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Title: Mycorrhizal and other Root Endophytic Fungi of Lupines in the Pacific Northwest

Abstract approved: _____
James M. Trappe

We investigated the root endophytic fungi of lupine using four approaches: (1) occurrence of fungal colonization in field-collected roots; (2) growth response of L. latifolius to inoculation with two types of fungi; (3) structure of root colonizations of Pinus and Lupinus by Phialocephala fortinii, a septate endophytic fungus of lupine; and (4) comparison root morphology, mycorrhizal colonization and natural ^{15}N-abundance N_2 fixation of three legumes. In part 1, three species of Lupinus were never observed to have fungal colonization; nine species were colonized by VA mycorrhizal fungi; seven species were colonized by fungi with septate hyphae which often formed intracellular sclerotia, here called septate endophytes. In part 2, shoot weight of 16 week old L. latifolius seedlings in the greenhouse was significantly reduced by Glomus spp. in one experiment; P. fortinii significantly increased nodule weight in one experiment and reduced it in the other. In part 3, P. fortinii colonized root epidermal and cortical

cells in the root hair zone on ultimate lateral pine roots, as well as cortical and epidermal cells of primary roots of Pinus and Lupinus. Fungal colonization was inter- and intracellular with sclerotia forming in cells of both hosts. Labyrinthine tissue, a type of fungal differentiation which occurs in the Hartig net of ectomycorrhizae, formed sporadically on pine roots. In part four, Roots of Lupinus albicaulis cv. hederma had a significantly larger proportion of coarse roots (> 1mm diameter) and significantly less mycorrhizal colonization than two other legumes, Medicago lupinula and Trifolium hybridum. Estimated aboveground N derived from fixation ranged from 6.1 to 39.9 kg per hectare (average = 22.0 kg/ha) and did not vary significantly among species.

Mycorrhizal and other Root Endophytic Fungi of Lupines in the Pacific Northwest

by

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"The most important learning, that of experience, can neither be summoned nor sought out... It comes in its own good time and in its own way to the man who will go where it lives, and wait, and be ready, and watch."

- Wendell Berry

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Mycorrhizal and other Root Endophytic Fungi of Lupines in the Pacific Northwest

Introduction

Legumes are among the most widely cultivated and economically important crop plants. They are grown for food, fodder, timber and fuel wood, and as cover crops. A major value of many legumes is their ability to host symbiotic nitrogen-fixing bacteria and thereby contribute N to natural and agroecosystems.

Lupinus (lupine) is a widespread legume genus in the Pacific Northwest. Franklin and Dyrness (1974) report species of Lupinus as components of more plant communities than any other legume genus. Although cropped commercially elsewhere (e.g. Australia) lupines are not used much in Pacific Northwest agriculture. There is interest in using lupines as N-fixing cover crops in forest plantations in the region and experiments are underway to assess their value in this regard.

Lupines tend to be colonized by vesicular-arbuscular mycorrhizal (VAM) fungi to a lesser degree than other legumes (Trinick, 1977; Bedmar & Ocampo, 1986). VAM are important for P uptake by many legumes, so the observation that lupines may be non-mycorrhizal led to discovery of the P-solubilizing capability of L. albus roots (Gardner & Boundy, 1983). Cover crops of

lupines can increase P as well as N availability to subsequent Pinus radiata plantations (Smethurst Turvey & Attiwill, 1986).

The conclusion that lupines are nonmycorrhizal came from observations of species in cultivation out of their native range. Our investigations of fungi in lupine roots began with a survey of native species to learn if they were colonized by VAM fungi in non-agricultural soils (Chapter 1). Lupine roots were colonized by VAM-type or other fungi in about half of the populations sampled. The non-VAM colonization's were formed by septate fungi (VAM fungi are coenocytic) and resembled fungal colonization's of roots described in reports scattered through decades of mycorrhizal research (Peyronel, 1923; Thomas, 1943; Read & Haselwandter, 1980; Currah & Van Dyk, 1986). These apparently symptomless colonization's variously called "dark-septate" or "rhizoctonia-like" occur on a wide variety of plants, are often intracellular and can occur in roots with typical VAM. Because these phenomena have rarely been investigated, we isolated one of the fungi involved and conducted experiments on its effect on growth and nodulation of Lupinus latifolius (Chapter 2) and studied the structures it formed on L. latifolius and Pinus contorta roots in dual culture (Chapter 3). The final study described herein is a comparison of N-fixation, root morphology and mycorrhizal colonization of a lupine and two other legumes planted in clearcut forest sites.

