the VA mycorrhizal fungus was found in roots supporting nodules, and often in Alnus rubra Bong. and Ceanothus velutinus

Table 2. Actinomycete-nodulated host, location, and the associated mycorrhizal fungi.

Host (# plants examined)	Location	Mycorrhizal Association	Vesicular-Arbuscular mycorrhizal Symbionts (Ave. total # spores/100 g)
BETULACEAE			
Alnus rubra (20)	Sand dunes, coastal Oregon and northern California	Ec, VA	*Acaulospora elegans*, *A. Laevis*; *Gigaspora calospora*, *G. pellucida*, *G. margarita* (> 100)
Alnus glutinosa (5)	Sandy alluvium, England	VA	*Glomus* spp. (15)
Alnus incana (5)	Vitrandept (pumice soil) eastern Oregon	VA	*Glomus* spp. (< 10)
Alnus sinuata (5)	Sandy river alluvium, eastern Oregon	Ec, VA	*Gigaspora calospora*, *Glomus* sp. (5)
MYRICACEAE			
Myrica californica (10)	Sand dunes, coastal Oregon and northern California	Ec, VA	*Acaulospora elegans*, *A. laevis*; *Gigaspora calospora*, *G. margarita*; *Glomus macrocarpus* v. *geosporus* (> 100).
Myrica gale (5)	Peat and sphagnum bogs	VA	Unknown
Myrica cerifera (4)	Sandy flatwoods, Florida	Ec, VA	*Gigaspora calospora*, *G. heterograma*; *Glomus monosporus* (10)

Table 2. cont.

Host (# plants examined)	Location	Mycorrhizal Association	Vesicular-Arbuscular mycorrhizal Symbionts (Ave. total # spores/100 g)
Myrica pennsylvanica (2)	Sandy woodlands, Massachusetts	VA	Glomus monosporus (< 10)
Comptonia peregrina (2)	Mine refuse and tailings, Massachusetts	Ec	none
ROSACEAE			
Purshia tridentata (20)	Volcanic ash and sand, Central Oregon	VA	Gigaspora calospora; Glomus gerdemannii, Glomus lacteus sp. nov* Glomus scintillans sp. nov* (16)
Dryas drummondii (2)	Glacial outwash, Alaska	Ec	none
Cercocarpus ledifolius (20)	Loamy sand and rock/out crops Central Oregon	VA	Glomus scintillans sp. nov (10)
DATISCACEAE			
Datisca glomerata (4)	River alluvium, central California	VA	Glomus sp. (< 5)
CASUARINACEAE			
Casuarina equisetifolia (4)	Marine sand dunes, Florida	VA	Gigaspora gigantea G. nigra (100)

Table 2. cont.

Host (# plants examine)	Location	Mycorrhizal Association	Vesicular-Arbuscular mycorrhizal Symbionts (Ave. total # spores/100 g)
Casuarina gunninghamiana (4)	Sandy woodlands Florida and Japan	VA	*Gigaspora coralloidea*, *G. gigantea*; *Glomus macrocarpus* v. *geosporus* (10)
ELAEAGNACEAE			
Hippophae rhamnoides (6)	Sand dunes, Gibralter Point, England	VA	*Acaulospora elegans*; *Glomus halonatus* sp. nov.* (75)
Shepherdia canadensis (5)	Volcanic ash and	Ec,VA	*Glomus* sp. (< 5)
RHAMNACEAE			
Ceanothus corduiatus (5)	Volcanic ash, western Oregon	VA	Unknown
Ceanothus cuneatus (5)	Serpentine soil, southwestern Oregon	VA	*Gigaspora calospora* (10)
Ceanothus integerrimus (10)	Serpentine alluvium, southwestern Oregon	VA	*Glomus* sp. (< 5)

Table 2. cont.

Host (# plants examined)	Location	Mycorrhizal Association	Vesicular-Arbuscular mycorrhizal Symbionts (Ave. total # spores/100 g)
Ceanothus prostratus (10)	Pumice and volcanic ash, western Oregon	VA	*Glomus* sp. (< 5)
Ceanothus pumilus (5)	Serpentine colluvium, southwestern Oregon	VA	*Clomus* sp. (< 5)
Ceanothus sanguineus (12)	Pumice and ash, western Oregon	VA	*Acaulospora* sp. (5)
Ceanothus thyrsiflorus (8)	Sandy soil, coast of southern Oregon and northern California	VA	*Glomus macrocarpus* v. *geosporus*, *G. monosporus*
Ceanothus velutinus (35)	Pumice and sandy loam, central Oregon, northern California	VA	*Gigaspora calospora*; *Glomus gerdemannii*, *G. lacteus* sp. nov.* *G. halonatus* sp. nov.* (20)

*Rose, S. and J.M. Trappe. Three new Endogonaceae associated with actinorrhizal shrubs. Manuscript in preparation.

Dougl. the hyphae extended into the nodular tissue of young nodules (5 mm dia.). In plants colonized by both ecto- and VA mycorrhizal fungi, VA mycorrhizal structures were found in the cortical cells and ectomycorrhizal hyphae, and mantles, and Hartig net surrounded the external surface of the root. It did not appear that colonization of one type of mycorrhizal fungus excluded infection by the other, however, pot culture experimentation with Alnus rubra suggest that if ectomycorrhizal fungi colonize first, a physical barrier to VA mycorrhizal penetration is established. All VA mycorrhizal structures were observed: hyphal haustoria and appresoria, simple and coiled hyphae, vesicles, and arbuscules. Not all characteristir structures were present in each host plant however. The presence or absence of a fungal structure seemingly varied with endophyte species, host, as well as soil type and soil moisture regime. This difference in colonization and structures present was most pronounced in 3 species of Myrica examined (Table 3): Myrica cerifera L. from the east coast exhibited thick hyphae and thick hyphal coils in the cortical root cells; Myrica gale L. from peat and sphagnum bogs was colonized by a fungal endophyte, possibly Glomus tenuis (Greenall) Hall, that produced many small vesicles in the central cortex and sparce fine inter- and intracellular hyphae; Myrica californica Cham. & Schlecht. from the sane dunes of the Pacific Northwest typically was both ecto- and VA mycorrhizal and root surfaces not colonized by extomycorrhizal fungi supported extramatrical vesicles commonly observed with species of Gigaspora.

Table 3. VA mycorrhizal colonization differences in three species of Myrica.

Host	Thick Hyphae (3 um) Hyphal Coils	Thin Hyphae (3 um)	Intracellular Vesicles (20 um dia)	Extracellular Vesciles
Myrica cerifera	X			
Myrica gale		X	X	
Myrica californica	X			X

The number of VA mycorrhizal fungal spores from the soil was low (from 1 to 10/100 g) in all natural wildland habitats except the marine sand dunes in the Pacific Northwest and in Florida, where numbers exceeded 100 spores per 100 g soil. Although relatively high for natural habitats, this figure is low when compared to the spore population in excess of 400/100 g soil reported by Nicolson (1974) for wheat fields and populations exceeding 1000 spores per 100 g soil for cultivated soil in Nigeria (Redhead, 1971). Low spore numbers have been reported under native perennial vegetation in New Zealand and Australia (Mosse and Bowen, 1968) and in Oregon by Gerdemann and Trappe (1974). In contrast, in this survey soil under Purshia tridentata growing adjacent to Milliken Flats, Oregon, supported high and diverse spore populations as compared to the populations associated with the same host at other locations. This difference could be related to the heavy grazing by cattle that occurs in this area, converting a wildland habitat to one of range, cultivated by animal activity.

The VA mycorrhizal fungi found associated with each plant host are listed in Table 2. The Glomus spp. were found in 16 of the 25 plant communities examined and was the most frequently isolated genus from soil samples. Gigaspora spp. were found in 9 of the 25 community-types; Acaulospora spp. occurred in only 3 of the sites. Williams and Aldon (1976) found Gigaspora spp., Glomus spp., and Acaulospora spp. to be common in four arid, wildland areas of New Mexico. No sporocarps were found in any site in this survey,

although Glonus monosporus and G. gerdemannii can be sporocarpic. Mosse (1973) reported that sporocarpic species were not found in logged areas she examined.

Gigaspora calospora (Nicol. & Gerd.) Gerdemann and Trappe was associated with four hosts in this study, sampled from a wide range of habitats. Its distribution in the east in coastal soils and in volcanic soils in the western United States and its association with Alnus, Purshia, Ceanothus, and Myrica spp. suggest little specificity to host, geography, and edaphic influences. Glomus macrocarpus variety geosporus (Nicol. & Gerd.) Gerdemann and Trappe was the next most commonly isolated spore. It was associated with Casuarina cunninghamiana in Florida and Japan, with Ceanothus thyrsiflorus Esch. in southern Oregon, and with Myrica californica in the coastal dunes of Oregon and northern California. Read et al. (1976) found a Glomus matching the description of G. macrocarpus in all the soils they examined in a large scale vegetation survey of the Sheffield region in England, and Hayman (1970) reported spores of Glomus macrocarpus in all of his study plots at Rothamsted, England.

Soil under Alnus rubra and Myrica californica in the marine sand dunes of Oregon and northern California supported high and diverse populations of Endogonaceous spores; five or more distinct species were isolated from these communities. Similarily, Koske et al. (1975) recovered over five species of endomycorrhizal species from the sand dunes near Lake Huron. Dune systems of England and Florida, respective habitats of Hippophae and Casuarina in this survey, were

characterized by low population density and species diversity. Nicolson (1960) and Redhead (1971) observed a similar low species diversity and corresponding low spore numbers in sand dunes of Gibralter Point and in moist forests of Nigeria. Mosse and Bowen (1968) suggest that the low spore counts in the sand dune soil of the east coast of Australia were in response to high soil moisture.

In habitats of the arid regions in central and western Oregon, in soil derived from pumice and volcanic ash, very low spore numbers were common and generally only one spore type was associated with each plant community, spores thus being confined to one host or habitat. For example, Glomus gerdemannii Rose, Daniels, and Trappe, has only been observed in pumice soil, associated with Ceanothus velutinus and Purshia tridentata (Rose et al., 1979). It is not clear whether this fungus is specific to host or to soil type and habitat, although pot-culturing work with Ceanothus velutinus seedlings indicates host specificity. Gigaspora margarita Becker and Hall was found only in the marine sand dunes of the Pacific Northwest and has not been previously isolated from soils in Oregon (Trappe, personal communication). Its occurrence at the coast may relate to the moisture regime and sandy soil. Three new taxa in the Endogonaceae were isolated from sand dunes in England and pumice soil of central Oregon (Rose and Trappe, in preparation).

The actinomycete-nodulated angiosperms are exceptional in their ability to invade disturbed, marginal habitats. All the nodulated plants in this survey occupy a pioneer niche in logged, mined, sand dune, or otherwise disturbed sites. All the plants examined in this

study were mycorrhizal, 23 of the 25 species being VA mycorrhizal. In contrast, investigators east of the Rocky Mountains report a lack of VA mycorrhizal colonization in pioneer non-nodulating weeds and grass species in severely disturbed communities in Wyoming and Colorado (Reeves et al., 1979; and Miller, 1979). Schramm (1966) observed that mycorrhizal plants not capable of fixing atmospheric nitrogen were absent from coal waste reclamation sites and that the nodulated plants were all mycorrhizal. The results of this survey agree with his findings. For certain habitats, a tripartite association, including a photosynthesizing green plant, a nitrogen-fixing endophyte, and a mycorrhizal fungus capable of maximizing nutrient uptake, may be essential for the successful natural invasion of stressed sites.

ACKNOWLEDGEMENTS

I would like to thank the following people who supplied materials for this study: K. Cromack, P. Dunn, A. Harris, L. Morris, B. Mosse, W. Pritchett, C. Schwintzer, W. Silver, W. Tiffney, J. Torrey, L. Viereck, L. Winship, and C. Youngberg. I would also like to thank Dr. J. Trappe for fungal species confirmation and identification and Dr. C. Youngberg for help in locating study sites and host plants. Special gratitude to Ms. D. Duff for her excellent thin section preparations and SEM microscopy done at the Forestry Sciences Laboratory, Corvallis, Oregon, 97331.

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CHAPTER 3

TRIPARTITE ASSOCIATIONS OF MOUNTAIN MAHOGANY: VA MYCORRHIZAE AND ACTINOMYCES [1/]

S. L. Rose and C. T. Youngberg [2/]

ABSTRACT

Mountain mahogany (Cercocarpus ledifolius Nutt) seedlings that had seeded in along a road bed from a stand in central Oregon were examined for the presence of nodules. Nodules were observed on 75% of the seedlings observed in September, 1978 and on 40% of those observed in May, 1979. Both nodulated and non-nodulated seedlings were colonized by vesicular-arbuscular mycorrhizal fungi.

Additional key words

Cercocarpus ledifolius, nodulation, vesicular-arbuscular mycorrhizae.

INTRODUCTION

Cercocarpus, one of 15 genera among eight families of actinomycete-nodulated plants (Torrey, 1978), is represented by 20 species.

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Vlamis et al. (1964) reported the occurrence of nodules on C. betuloides Nutt. Hoeppel and Wollum (1971) reported nodulation in C. montanus Raff. and C. paucidentatus Butt. Nodules have been observed on mountain mahogany (C. ledifolius Nutt.) in the Mahogany Mountains of eastern Oregon (Personal communication, E. Dealy, 1978). Non-nodulated C. ledifolius seedlings from a stand in central Oregon were transplanted into pots of "high nodulation potential" soil (Youngberg and Wollum, 1970). After six months in the greenhouse, 46% of the plants were nodulated (Youngberg and Hu, 1972).

Trappe (1964) observed that the ectomycorrhizal fungus Cenococcum graniforme forms mycorrhizae with Cercocarpus ledifolius in the Pacific Northwest. Cercocarpus montanus from sites in New Mexico was ectomycorrhizal and C. paucidentatus Britt. can be both nodulated and ectomycorrhizal under greenhouse situations (Hoeppel and Wollum, 1971).

As part of a survey of the mycorrhizal association with actinomycete-nodulated flowering plants, several stands of C. ledifolius in central and eastern Oregon were examined and sampled for nodules and mycorrhizae.

METHODS AND MATERIALS

In September, 1978, and in May, 1979, a total of forty seedlings was sampled from the same stand sampled by Youngberg and Hu (1972) in Central Oregon. The stand is on soil of the Waha series, a fine loamy, mixed Pachic Haploxeroll (Table 1). The presence of nodules was noted and the seedlings were then placed in paper bags, sealed, and returned to the laboratory for analysis for mycorrhizal fungal

Table 1. Some properties of the Waha soil series.

Horizon	Depth	pH	P	Total N	OM	CEC	NO_3-N
	cm		ppm	%	%	me/100g	ppm
A	0-20	6.6	36	.10	3.35	23	11.4
B1	20-30	6.5	14	.07	1.75	22	4.2
B2	30-56	6.6	10	.04	.82	22	4.9

Table 2. Nodulation and number of spores of VA mycorrhizal fungi associated with roadside seedlings.

Collection period	% nodulation	VA mycorrhizal fungal spores (Total # spores/100 g soil)
September, 1978	75	9
May, 1979	40	11

colonization. Soil (1000 g) was collected from the surface to a depth of 15 cm around the main roots, stored in sealed paper bags and returned to the laboratory for analysis for fungal spores.

To assess for mycorrhizae, the entire root systems of the seedlings were washed, cut into 1 cm segments, cleared and differentially stained with 0.05% trypan blue in lactophenol following the procedure by Phillips and Hayman (1970). The root segments were mounted on microscope slides in clear lactophenol to assess for the presence of vesicles, arbuscules, hyphae, mantles and Hartig net for vesicular-arbuscular (VA) and ectomycorrhizae.

Spores of vesicular-arbuscular fungi (VA) were recovered from 100 g soil subsamples by wet-sieving and decanting (Gerdemann and Nicolson, 1963).

Seeds of C. ledifolius were surface sterilized in 25% H_2O_2 for 20 min and rinsed in sterile, distilled water. The seeds were asceptically transferred to sterile water agar slants and stored at 4° C for 2 months. After germination 10 seedlings were transferred to 4 inch greenhouse pots with field soil collected from the stand and grown for 6 months in the greenhouse under a 16 hr light regime.

RESULTS AND DISCUSSION

Nodules were observed on 75% of the seedlings sampled in September, 1978 and on 40% of the young plants observed in the May, 1979 collection period (Table 2). The seedlings were approximately 1 year old at the time of sampling and the nodules were too young to demonstrate the coralloid nodular morphology as reported by Youngberg and

Hu (1972) from greenhouse grown plants, rather the nodules were dichomously branched and commonly composed of several lobes.

Williams and Aldon (1976) and Rose (Mycorrhizal associations of some actinomycete nodulated nitrogen-fixing plants. M.S. in preparation) report the occurrence of VA mycorrhizae on C. montanus Raf. and C. ledifolius, respectively. The young plants examined in this survey, from both collecting periods, were VA mycorrhizal. No ectomycorrhizal structures were observed.

Spores of the genus Glomus were recovered from the soil subsamples. Only one spore type was recovered. These spores occurred in low populations of approximately 10/100 g of soil. The spores were not recognized as being from a known species. Subsequently, it has been determined as Glomus scintillans sp. nov. Rose and Trappe (Rose, S. and J.M. Trappe. Three new Endogonaceae associated with actinorrhizal shrubs: Glomus halonatus, Glomus lacteus and Glomus scintillans. Manuscript in preparation). This species has also been found in association with Purshia tridentata (Pursh) DC, another nodulated member of the Rosaceae native to the ponderosa pine and high desert regions of Oregon.

Fifty percent of the seedlings transplanted to field collected soils were nodulated at the end of six months. This suggests that the nodule forming endophyte does survive in the soil and is some what effective at initiating nodulation when soil is used as the inoculum.

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CHAPTER 4

TRIPARTITE ASSOCIATIONS IN SNOWBRUSH (*CEANOTHUS VELUTINUS*): EFFECT OF VA MYCORRHIZAE ON GROWTH, NODULATION, AND NITROGEN FIXATION [1/]

S. L. Rose and C. T. Youngberg [2/]

ABSTRACT

Symbiotic associations were established between nitrogen-fixing nonleguminous (actinorrhizal) snowbrush (*Ceanothus velutinus* Dougl.) seedlings and two categories of microorganisms: vesicular-arbuscular (VA) mycorrhizal fungi and a filamentous actinomycete capable of inducing nodule formation. The actinomycete is housed in nodules where fixation of atmospheric dinitrogen occurs and is made available to the host plant; the mycorrhizal fungus is both inter- and intracellularly within the root tissue, and may be found within the nodules. The two major nutrients, N and P, can be supplied to the host plant by means of these two symbiotic microorganisms. The root system of snowbrush seedlings were dually colonized by VA mycorrhizal fungi and a nitrogen-fixing actinomycete and the possibility of a direct interaction between the endophytes in the symbioses was investigated.

[1/] Technical Paper No. _____. Oregon Agricultural Experiment Station, Corvallis, Oregon.

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Dually infected plants showed increases in total dry weight of shoots and roots, number of nodules, weight of nodular tissue, as well as higher levels of N, Ca, and P, and an increase in nitrogenase activity as measured by acetylene reduction.

INTRODUCTION

Legumes are commonly tripartite associations (Jones, 1924; Asai, 1944). Asai (1944) first suggested that mycorrhizae were a necessary precondition for nodulation in many legumes. Crush (1974) and Daft and El Giahmi (1974, 1976) found that plant dry weight, weight of nodules, amount of nodular tissue, N and P content, concentration of leghaemoglobin, and rate of acetylene reduction were greater in mycorrhizal nodulated plants than in nodulated-only plants.

In alfalfa, beans, and peanuts, shoot and root size, weight of fruit, and numbers of fruit were significantly higher in mycorrhizal-nodulated plants than in nodulated-only plants (Daft and El Giahmi, 1976). Carling et al. (1978) demonstrated that the nitrogen-fixing capability of soybeans increased in response to added increments of P or mycorrhizal infection, the mycorrhizal plants having five times more nodular dry weight than the non-mycorrhizal plants. It has been suggested that mycorrhizae aid in the uptake of P, S, and minor elements such as Co, Cu, and Zn (Mosse, 1973; Mosse et al., 1976; Stark, 1971) and that enhanced nodulation and nitrogen-fixation is a response to improved nutrition of the host rather than a direct effect of the nitrogen uptake by the host (Carling et al., 1978; Crush, 1974).

Hewitt (1958) reported that the P and Cu concentration in plant tissue influences the infectivity of the Rhizobium strain and the rate of nitrogen fixation of legumes in the field. The intensity of mycorrhizal colonization positively influences development of nodules in legumes and favors an effective symbiosis (Crush, 1974).

The symbiosis in actinorrhizal plants is less well known than that of the Rhizobium-legume interaction. About 160 species in 15 genera representing 8 families of flowering plants have been reported to fix atmospheric dinitrogen in actinomycete induced nodules (Torrey, 1978). As with legumes, actinorrhizal plants are typically tripartite associations; 25 species of nodulating actinorrhizal plants representing 11 genera were examined and found to be colonized by either ectomycorrhizal fungi, VA mycorrhizal fungi, or both ecto- and VA mycorrhizal fungi (Rose. Mycorrhizal associations of some actinomycete nodulated nitrogen-fixing plants. Manuscript in preparation).

Harley (1970; 1973) suggests that dual symbiotic associations are particularly successul as primary colonizers due to their ability to compensate for the infertility of the habitat and may be well adapted to habitats low in nitrogen and phosphorus. The efficiency of colonization of a marginal habitat depends on the plant, the physical parameters of the environment, the infectivity of the nitrogen-fixing endophyte, the rate of nitrogen fixation, and the degree of dependence on mycotrophy by the invading plant.

Eight species of nodulating Ceanothus occur in Oregon, all early colonizers in edaphically or climatically stressed sites. These shrubs contribute to the nitrogen balance of the ecosystem through

their associations with the endophytic nitrogen fixing microorganisms. Delwichs et al. (1965) reported that C. velutinus can improve depleted soil by adding up to 60 kg/ha/yr of nitrogen to the shrub community. Youngberg and Wollum (1976) have shown that accretion of nitrogen in the 0-23 cm depth of soil in a C. velutinus stand was 556 kg/ha in a 10-year period. In xeric forest habitats, particularly on logged and burned sites, C. velutinus is able to establish, grow, and improve infertile soil.