Chapter 1. Root Endophytes of Lupine I: Colonization in the Field

By T. E. O'Dell and J. M. Trappe

Summary

Roots of ten species of Lupinus and three other legumes collected from field soils were cleared, stained, and examined for fungal colonization. Three species were not observed to have fungal colonization. Nine species had colonization's with aseptate hyphae and vesicles attributable to vesicular-arbuscular mycorrhizal fungi. Seven species were sometimes colonized by fungi with septate hyphae, which often formed intracellular sclerotia, here called septate endophytes. The associations observed are compared with previous reports and discussed in relation to legume systematics. Septate colonizations are compared with similar reports from a wide variety of hosts and habitats.

Keywords: Lupinus, mycorrhiza, tripartite associations, fungal endophytes

Introduction

Legumes have world-wide importance for food, fodder, fuelwood, and as a nitrogen source for natural and agro-ecosystems. The Fabaceae is the most important family of food plants (Mabberly, 1989) and, despite widespread use of petroleum-based fertilizers, symbiotic nitrogen fixation by

legumes remains the main source of agricultural N. Many legumes are early colonizers of disturbed lands and some facilitate subsequent vegetation establishment (Morris & Wood, 1989). They are used in agriculture and forestry (Gadgil, 1971; Smithurst, Turvey & Attiwill, 1986) to improve growth of companion crops and should be useful in land restoration for their nitrogen fixing ability and because some increase phosphorus availability to other plants (Gardner & Boundy, 1983; Smithurst et al., 1986).

Most legumes growing in soil have vesicular-arbuscular mycorrhizae (VAM), some (especially subfamily Caesalpinioideae) are ectomycorrhizal (EM), and a few lack mycorrhizae (Peyronel, 1923,1924; Jones, 1924; Asai, 1944; Trinick, 1977; Alexander & Högberg, 1986; Currah & Van Dyk, 1986; Lesica & Antibus, 1986; Newman & Reddell, 1987). VAM and EM increase the growth and survival of many legumes (Asai, 1944; Crush, 1974; Daft & El Giahmi, 1976), usually by enhancing P-availability and interacting in other physiological ways. Occasionally growth decreases are attributed to mycorrhizal colonization (Crush, 1976; Bethlenfalvay, Brown & Pacovsky, 1982). Ames & Bethlenfalvay (1987) used a split-root technique to provide the first strong evidence of increased N-fixation in VAM legumes by some mechanism other than enhanced phosphorus nutrition.

Most investigations of legume mycorrhizae involves crop plants; relatively few have examined the occurrence of

mycorrhizae in non-crop legumes (Thomas, 1943; Currah & Van Dyk, 1986; Alexander & Högberg, 1986). Knowledge of the occurrence and function of tripartite associations is therefore biased in favor of plants with a long history of human selection. A focus on crop plants ignores the majority of legumes and potentially overlooks other adaptations to low nutrient availability (Pohill, Raven & Stirton, 1981).

Lupinus spp. are less frequently colonized by mycorrhizal fungi than other legumes. The first reports that lupines might be nonmycorrhizal were those of Schlicht (1889) and Jones (1924). Trinick (1977) reported that, in Australia, Lupinus angustifolius L., L. cosentinii Guss. and L. luteus L. were poorly colonized (<10% of root length) by VAM. But J. Zak (personal communication) found intense colonization of L. texensis Hook., and R. W. Pacovsky (personal communication) found intense colonization of Lupinus spp. Bedmar & Ocampo (1986) found that VAM colonization of lupines varied between species and cultivars. Three varieties of L. albus L. and one of L. luteus were weakly colonized (< 3% of root length) when inoculated with Glomus mosseae (Nicol. & Gerd.) Gerd. & Trappe); four varieties of L. albus, three of L. angustifolius, and one each of L. luteus and L. pilosus L. remained uncolonized. Morley & Mosse (1976) found that L. cosentinii resisted colonization by G. mosseae and G. fasciculatum (Thaxt.) Gerd. & Trappe and that the presence of L. cosentinii in the same pot reduced colonization of Trifolium pratense L. by these fungi. Snyder (1984) grew fields of Liquidambar styraciflua L.