Our objective was to investigate the enhancement of growth and nitrogen fixation rates in response to dual infection of C. velutinus along with the effects of VA mycorrhizal formation on nitrogen fixation and growth in nodulated and non-nodulated snowbrush.

METHODS AND MATERIALS

Seed

Seeds were collected from mature Ceanothus velutinus plants at the Fort Benham site, Deschutes Co., central Oregon, at an elevation of 1400 m. Seeds were hulled by hand, rinsed in water, and placed in a plastic vial in which holes had been bored. Snowbrush seeds require a heat treatment followed by cold stratification to induce germination (Quick, 1935). To ensure this, the seed vial was placed in one liter of water at 95° C and immediately allowed to come to room temperature and imbide water for 24 hours. After imbibition, the seed vial was transferred to a container with 30% hydrogen peroxide and placed on a reciprocal arm shaker for 20 min, then aseptically rinsed with 3 liters of sterile distilled water. Individual

seeds were transferred to PDA slants (10 g/l Bacto Agar) in 15 ml vials, four seeds per vial; the seeds were imbedded 1/3 of their length into the agar. Vials were stored in the dark at 5° C for 90 days.

Soil

Soil samples (200 g) were collected from the Fort Benham site and returned to the Corvallis laboratory and stored at 2° C until processed. For extracting chlamydospores of VA fungi from soil samples, the soil subsamples (50 g) were washed through a series of fine mech screens. Particles ranging from 600 um to 2 mm resulting from such sievings were saved and stored at 2° C. Ten liters of this soil component was steam pasteurized at 100° C for 10 hrs, allowed to cool to room temperature, and the pasteurization process repeated on two succeeding days. Two liters of field soil were also pasteurized in the manner described. The two soils were mixed and 150 ml aliquots placed into 160 ml plastic grow tubes. Properties of the soil, pasteurized soil, and soil sievings are listed in Table 1. All soil and plant analyses were determined by the Soil Testing Laboratory, Oregon State University, Corvallis, Oregon (Berg and Gardner, 1978).

Nodule Inoculum

Ceanothus velutinus plants growing in the greenhouse in Fort Benham soil for two years were used as source plants for nitrogen-fixing nodules. The root systems were exposed, soil shaken loose, and nodules picked off and placed in a sterile container and weighed.

Table 1. Chemical analysis for pasteurized soil used in greenhouse studies.

Determinations	Soil	Sand Sievings
pH	6.0	6.0
TN(%)	.97	.66
NH_4N (%)	48.65	44.33
P (ppm)	9	11
K (ppm)	300	207
Ca (meq/100 g)	3.7	4.0
Mg (meq/100 g)	.64	.60
CEC	13.21	9.92
OM (%)	6.37	4.16

Nodules were placed in 95% ethanol for 1 min, rinsed in 1% Hyamine detergent for 10 min, rinsed in 2 liters of sterile distilled water and placed in 0.1% mercuric chloride for 1 min, followed by a rinse with 3 liters of sterile distilled water. The surface sterilized nodules were ground in a sterile tissue grinder and 5 ml aliquots, containing approximately 0.25 g nodule tissue, transferred to sterile dram vials.

Spore Inoculum

Spores of the vesicular-arbuscular fungus Glomus gerdemannii Rose, Daniels, and Trappe (1979) were recovered from the Fort Benham, Oregon soil samples following the sieving and decanting method of Gerdemann and Nicolson (1963) as modified by Smith and Skipper (1979). Spores were placed in sterile vials with 0.1% Hyamine detergent and shaken at high speed for 5 min on a reciprocal arm shaker, rinsed with sterile distilled water and recleaned in 10% Chlorox over a constant flow of sterile water in a Buchner funnel apparatus. Spores were rinsed with an additional two liters of sterile distilled water and transferred to sterile dram vials containing 5 ml sterel distilled water to a final concentration of 25-30 spores/vial.

After 90 days, seed vials were removed from cold incubation, placed under 10,000 lux and upon germination, seedlings were transferred to plastic tubes containing 150 ml sterile soil. Each treatment described below consisted of ten tubes. Control tubes contained seedlings in pasteurized soil plus 10 ml spore rinse water. For nodulated-only seedlings, 5 ml aliquot of nodule tissue was poured on

the root system of the seedling and pasteurized soil pressed around to cover the roots. Dual-infection seedlings contained the 5 ml crushed nodule tissue plus the 5 ml spore suspension, combined and poured over the roots and pasteurized soil pressed around to cover the roots. All inoculations took place on 15 September, 1978. Seedlings were grown at 11,000 lux under a 16 hr light photoperiod for one year. During the first month of growth, plants were watered by hand with a light spray and supplied with a nutrient solution containing essential minor elements plus 12.3 mg/l KH_2PO_4 and 15.7 mg/l NH_4NO_3. The nutrient solution was discontinued after one month and the water was changed from hand application to an automated mist supplied on alternate days.

Nitrogenase Activity

Nitrogenase activity was determined according to the methods of Hardy et al. (1968) as modified by McNabb and Geist (1980). The roots were rinsed in sterile water and placed in 50 ml flasks. After the flasks were sealed, 5 ml of air was removed and 5 ml acetylene added through a rubber serum stopper. Samples were incubated at room temperature for 2 and 4 hr periods. One ml gas samples were analyzed on a gas chromatograph equipped with a flame ionization detector and a column packed with Porapak R, 80-100 mesh. Ethylene was quantified from a standard curve.

Mycorrhizal Colonization

The percentage of the root systems infected by VA mycorrhizal

fungi was determined by preparing root samples according to the method of Phillips and Hayman (1970): 10 mm segments were cut from each root system approximately 10 cm below the root crown. The segments, stained and cleared in lactophenol, were examined under a light microscope at 400 X and the percentage calculated thus (Read et al., 1976):

$$\%\text{VA infection} = \frac{\text{No. of infected segments}}{\text{Total No. of segments examined}} \times 100$$

Weight and Nutrient Determinations

Root, nodule, and shoot tissue remaining after other analyses were oven dried at 50° C to a constant weight. Following dry weight determinations, samples were processed in a Wiley mill and ground to a fine mesh consistency.

RESULTS AND DISCUSSION

One-year-old Ceanothus velutinus seedlings inoculated with spores of Glomus gerdemannii were colonized by the VA fungus; each mycorrhizal-only plant had an average colonization of 45%, whereas mycorrhizal-nodulated plants had an average infection of 80% per each root system (Table 2). Neither the control or the nodulated-only plants became mycorrhizal fungi.

Seedlings became nodulated within 1 yr when inoculated with the crushed nodule suspension; nodulated-only plants had a mean of 3 nodules per plant and mycorrhizal-nodulated plants had a mean of 5 nodules per plant. Neither the control nor the VA-mycorrhizal-only plants became nodulated.

Table 2. Effect of Glomus gerdemannii and actinomycete-induced nodules on growth and nitrogen fixation of Ceanothus velutinus seedlings.

	Control	+VA	+Nodules	+VA Nodules
Mean dry weight shoot (mg)	72.8^{+a}	84.4^{+a}	392.9^{+b}	1028.8^{+c}
Mean dry weight root (mg)	166.4^{+a}	183.4^{+a}	285.0^{+b}	904.4^{+c}
Mean number of nodules per plant	0	0	3	5
Mean nodule dry weight (mg)	0	0	10.5^{+a}	44.6^{+b}
Mean nMoles C_2H_2 reduced/mg nodule hour	0	0	27.85^{+a}	40.46^{+b}
Mean nMoles C_2H_2 reduced per plant hour	0	0	374.8^{+a}	1014.16^{+b}
%VA colonization (mean)	0	45^{+a}	0	80^{+b}

Mean of ten replicates.

$^{+}$a,b,c, - significant at .01 level.

Significant dry weight increases were observed in the nodulated-only and the mycorrhizal-nodulated plants (Table 2). Nodulated plants were about twice as large as non-nodulated plants (Fig. 1), and mycorrhizal-nodulated plants displayed a three-fold increase in shoot and root dry weight as compared to the nodulated-only plants.

Acetylene reduction rates, an indirect measurement of the nodules' ability to fix atmospheric nitrogen, was significantly greater in the mycorrhizal-nodulated plants as compared to the nodulated-only plants; 40.46 nMoles acetylene/mg nodule hr was reduced by mycorrhizal-nodulated plants as compared to 27.85 nMoles acetylene/mg nodule/hr reduced by nodulated-only plants. On a per plant basis, 1014.2 nMoles C_2H_2 was reduced/hr per plant for dually-infected plants as compared to 374.8 nMoles C_2H_2 reduced/hr for nodulated-only plants.

The number of nodules, nodule dry weight, efficiency of nitrogenase activity, and the total amount of plant tissue significantly increased in response to a tripartite association. VA mycorrhizae, in concert with the nodule endophyte and the host plant, enhanced the nitrogen-fixing capabilities of snowbrush seedlings. This respone to VA mycorrhizal infection has been reported for dually-colonized legumes (Asai, 1944; Carling et al., 1978; Crush, 1974; Mosse et al., 1968). Carling et al. (1978) and Daft and El Giahmi (1976) have demonstrated that enhancement is due to an improved nutrient status of the host rather than a direct interaction between the VA fungus and the nitrogen-fixing endophyte. Daft and El Giahmi

Fig.1 Snowbrush seedlings grown for one year. The control plant is on the left. Nodules-only plant was inoculated with 0.25 g crushed nodule tissue. VA- and nodule seedling was inoculated with 0.25 g crushed nodule tissue and 5 ml spore suspension (25-30 spores) of Glomus gerdemannii.

(1976) reported that in peanuts infected with mycorrhizae, all organs contained higher amounts of P and Mg than non-mycorrhizal plants. Carling et al. (1978), Crush (1974) and Mosse et al. (1976) have demonstrated that mycorrhizal-nodulated plants have higher levels of P than non-mycorrhizal nodulating plants.

Mycorrhizal-nodulated snowbrush seedlings had somewhat greater concentrations of N and Ca in the shoot tissue and significant increases of %N and %Ca in the root tissue as compared to the other treatments (Table 3). The nodulated-only plants and mycorrhizal-nodulated plants had significantly greater N and P concentrations than the control and mycorrhizal-only seedlings. The control and mycorrhizal-only seedlings showed symptoms of foliar N deficiency, and chemical analyses of the plant tissue confirmed this condition (Table 3). Nitrogen appears to be the limiting factor in the growth medium as plants without the ability to fix atmospheric nitrogen were stunted and chlorotic whether or not they were mycorrhizal. Actinorrhizal plants probably have a higher demand for N as Alnus rubra Bong. plants became nitrogen deficient in substrates that are not nitrogen deficient for conifer growth (Trappe, personal communication).

Applications of phosphorus fertilizers will enhance the degree of nodulation and increase the nitrogen-fixing capabilities of legumes (Crush, 1974; Mosse et al., 1976). In this experiment the enhancement of nodulating frequency and acetylene reduction rates were achieved through VA mycorrhizal infection. Mycorrhizal fungi are possibly able to improve the P supply to the host by increasing the

Table 3. Effect of VA mycorrhizae on nutrient content of shoots and roots of snowbrush seedlings. Plants inoculated with mycorrhizal spores only (VA), plants inoculated with crushed nodule tissue only (NOD), plants inoculated with mycorrhizal spores and crushed nodule tissue (VA + NOD).

Treatment	%N	%P	%Ca	%Mg	%K
Shoot					
Control	$.32^{+a}$	$.08^{+a}$	*	*	*
VA	$.30^{+a}$	$.07^{+a}$	*	*	*
NOD	1.24^{+b}	$.25^{+b}$	1.07^{+a}	.24	.10
V +NOD	1.31^{+c}	$.25^{+b}$	1.15^{+b}	.22	.11
Root					
Control	$.48^{+a}$	$.09^{+a}$	*	*	*
VA	$.47^{+a}$	$.09^{+a}$	*	*	*
NOD	$.89^{+b}$	$.19^{+b}$	.55	$.52^{+a}$	.14
VA+NOD	1.36^{+c}	$.20^{+b}$	.50	$.36^{+b}$	.11

*Insufficient plant tissue for analysis of nutrient content of Ca, Mg and K.

+a,b,c - significant at the .01 level.

zone of contact between root and soil phosphorus by means of hyphal extension and ramifications through the soil (Gerdemann, 1968) and serving as an auxillary absorption system which operates in low nutrient regimes (Nicolson, 1967). The VA mycorrhizal do not mobilize phosphorus, but greatly increase the utilization of that which is available (Mosse et al., 1976). By improving the nutrient balance, particularly the N, P, and Ca^{++} supply, the VA mycorrhizae were able to stimulate snowbrush seedlings to produce greater leaf, shoot, and root mass, and in concert with the nitrogen-fixing actinomycete, increased nodule mass and higher acetylene-reduction rates. Both symbionts interact in improving the nutrition of the host.

Maximum benefits can be exploited from tripartite associations of actinorrhizal plants with improved understanding of the contribution of each endophyte to the host, the interactions of the endophytes in the symbioses, and an evaluation of the environmental parameters which set physical as well as biological limitations on the tripartite associations.

AKNOWLEDGEMENTS

The assistance of L. Schardine and T. Walsh is greatly appreciated. A. Todd, K. Cromack, and D. Binkley helped with gas chromatography and acetylene reduction determinations.

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CHAPTER 5

GLOMUS GERDEMANNII SP. NOV.

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GLOMUS GERDEMANNII Rose, Daniels & Trappe sp. nov.

Figs. 1-3

Sporae globosae, subglobosae, vel ellipsoideae, 140-198 x 149-230 um, juventute haylinae, laeves, deinde pallide avellaneae, asperae. Sporae tunica 5-10 (-13) um crassa, stratis quinque: exteriore 0.5-1.0 um crasso, hyalino; secundo 2-5 um crasso, hyalino, lamellato; tertio 1-2 um crasso, hyalino, secedenti; quarto ± 0.1 um crasso, hyalino; interiore 2-3 um crasso, luteo. Hypha affixa recta, 7-12 um diam, hyalina.

Spores naked, formed singly or in loose clusters or small sporocarps in soil, globose to subglobose or ellipsoid, 140-198 x 149-230 um (broader than long when not globose) hyaline and smooth in youth, becoming pale yellow brown and roughened with age. Spore

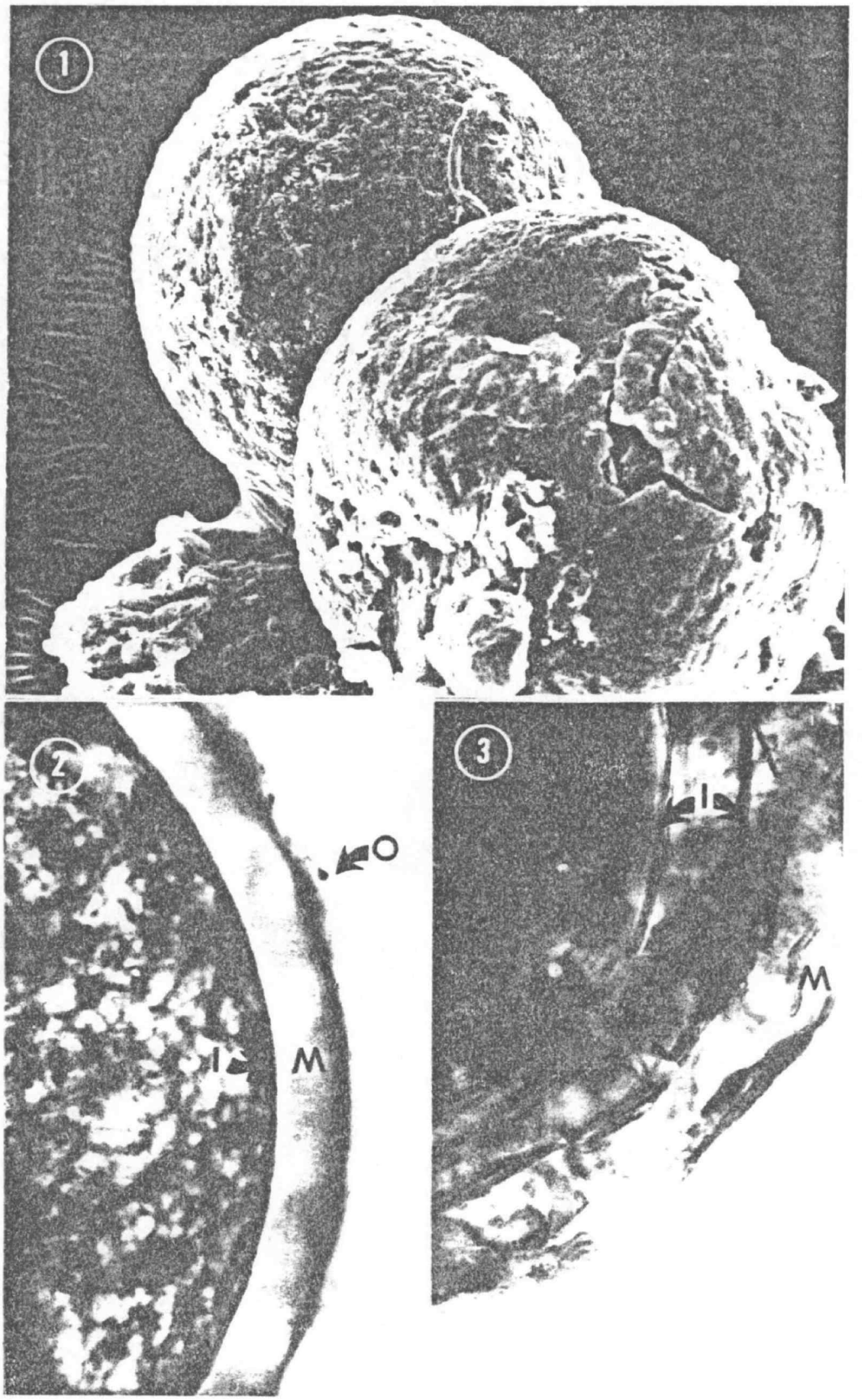
walls 5-10 (-13) um thick, of 5 layers: the outermost ± 0.5-1.0 um thick, hyaline, and smooth in youth, with age becoming roughened and cracked and flaking away in pieces; the 2nd layer inward 2-5 um thick, hyaline and fused-laminated in youth, with age becoming pale yellowish brown and degrading progressively inward to flake away as amorphous pieces of laminations; the 3rd layer inward 1-2 um thick, hyaline smooth, separable; the 4th layer inward ± 0.1 um thick, hyaline, adherent to the 5th and innermost layer 2-3 um thick and yellow. Spore contents of hyaline oil globules 7-25 (-50) um diam. Attached hypha straight, readily detaching, 7-12 um diam, hyaline, the walls thickened a short distance from the point of attachment, occluded by thickening of the 2nd spore layer. Reaction to Melzer's reagent not distinctive.

DISTRIBUTION AND HABITAT: Cascade Range and Siskiyou Mountains of Oregon on volcanic soils in climatically stressed and pioneer sites.

MYCORRHIZAL ASSOCIATIONS: Associated in the field with vesicular-arbuscular mycorrhizae of Ceanothus velutinus Dougl., C. prostratus Benth., and C. integerrimus Hook & Arn.; forming mycorrhizae in pot culture with C. velutinus. No other host genera have been discovered thus far.

ETYMOLOGY: In honor of Dr. James W. Gerdemann for his contributions to knowledge of the Endogonaceae, particularly those of Oregon.

COLLECTIONS EXAMINED: TYPE: OREGON, Deschutes Co., ca. 1 km north of Benham Falls at Fort Benham, elev. 1100 m, July 1976, 15 cm deep in soil under Ceanothus velutinus Dougl. Rose S101(OSC).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Figs. 1-3. Glomus gerdemannii. 1. Two spores by scan electron microscopy; the foreground spore shows the outer wall layer cracked and separating from the adjacent inner layer. x425. 2. Young spore in cotton blue-lactophenol, with early stage of outer layer (0) formation, thick middle layer (M), and a single, thin inner layer (1). x1,000. 3. Crushed mature spore in cotton blue-lactophenol with outer layer missing, thick middle layer (M) degraded to separable, amorphous flakes, and 2 thin, separable inner layers (1), the innermost composed in turn of 2 nonseparable layers. x400.

OTHER COLLECTIONS: OREGON--Douglas, Jackson, and Lane Counties--used in experiments and thus not available for herbarium deposit.

The complex layering of spore walls of G. gerdemannii strikingly separate it from all other known Glomus spp. The outermost, thin layer is apparent only in relatively young, smooth spores, because it flakes off soon after spores have reached full size and the 2nd layer inward has begun to thicken. At this stage, the inner 3 layers are not distinctly differentiated. As the outer 2 layers begin to flake away, however, the inner 3 differentiate clearly. The inner 3 layers persist, so that well-matured spores appear to have 3 thin wall layers enclosed in the rough, amorphous remnants of the degenerating outer walls.

The complex wall structure of G. gerdemannii resembles azygosporic species in Acaulospora and Gigaspora more than other species of Glomus (Gerdemann and Trappe 1974). Its hyphal attachment, however, places it in Glomus as an apparent chlamydospore. Sexual fusion could, of course, take place some distance below the attachment

to the spore, but we have not observed it. In any event, G. gerdemannii is morphologically suggestive of a relationship between "chlamydosporic" Endogonaceae and azygosporic species.

Spores of G. gerdemannii sink rapidly in water. In retrieving them from soil by wet-sieving and decanting, the soil suspension must be decanted within less than a minute after stirring or most of the spores will have settled to the bottom.