(sweetgum) seedlings following cover crops of Avena sativa L., Lupinus albus, Secale cereale L., or Trifolium vesiculosum Savi. He reported lower VAM colonization but greater height and root collar diameter of sweetgum following the L. albus rotation than the other crops; L. albus was equally colonized by VAM as the other cover crops. Data on growth response of lupines to VAM is lacking, except for a report (unpublished) by T. Wood that L. texensis responded to inoculation with commercial VAM inoculum.

Much attention has been given to VAM and some to EM of legumes, but other non-pathogenic root-colonizing fungi have been observed (Peyronel, 1923, 1924; Thomas, 1943; Haselwandter & Read, 1982; Currah & Van Dyk, 1986). We surveyed the roots of some native legumes in Oregon and Washington for colonization by all types of fungal endophytes. Because of their widespread occurrence, ecological importance, and unusual status as potentially nonmycorrhizal legumes, we concentrated on the genus Lupinus.

Methods

Plants were identified by use of Hitchcock & Cronquist (1976); vegetation zones and physiographic provinces follow Franklin & Dyrness (1973). Voucher collections of most species were deposited in the Oregon State University Herbarium. Roots were collected by careful excavation; tap root and branches were followed as far as possible. In most cases roots of five individuals were collected at each site. Though we attempted to

collect entire root systems, this was not possible with deeply taprooted species. On return to the laboratory, roots were stored at 5°C until processing. Roots were cleared in 3% KOH for 12 hours at 60 °C, stained in 0.01% trypan blue and examined at 5-50x with a stereomicroscope. Fungal colonization was confirmed by examination at 100-1000x magnification with a compound microscope.

Septate fungi were isolated from roots by surface disinfecting in 0.01% HgCl or 5.0% H_2O_2 for five to 15 minutes followed by plating on 1.0% malt extract or yeast extract-mannitol agar.

Roots colonized by intra- or intercellular aseptate hyphae staining in trypan blue usually contained vesicles or hyphal coils typical of VAM. We refer to these as "VA-type" colonizations, even though not all VAM structures were always present in a given root. Roots colonized by inter- and intracellular septate hyphae (SE) included some that were strongly pigmented, the "dark-septate" endophytes of Read & Haselwandter (1981), as well as others which remain hyaline or stain in trypan blue (cf. Stoyke & Currah, 1991).

We attempted to collect from areas undisturbed by humans, using Research Natural Areas (RNA's), but rarely found legumes at these sites, even where they were reported to be abundant. Perhaps lessened disturbance from years of fire suppression is

reducing the presence of legumes in RNA's. The geographic range of our survey is illustrated in Figure 1.

Results

Three of 13 species collected, Lupinus micranthus, L. rivularis, and L. sulphureus, were never colonized (species data and authorities are presented in Table 1). Three, Astragalus cottonii, L. polyphyllus, and Trifolium repens, had only VA-type colonization. One, L. wyethei, had only SE or no colonization. Six were dually colonized (VA-type and SE) in at least some collections. Forty-four collections contained some fine roots. Of these ten were colonized only by VA-type, seven only by SE; twelve were dually colonized, and fifteen lacked endophytes. Thirteen other collections lacked fine rootlets on the portions of the root system that could be excavated and consequently could not be evaluated for mycorrhizal colonization.

Except for two species (the annual Lupinus micranthus and perennial Trifolium repens) the legumes examined had deep tap roots, often over a meter deep, with few fine roots, so complete root systems were rarely obtained. Evaluation of colonization was consequently based on a sample of an individual's upper roots rather than the complete system.

VA-type colonization of Lupinus spp. was sparse, generally less than five percent of root length examined; roots of Trifolium and Astragalus were more intensely colonized and contained

arbuscules in addition to vesicles and intercellular hyphae. Arbuscules were not observed in any lupine collections. Many of the VA-type collections were colonized by a fine endophyte (cf. Glomus tenue (Green.) Hall), sometimes in addition to typical VA-type hyphae and SE colonization. The fungi isolated from lupine roots included more than one entity, judging from differences in growth rate and colony morphology. Only one isolate sporulated in culture; it proved to be Phialocephala fortinii Wang & Wilcox.