Spores colonized by other fungi have been observed fairly often. One relatively frequent colonizer grows as brown, septate hyphae appressed to the degenerated surface of maturing spores (the outer walls of the spores degenerate whether or not microfungi colonize the surface). The brown, septate hyphae produce globose to irregular structures 10-25 um diam. These structures, of undetermined function, remain attached to the Glomus spore walls. Hyaline globose cells 35-45 um diam are attached to the surface of occasional spores of G. gerdemannii. These resemble the sporangia of Rhizidiomycopsis stomatosa Sparrow, reported by Schenck and Nicolson (1977) to parasitize Endogonaceae.

ACKNOWLEDGEMENTS

The work reported in this paper was supported in part by National Science Foundation grant no. DEB 74-20744-A06 to the Coniferous Forest Biome, Ecosystem Analysis Studies. This is Contribution No. 335 from the Coniferous Forest Biome and Oregon Agricultural Experiment Station Paper No. 4876. Facilities for the research were generously provided by the U.S. Department of Agriculture, Forest Service, Pacific Northwest Forest and Range Experiment Station, Forestry Sciences Laboratory. Darr M. Duff of that laboratory performed the scan electron microscopy.

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CHAPTER 6

THREE NEW ENDOMYCORRHIZAL GLOMUS SPP. ASSOCIATED WITH ACTINORRHIZAL SHRUBS

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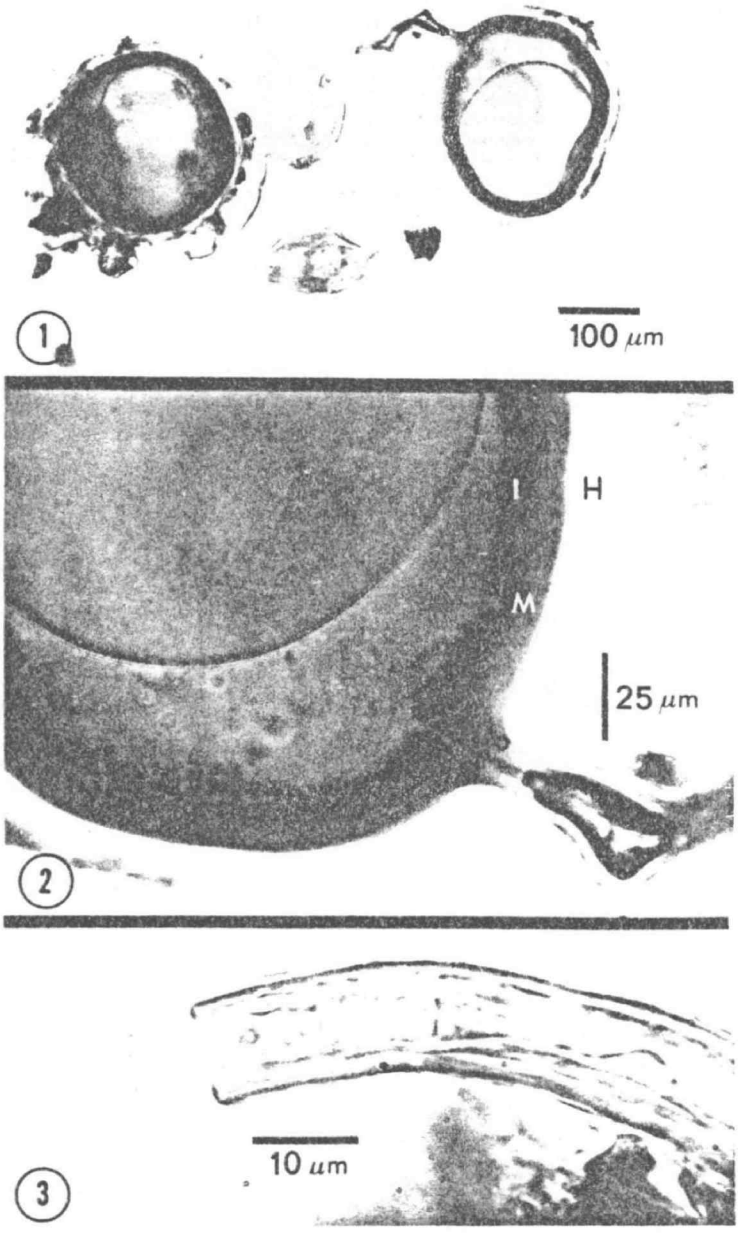
In assessing mycorrhizal associations of non-leguminous dinitrogen-fixing (actinorrhizal) plants, we sampled soil for Endogonaceae by wet-sieving and decanting (Gerdemann and Nicolson, 1963) as modified by Smith and Skipper (1979). The three new species described below were associated with actinorrhizal shrubs in central Oregon and coastal England. The collections are deposited in the herbarium of Oregon State University (OSC).

GLOMUS HALONATUS Rose & Trappe sp. nov. (Figs. 1-3)

Chlamydosporae signulae vel laxe fasciculatae in solo efformatae, globosae vel subglobosae, 200-280 um in diam, brunneolae vel tabacinae, Sporae tunica 18-35 um crassa, stratis duobus: exteriore 8-12 (-20) um crasso, hyalino, mucilagino, juventute laevi, deinde scabro; interiore 10-15 um crasso, brunneo, lamellato, minute echinulato. Hypha affixa ad tunicam sporae constricta, infra constrictionem inflata, septo.



Figs. 1-3. Glomus halonatus. 1. Two mature spores in poly-vinyl-lactophenol (PVL) showing the typical outer "halo" as seen in transmitted light. 2. Wall layering in mature spores; (H) outermost hyaline layer forming a halo, (M) middle lamellate layer, and (I) inner layer found in mature specimens only. 3. Germination by regrowth of subtending hypha.

Chlamydospores borne singly in soil or in small, loose clusters of 3-7 spores within a loosely wefted peridium, globose to subglobose, 200-280 um in diam, light brown to brown. Spore walls 18-35 um thick, of two layers: the outer 8-12 (-20) um thick, hyaline, amorphous, sometimes with obscure radial striations, in youth smooth, with age becoming roughened; the inner 10-15 um thick, brown, often prominently lamellate, ornamented with crowded spines 0.5 x 0.2 um that extend into the outer layer. Old spores sometimes with a third dark brown innermost layer ± 5 um thick. Attached hypha straight, extending through the outer hyaline wall wherein it is constricted to 5-6 um in diam. At the surface of the outer hyaline wall, subtending hypha expanded to 11-13 um in diam, and at ± 10 um below the outer hyaline wall inflated to as much as 17 um in diam; hyphal walls near the spore totaling 5-8 um thick, with a thick hyaline outer layer and a thin brown inner layer; hypha often with a septum ± 30 um below the attachment. Spore contents of oil globules of varying size.

DISTRIBUTION AND HABITAT: Central Oregon in arid, volcanic soils, in sand dunes in coastal England, and grasslands in Veracruz, Mexico.

MYCORRHIZAL ASSOCIATIONS: Associated in the field with vesicular-arbuscular (VA) mycorrhizae of Ceanothus velutinus Dougl. and Hippophae rhamnoides L.

ETYMOLOGY: Latin, "haloed"; in transmitted light in optical cross section, the thick, hyaline outer spore wall appears as a bright ring around the spore.

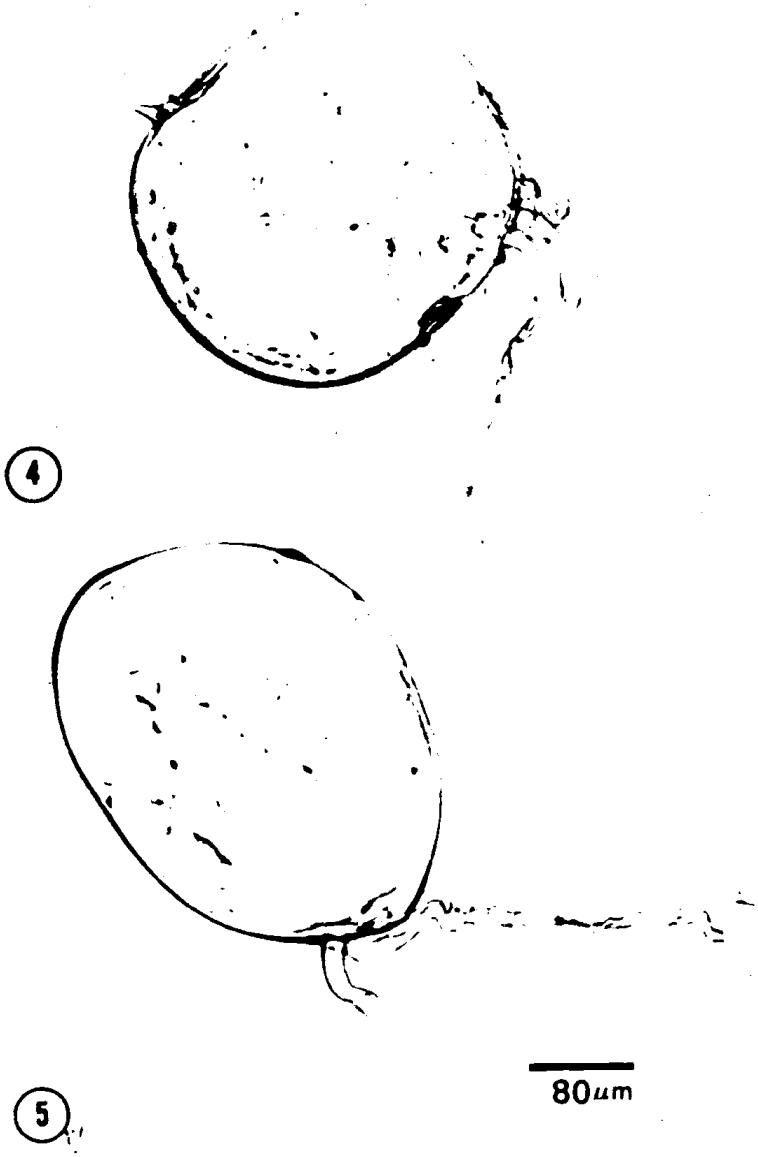
COLLECTIONS EXAMINED: HOLOTYPE-ENGLAND, Lincolnshire, Gibralter Point, under Hippophae rhamnoides, Nov. 1978, col. C. T. Youngberg, Rose S-225 (OSC). PARATYPE - UNITED STATES, Oregon, Deschutes Co., under Ceanothus velutinus, May 1979, Rose S-250. Mexico - Veracruz, with roadside grasses, 1977, no. 3594.

Glomus halonatus differs from G. caledonius (Nicol. & Gerd.) Trappe & Gerd. in having an amorphous poorly separable outer wall and echinulate inner wall vs. the nonmucilagenous, separable outer wall and smooth inner wall of the latter. The echinulations of the inner wall of G. halonatus resemble those of G. monosporus spores which, however, have but a thin outer wall, a hyphal mantle partially to totally enclosing the spore, and a subtending hypha that typically recurves along the spore surface. The spores of G. halonatus are cyanophilous in cotton blue but do not react distinctively to Melzer's reagent. The halo effect created by the outer wall in transmitted light in optical cross section is striking. The radial striations in the outer wall appear to extend from the spines on the inner wall and can be seen clearly only in some spores.

GLOMUS LACTEUS Rose & Trappe sp. nov. (Fig. 4 & 5)

Chlamydosporae singulae in solo efformatae, globosae vel subglobosae, 150-220 um in diam, lacteae. Sporae tunica una, 3-5 um crassa, hyalina, laevis. Hyphae affixae 1-3, 6-12 um in diam, hyalina, tunicis parum incrassatis. Contentum sporae granulatum vel globulsum.

Chlamydospores borne singly in soil, globose to subglobose, 150-220 um in diam, opaque, milky white, shiny smooth. Spore walls



Figs. 4 & 5. Glomus lacteus with arrangement of two merging hyphae at the spore base and a third hypha situated some distance away.

single, 3-5 um thick, hyaline. Attached hyphae 1-3 per spore, 6-12 um in diam, straight, hyaline, with walls slightly thickened only for a short distance from the spore; in most spores two hyphae merge near the spore to form a single attachment. Spore contents hyaline, granular or of globules of varying size.

DISTRIBUTION AND HABITAT: Central Oregon in arid, volcanic soil in edaphically stressed sites.

MYCORRHIZAL ASSOCIATIONS: Associated in the field with VA mycorrhizae of Ceanothus velutinus and Purshia tridentata (Pursh) D.C. Forming VA mycorrhizae with Bromus tectorum L. in pot culture.

ETYMOLOGY: Latin, "milk-white", referring to the opaque milky appearance of the spores under incident light.

COLLECTIONS EXAMINED: TYPE: UNITED STATES, Oregon, Deschutes Co., 1 km north of Benham Falls, elev. 1100 m., 1-15 cm keep in soil under Ceanothus velutinus, April 1978, Rose S-210 (OSC). PARATYPE: Oregon, Deschutes Co., 1 km west of Pine Mtn., elev. 1500 m, 1-15 cm deep in soil under Purshia tridentata, Sept. 1978, Rose S-219 (OSC).

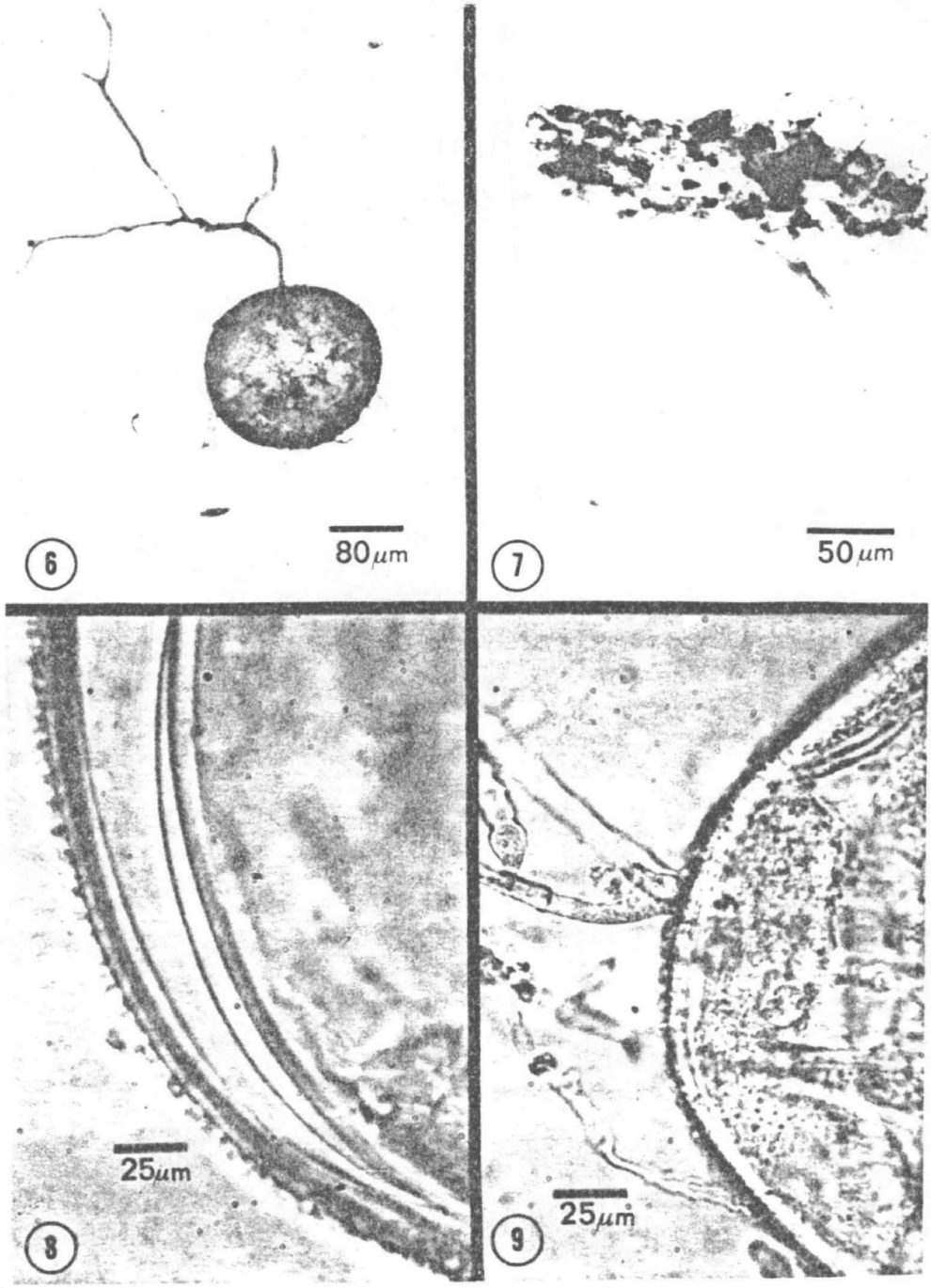
Glomus lacteus spores are distinctive in their combination of frequently multiple hyphal attachments, white color, and a thin, smooth, singly layered spore wall. Spres of G. lacteus closely resemble spores of Glomus albidus sp. nov. (Walker and Rhodes, manuscript in preparation) but can be differentiated by wall morphology. Young spores of G. albidus posses two walls of equal thickness, each 0.5-2.0 um, whereas only one thin spore wall, 3-5 um thick, appears on G lacteus regardless of spore age. Spores of G. albidus and G. clarus both possess an outer spore wall that sloughs off at maturity,

leaving a roughened outer surface on most spores. The spore wall of G. lacteus does not slough off; the spore surface is always smooth. Glomus multicaulis Gerd. and Bakshi spores frequently have more than one hyphal attachment but the spore walls are dark brown and ornamented with rounded projections in contrast to the white, smooth G. lacteus spores (Geredemann & Bakshi, 1976). Occasional spores of other Glomus spp. have two hyphal attachments (e.g. G. fasciculatus, G. microcarpus, G. monosporus, G. mosseae, and G. albidus) but the phenomenon in these cases is atypical (Gerdemann & Trappe, 1974; Gerdemann & Bakshi, 1976; Walker and Rhodes, manuscript in preparation).

In G. lacteus two hyphae often grow parallel to each other for some distance, then merge near the spore to form a single attachment. Another hypha often is attached 10-20 um away from the attachment point of the merged hyphae, and sometimes yet another hypha is attached at a still greater distance from that of the merged hyphae. These multiple attachments resemble the progametangia of zygospores of Endogone multiplex Thaxter (1922) and of some Kickxellaceae (Benjamin, 1966). It is thus possible that G. lacteus as we describe it is zygosporic rather than chlamydosporic. As presently circumscribed the genus Endogone contains only sporocarpic species and is not known to be VA mycorrhizal. The assignment of this new species to Glomus seems to be best until more is known of its life cycle.

GLOMUS SCINTILLANS Rose & Trappe sp. nov. (Figs. 6-9)

Chlamydosporae singulae in solo efformatae, globosae vel subglobosae, 180-210 um in diam, hyaline. Sporae tunica 7-10 um in diam,



Figs. 6-9. Glomus scintillans. 6. Spore stained with Cotton Blue showing cyanophilous reaction and multiple hyphal attachments. 7. Spore in PVL, wall layering and surface ornamentation is visible. 8. Detail of surface protrusions and wall layering. 9. Germination directly through the spore wall; spore contents aggregates near the point of germination.

stratis tribus: exteriore 2-4 um crasso, hyalino, nodulis congestis, hyalinis, 1-3 x 0.4 - 1.2 (-3) um ornatis; medio 2-3 um crasso, hyalino, ex strato exteriore separabili; interiore 2-3 um crasso, ad stratum medium adherenti. Hypha affixa 7-9 um in diam, hyalina.

Chlamydospores borne singly in soil, globose to subglobose, 180-210 um in diam, hyaline. Spore walls 7-10 um thick, of three layers; the outer 2-4 um thick, hyaline, with a surface ornamentation of hyaline knobs 1-3 x 0.4 - 1.2 (-3) um; the middle layer 2-3 um thick, hyaline, separable from the outer layer; and the inner layer, 2-3 um thick, hyaline, adherent to the middle layer. Attached hypha straight, 7-9 um in diam, hyaline; occasionally 2 hyphae merging near the spore to form a single attachment. Spore contents of hyaline globules 7-20 um in diameter. Spores strongly cyanophilous in cotton blue but do not react distinctively to Melzer's reagent.

DISTRIBUTION AND HABITAT: Central Oregon in loamy pumice soil in desert areas with typically hot, dry summers.

MYCORRHIZAL ASSOCIATIONS: Associated in the field with VA mycorrhizae of Cercocarpus ledifolias Nutt. and Purshia tridentata.

ETYMOLOGY: Latin, "sparkling", referring to the way the spores sparkle under incident light due to reflections off the surface ornamentation.

COLLECTIONS EXAMINED: TYPE: UNITED STATES, Oregon, Lake Co., near Picture Rock Pass, 1500 m elev., 1-15 cm deep in soil under Cercocarpus ledifolias, Sept. 1978, Rose S-220 (OSC). PARATYPE: Oregon, Deschutes Co., 1 km west of China Hat Mtn., in soil under Purshia tridentata, May 1979, Rose S-251 (OSC).

Glomus scintillans closely resembles Glomus clarus in size and color, but differs in having a knobby surface, in lacking a bulging pore septum in the subtending hypha at the spore base, and the spores do not turn yellow with age as is commonly the case with G. clarus. It differs from Complexipes moniliformis gen. et sp. nov. (Walker, 1979) ("crenulate spore", Mosse & Bowen, 1968a) by its lack of color and hyphal septation.

Glomus scintillans will key out to spore WUM 4 (couplet #59) in the key to the Endogonaceae (Hall and Fish, 1979). No samples of WUM 4 were compared with Glomus scintillans spores for this description.

Spore germination is by hyphal extension directly through the spore wall (Fig. 9) as is commonly observed in Glomus pallidus and in species of Gigaspora (e.g. G. margarita and G. rosea).

ACKNOWLEDGEMENTS

Drs. B. Mosse and C. T. Youngberg kindly furnished spore collections from England. S. Morris, J. Fuller, M. Guilemette, and G. Spiro generously contributed their time and help during sampling periods. This is Technical Paper No. 5355, Oregon Agricultural Experiment Station. Facilities for the research were provided by the U.S. Department of Agriculture, Forest Service, Pacific Northwest Forest and Range Experiment Station, Forestry Sciences Laboratory.