Discussion

So far as we can determine, this is the first report of mycorrhizal status of most of these species. Although observations of fungal structures in field-collected roots may indicate the potential for plant-fungal interactions, such observations reveal little of function. Typical VAM colonization is sometimes associated with reduced growth of legume hosts under experimental conditions (Crush, 1976). We do not yet know how lupines respond to VAM or SE; these associations may be mutualistic, parasitic, or commensal, or may vary with environmental conditions and phenology of hosts and fungi.

Our results support Bedmar & Ocampo's (1986) conclusion that mycotrophy varies among species of Lupinus. Eight species have previously been reported as nonmycorrhizal at least in some samples (Table 2). Of the species we collected, most can host VA-type endophytes under some conditions; how they respond to this association remains undetermined. Having examined entire

root systems of fifteen individuals of the annual species L. micranthus, we conclude that it is nonmycorrhizal. We are less certain of the status of L. rivularis and L. sulphureus; these species had deep tap roots, so complete root systems could not be collected. Although we often observe VA-type colonization of field-collected L. latifolius, we have failed to observe colonization by inoculation of this species with VAM fungi in greenhouse experiments (O'Dell & Trappe, unpublished data). VAM fungi may need to be established on a companion host before colonizing roots of lupines (Morley & Mosse, 1976). The ability of some Lupinus species to increase availability of phosphorus in soil (Gardner & Boundy, 1983) suggests independence of mycorrhizal fungi.

Nonmycorrhizal individuals have been frequently observed in the papilionoid legumes (Table 2), although VAM colonizations are by far more common. Astragalus and Oxytropis spp. have been reported as nonmycorrhizal a number of times (Table 2), but our examination of A. cottonii and O. campestris showed VAM, as is also commonly reported for both genera (Currah & Van Dyk, 1986; Jones, 1924; Pendleton & Smith, 1983; Selivanov, Kryuger & Khatskelevich, 1966; Strelkova, 1956). Many genera of papilionoid legumes, occurring in widely dispersed tribes, include species sometimes reported as nonmycorrhizal (e.g. Sophoreae (Ammodendron); Robineae (Robinia); Galegeae (Astragalus); and Genisteae (Lupinus)) (Table 2). These tribes have polyphyletic origins within the Fabaceae (Lavin, Doyle & Palmer, 1990; Pohill

et al., 1981), indicating that, if it is a derived condition (see Trappe, 1987), loss of obligate mycotrophy has occurred numerous times in the evolution of the family.

SE colonizations have been reported for 22 species representing 12 genera of papilionoid legumes (Table 3), but ours are the first reports for Lupinus. Forty-three per cent of our lupine collections and 60 percent of the lupine species we sampled had SE colonizations at least some of the time (Table 1). Exactly the same proportions hold true for VAM with the lupines (Table 1), so in our samples SE is as common as VA-types. Peyronel's (1924) long ignored contention that SE colonizations were common and potentially important in plants clearly holds true in our data. What kind of response they produce remains to be demonstrated, but our observations revealed no suggestion of harm to the host.

Oxytropis spp. have been previously reported to have SE colonizations (Table 3). Although our sample of Trifolium repens lacked SE, SE has been reported for that and other species of the genus (Table 3). Our own experience suggests that SE has been disregarded by most past workers. We predict that as more notice is paid SE colonizations, they will prove to be widespread around the world in affirmation of Peyronel's (1924) prescient observations, and their significance will be experimentally examined.