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CHAPTER 7

A STREPTOMYCETE ANTAGONIST TO *PHELLINUS WEIRII* *FOMES ANNOSUS*, AND *PHYTOPHTHORA CINNAMOMI* [1/]

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ABSTRACT

An actinomycete isolated from the rhizoplane of nitrogen-fixing nodules of Ceanothus velutinus was identified as a variety of Streptomyces griseoloalbus. S. griseoloalbus is a strong antagonist to three destructive root pathogens: Phellinus weirii, Fomes annosus, and Phytophthora cinnamomi, inhibiting all three on several culture media and preventing establishment of F. annosus on hemlock wood disks. The stability and longevity of the antimicrobial substance produced by it, its consistent effect on the pathogens on all substrates, its ability to colonize wood, and grow at 10° C suggest biological control possibilities for this organism in the Pacific Northwest.

INTRODUCTION

A number of investigators have reported antagonism of fungi, actinomycetes, and true bacteria to root rot pathogens (Hutchins, manuscript in preparation; Nelson, 1969; Pedziwilk, 1967; Pratt, 1971). Inhibition of the growth of fungal pathogens by actinomycetes and true bacteria has been demonstrated by Broadbent et al. (1971). Among bacteria, mycolytic properties have been mainly observed in the genera Bacillus, Pseudomonas, and Streptomyces. Much of the reported inhibition is due to a response by the pathogen to an antimicrobial substance or antibiotic produced by the actinomycete or bacterium (Ballesta and Alexander, 1972). Antibiotics are thought to be restricted to the rhizosphere where there is a higher concentration of roots and organic substances (Soulides, 1969). Brian (1967) and Lingappa and Lockwood (1961) have also reported stable and continued antibiotic production by soil microorganisms isolated and cultured under laboratory conditions.

Recent work has demonstrated that biological control of root disease organisms in living trees may be possible by treating wounds with microorganisms or by artificial inoculation of soil microorganisms that can be stimulated to multiply and subsequently replace the established pathogen (Etheridge, 1972). Actinomycetes have been found to inhibit the growth of Fomes annosus (Fr.) Cke., a destructive root rot pathogen of hardwoods and conifers in many parts of the world (Gunderson, 1963; Nissen, 1956). The fungus Peniophora gigantea (Fr.) Massee is a vigorous competitor and has been used successfully

as a stump protectant on Pines in Europe and the southeastern United States, but it does not satisfactorily inhibit the growth of F. annosus on western hemlock (Tsuga heterophylla Raf. Sarg.) in western North America (Wallis and Morrison, 1975).

The most commonly used means of control of F. annosus root rot has been the application of chemicals to stump surfaces to prevent fungal colonization. Dry Borax, 10% zinc chloride, and 20% ammonium sulphamate are effective inhibitors (Wallis and Morrison, 1975). Borax, however, fails to control the decay fungus during periods of high precipitation and ammonium sulphamate and zinc chloride are relatively costly and toxic to man.

Phellinus weirii (Poria weirii) (Murr.) Gilb. is a serious root rot pathogen in the western conifer regions of North America where it causes considerable financial loss to the timber industry each year. Phytophthora cinnamomi Rands. is responsible for serious nursery loss and hardwood damage in many parts of the world as well as considerable financial loss in crop production of ornamental flowers, avocado and pineapple (Malajczuk and Glenn, 1978; Pegg, 1976).

This report describes a Streptomyces repeatedly isolated from the rhizoplane of nitrogen-fixing nodules of Ceanothus velutinus Dougl. collected from central Oregon. This isolate produces a diffusable antimicrobial substance inhibitory to the growth of three important Northwest root rot fungi: P. weirii, F. annosus, and P. cinnamomi. This isolate is effective in culture media and colonizes and inhibits F. annosus on wood disks.

METHODS AND MATERIALS

Media

Each solid medium used for isolation and cultivation contained 1.5% agar. Glucose nutrient agar (GNA) consisted of nutrient agar from Difco plus 1% glucose. Starch casein agar (SCA) contained 1% soluble starch, 0.1% vitamin-free casein and 0.05% K_2HPO_4 adjusted to pH 7.3. Malt yeast peptone agar (MYP) consisted of 3% malt extract, 0.5% peptone and 0.1% yeast extract. MYP-B was a malt yeast peptone agar buffered to pH 5.8 with potassium phosphate.

Isolation and Culture

Nitrogen-fixing nodules were excised from lateral roots of C. velutinus growing at a depth of 15 cm, placed in bags, and stored at 4° C until processed. Within 2 days of collection, nodules were separated from root tissue, washed in 1% Hyamine detergent for 20 min and rinshed 3 times in sterile distilled water. After rinsing, nodules were either shaken 8 min in 20% hydrogen peroxide and then rinsed in sterile water, or immersed in 1% mercuric chloride for 3 min followed by 3 rinses in sterile distilled water. After rinsing, nodules were transferred to the surface of GNA in petri dishes. The petri plates were incubated at room temperature (22-25° C) for 5 days. A Streptomyces with a distinctive diffusable pigment appeared among the several colonies of fungi and bacteria. This Streptomyces isolate was subcultured to GNA and SCA slant tubes and stored at 4° C for future studies.

Identification and Taxonomy

The Streptomyces isolate was identified by the description and methods of Shirling and Gottlieb (1966, 1968a, 1968b) as modified by Kuster (1972) followed by comparisons with cultures from the American Type Culture Collection (ATCC). The criteria for identification were rate of melanin production, spore surface characteristics, morphology and color of aerial mycelium, color of substrate mycelium, number and kinds of soluble pigments, carbon utilization, and ability to fix atmospheric nitrogen as assayed by the acetylene-reduction technique (Hardy et al., 1973).

Antagonistic Determinations

Antagonism of the Streptomyces isolate against F. annosus, P. weirii, and P. cinnamomi was tested on MYP, MYP-B, and SCA by the cross-streak method (Johnson and Curl, 1972). An agar plug from the margin of an actively growing fungal culture was placed on the agar surface opposite a streak of the Streptomyces isolate. The plates were examined at weekly intervals for a clear zone, devoid of fungal growth, indicative of inhibition between the organisms. Agar plugs of the fungal pathogen placed on the three media without the Streptomyces isolate served as controls. All plates were incubated at 26° C under dark conditions. The inhibition trials continued over a six month period (6-78 and 12-78) using cultures originally isolated in September, 1977. During this period, our isolate maintained its ability to inhibit the growth of the three pathogens under laboratory conditions.

The following procedure was used to determine if the Streptomyces isolate was able to colonize wood and antagonize F. annosus on this substrate. Stem disks, 7-7.5 cm in diameter and 2.5 cm in length were cut from 11-13 year old living western hemlock and immediately brought to the laboratory. Bark was removed and surfaces of the disks were sterilized for 1 hr with ultraviolet light (254 nm). One flat surface of each disk was dipped in melted paraffin and placed downward on the bottom half of a sterile 50 x 90 mm glass petri dish. The non-paraffin coated surface was brushed with a spore suspension (25,000 spores/ml liquid) of the Streptomyces isolate either in actinomyces broth (Difco no. 9) or in water. Afterward, the cut surface of each disk was inoculated with a spore suspension of F. annosus in water. Disks treated with paraffin only, disks inoculated with spores of F. annosus but not with sterile distilled water were used as controls. Each treatment was replicated 10 times. Ten ml of sterile distilled water were poured into each petri dish to maintain a high relative humidity. A lid, which fit well but did not prevent gas exchange, was placed on each petri dish.

Disks were incubated at 22-24° C for one week and examined for the presence of mycelium and the Oedocephalum spore stage of F. annosus. Those disks that showed no signs of F. annosus were split; 4 chips from the split surface of one of the resulting halves and one from the upper surface of the disk were taken with a pair of chisel forceps and transferred to a medium selective for F. annosus (Kuhlman and Hendrix, 1962).

RESULTS AND DISCUSSION

Taxonomy and Identification

Spore chain morphology: Sporophores are flexed and included in the section Rectiflexibilis. The spore surface is smooth with about 50 spores per chain. Although spore production is generally good on oatmeal agar, the number of spores produced varies considerably on salts-starch agar and on yeast-malt agar.

Color characteristics: Aerial mycelium is white in mass on oatmeal agar, asparagine glucose agar, yeast-malt agar, and salts-starch agar. The reverse of colony is pigmented with a color varying from brownish-orange on oatmeal agar to bright orange-yellow on yeast-malt agar. Diffusion of the orange pigment around the colonies was evident on all media; however, production of the pigment decreased with repeated transfers. Melanoid pigments are not formed on peptone-yeast-iron agar nor on tyrosine agar.

Carbon utilization: Good growth was observed on the following carbon sources: L-arabinose, sucrose, D-xylose, D-mannitol, D-fructose, rhamnose, raffinose, L-inositol, and glucose. No growth was observed on cellulose nor on the negative control (without a carbon source).

The isolate was unable to fix atmospheric nitrogen after a 2-week incubation period on a nitrogen-limiting medium (Hino-Wilson broth).

Antibiotic producing properties: This isolate produced a diffusable antimicrobial substance inhibiting *P. weirii*, *F. annosus*, and *P. cinnamomi* in culture media.

Temperature requirements: The optimum temperature for this isolate is 28° C on oatmeal agar with a maximum of 32° C, and a minimum of 10° C.

Based on the above and on the taxonomic descriptions of the type culture as described by Shirling and Gottlieb (1966, 1968a, 1968b, and 1969), we have identified the Streptomyces isolate as a variety of Streptomyces griseoloalbus Kudrina.

The isolate was compared to S. griseoloalbus ATCC No. 23624. The two organisms were similar in most cultural and morphological properties but differed in several behavioral characteristics. Our isolate grew faster and produced aerial mycelium, spores, and pigments more rapidly than did the ATCC culture. For example, the ATCC culture required an incubation time of 2 weeks to produce aerial mycelium and spores was compared to 5 days for our isolate. The ATCC organism would not grow at 10° C but did grow at 36° C while our isolate would not tolerate temperatures above 32° C.

Antagonism Toward Root Pathogenic Fungi

Our isolate of S. griseoloalbus inhibited all three pathogens on MYP, MYP-B, and SCA agar (Fig. 1A, B, C). An inhibition zone, 10-19 mm depending on the medium used, was produced between this isolate and P. weirii. An orange-brown pigment produced by the isolate diffused throughout the medium but stopped at the edges of the fungal colony. A dark melanoid zone was produced on the reverse side of the fungal colony at the interface of the pigment and the fungal mycelium. Along this edge premature sporocarps, including a hymenial layer,

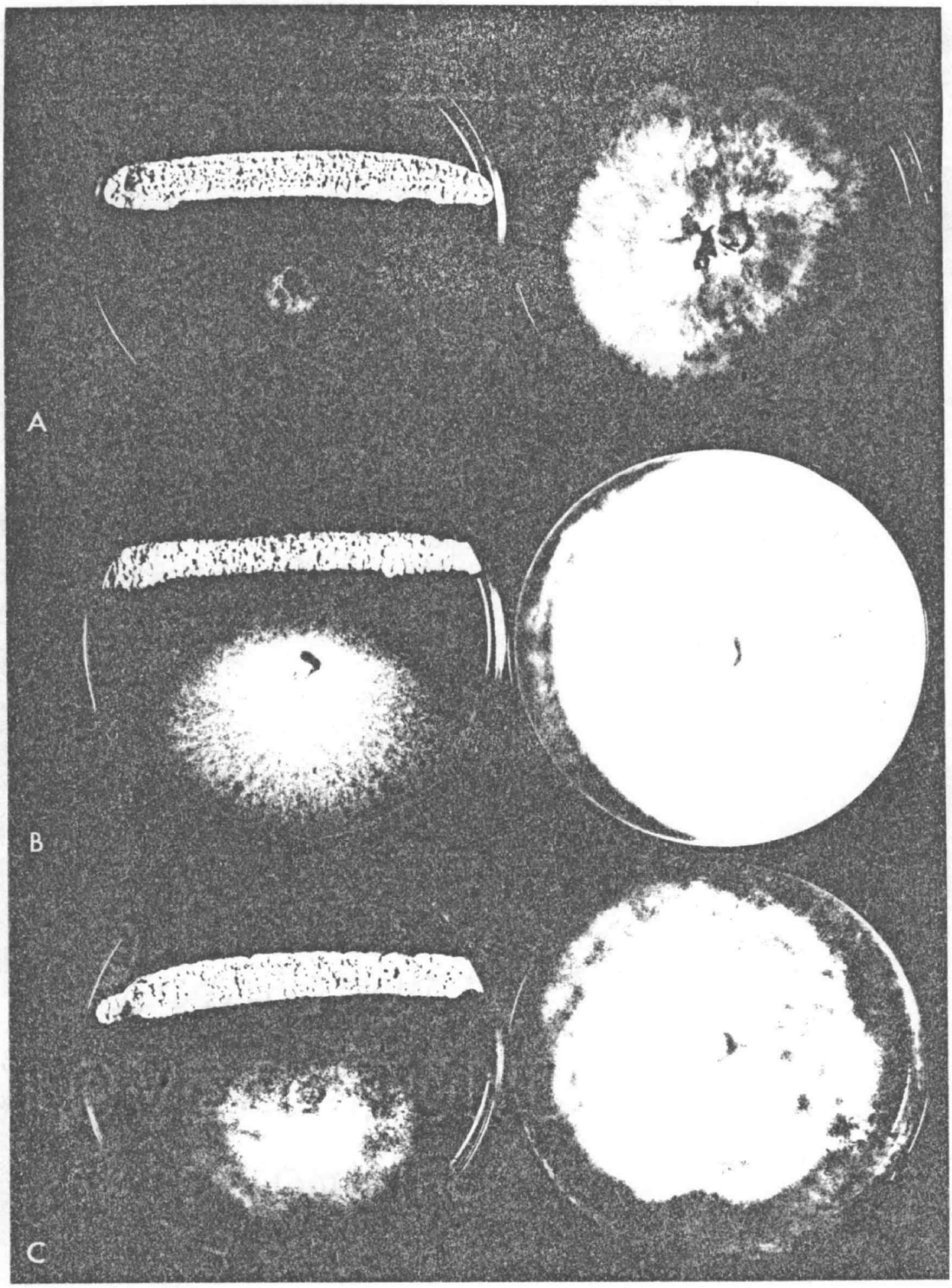

Fig. 1. A. Inhibition of three root rot pathogens by Streptomyces griseoloalbus: Two-week old culture of Phellinus weirii and S. griseoloalbus on malt-yeast-peptone agar. Plate on right is the control of P. weirii.

were observed; however, no basidiospores were produced over a 2-month period. P. weirii (T-55) did not produce basidiocarps on the control plates although we have observed it to do so under laboratory conditions within 2 months incubation. Phellinus mycelium appeared similar on test and control plates.

A 10 mm inhibition zone was produced between our S. griseoloalbus isolate and F. annosus on SCA medium. An orange-brown pigment originating from the Streptomyces colony diffused toward the fungus but stopped at the margin of the fungal colony. At this margin, the conidiophores grew back upon themselves, forming a tangled mass of convoluted hyphae. Control colonies did not exhibit this response. Conidia did not differ morphologically between test and control plates.

Inhibition zones averaging 5 mm developed between our Streptomyces isolate and P. cinnamomi. A brown diffusable pigment was produced by the isolate seemingly stimulating the production of chlamydospores or vesicles (Tucker, 1931) where the pigment contacted the Phytophthora colony. These structures appeared as red protuberances under the agar surface. The red color, upon microscopic examination, was due to a pigmented granular material inside the hyphae. Neither pigmentation nor chlamydospore production occurred on the control plates.

When applied to the surface of the wood disks in Difco actinomyces broth, our isolate of S. griseoloalbus grew rapidly over the surface without substantially altering the wood's properties or physical appearance, effectively preventing F. annosus from colonizing the disks (Fig. 2). Wood chips inoculum taken from split disks

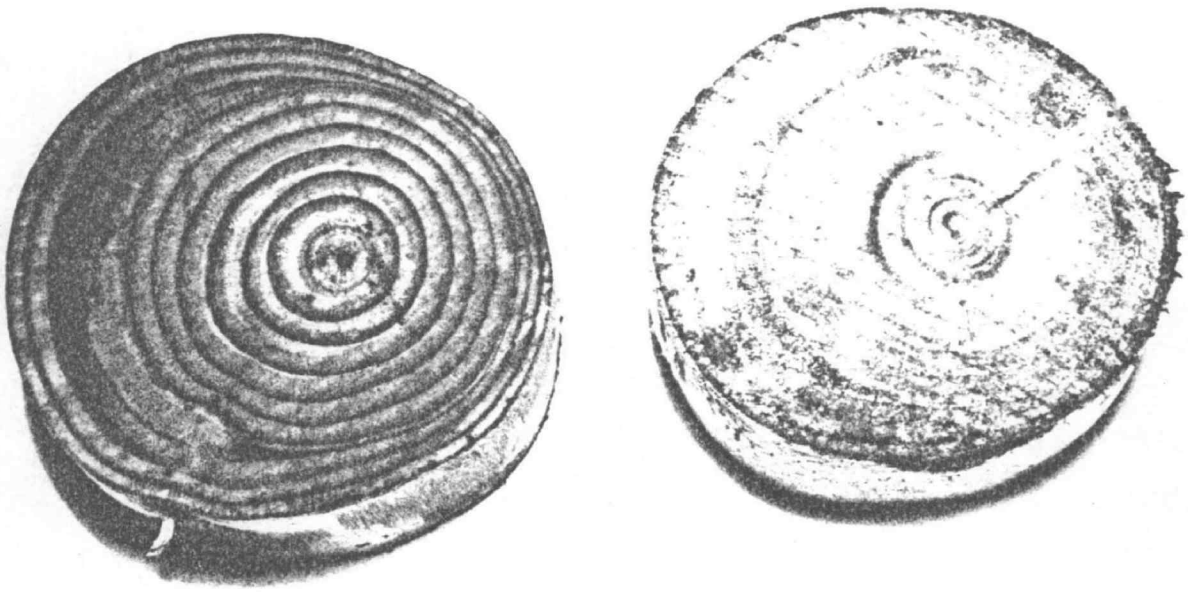

Fig. 2. Establishment of Streptomyces griseoloalbus on wood disk of western hemlock two weeks after being inoculated with liquid spore suspension. The disk on the left has not been inoculated with spores of S. griseoloalbus.

and disk surfaces failed to produce F. annosus colonies on the selective medium. F. annosus colonies did grow from the wood chip inoculum taken from the control disks. The actinomycete however, was unable to retard development of F. annosus when applied as a water suspension. These results suggest that our Streptomyces isolate depends upon nutrients from the actinomyces broth for growth and establishment on the wood. Continued survival on the wood and the non-collapsing appearance of the wood cells under microscopic observation suggests that our isolate was able to utilize non-structural carbohydrates such as simple sugars which have been identified in wood (Smith and Zavarin, 1960).

This organism not only produces an antimicrobial agent to retard the growth of F. annosus but also inhibited the development of the pathogen by possibly rapidly removing non-structural carbohydrates from wood which seem to be necessary for rapid hyphal progression. The effectiveness of S. griseoloalbus as a stump protectant against F. annosus under field conditions is currently being investigated.

ACKNOWLEDGEMENTS

The technical assistance of Mr. H. Fay and Mr. B. Addison is greatly appreciated.

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CHAPTER 8

SUMMARY

The actinomycete-nodulated angiosperms are exceptional in their ability to invade disturbed, marginal habitats. All the nodulated plants in this study occupy a pioneer niche in logged, mined, or otherwise disturbed sites. All the plants examined in this survey were mycorrhizal; 23 of the 25 plants assessed were colonized by vesicular-arbuscular (VA) mycorrhizal fungi. For certain habitats, a tripartite association, including a photosynthesizing green plant, a nitrogen-fixing endophyte, and a mycorrhizal fungus capable of maximizing nutrient uptake, may be essential for the successful natural invasion of stressed sites.

The possibility of an interaction between the mycorrhizal endophyte and the actinomycete endophyte in actinorrhizal plants was investigated. It was speculated that the presence of VA mycorrhizae might increase nodule formation, favor greater growth of the plant, increase nutrient content, and increase the rate of nitrogen fixation. To determine the effect of VA mycorrhizae on these physiological parameters, sterile snowbrush (Ceanothus velutinus Dougl.) seedlings were inoculated with VA mycorrhizal fungal spores and with crushed nodule suspensions. The response to the tripartite symbiosis demonstrated the same level of enhancement as found in dually infected legumes. The importance of VA mycorrhizae to the effectiveness of the nitrogen-fixing endophyte and to the rates of nitrogen

fixation was demonstrated for snowbrush. From these results, and from the reports of enhancement to nitrogen fixation in legumes, it is suggested that tripartite associations will benefit other actinorrhizal hosts. Economically important species of Alnus and Casuarina will probably grow taller and fix more nitrogen when dually infected than when nodulated only. This research suggests that more work be done in determining the contribution of VA and ectomycorrhizae to the growth and economic potential of these plants to both forestry and agriculture.

A strongly pigmented streptomycete was found in the rhizoplane of snowbrush nodules. The antagonistic capabilities were determined for this organism. The isolate strongly inhibited the growth of three root-rot pathogens; Poria weirii, Fomes annosus, and Phytophthora cinnamomi. This antibiotic producing microorganism confers protection from pathogens and competitors to the nodule at the soil-nodule interface.

The rhizosphere of actinorrhizal shrubs was examined for mycorrhizal fungal spores. Four new species of Glomus, a genus of vesicular-arbuscular mycorrhizal Mucorales, were isolated from Oregon, England, and Mexico. These species have not been found associated with non-actinorrhizal hosts, suggesting a specialization to habitat or a specificity between fungus and host.

This research demonstrates that actinorrhizal plants are heavily mycorrhizal. The tripartite association has been shown to increase the growth of the host, increase nodulation and the activity of

nitrogenase, and to favor increased nutrient uptake. For actinorrhizal plants which inhabit marginal habitats, mycotrophy may be a necessity.