Acknowledgments

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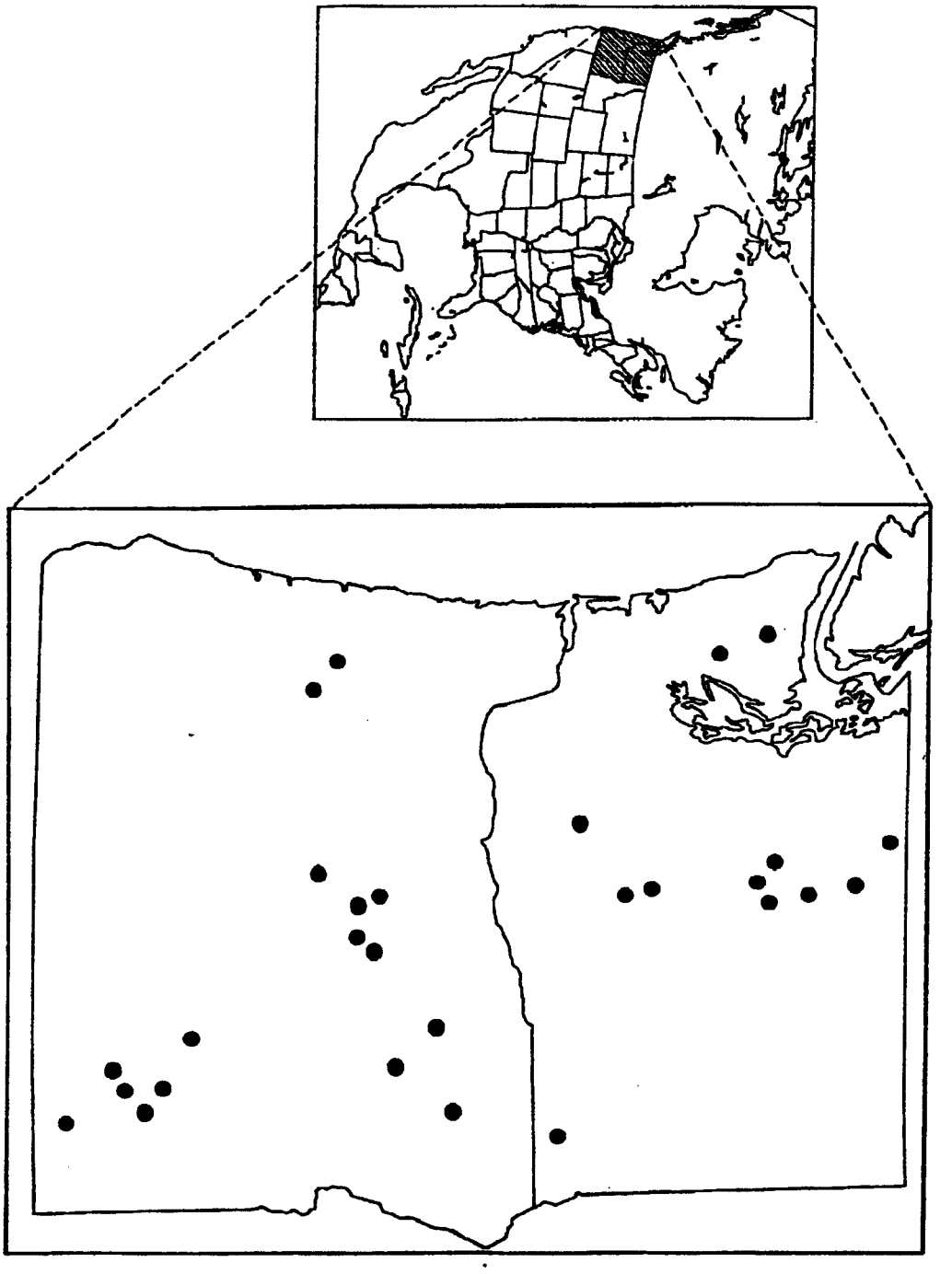

Figure 1.1 Approximate locations of collecting sites

Table 1.1. Fungal colonization of roots of native legumes.

Species		VA-Type	SE	Elevation (m)	Vegetation zone [1]	Physiographic province [2]
Astragalus	cottonii Jones	+	0	1829	Alpine	Olympic Peninsula
Lupinus	caudatus Kell.	0	+	1524	Pi po	Blue mtns
Lupinus	caudatus	0	+	1524	Pi po	Blue Mtns
Lupinus	caudatus	+	+	2377	Shrub steppe	Basin and Range
Lupinus	caudatus	+	+	1280	Shrub steppe	Basin and Range
Lupinus	latifolius Agardh	+	+	1311	Ps me	Blue Mtns.
Lupinus	latifolius	+	0	1737	Ab gr	Blue Mtns
Lupinus	latifolius	0	0	1676	Pi po/Ab gr	Blue Mtns
Lupinus	latifolius	+	0	1372	Alpine	North Cascades
Lupinus	latifolius	+	0	1372	Ab la	North Cascades
Lupinus	latifolius	+	0	1524	Ts me	Olympic Penn
Lupinus	latifolius	0	+	1554	Alpine	S. Wa Cascades
Lupinus	latifolius	0	+	1676	Ab am	S. Wa Cascades
Lupinus	latifolius	+	+	1768	Alpine	S. Wa Cascades
Lupinus	latifolius	0	0	900	Ab la	North Cascades
Lupinus	latifolius	+	+	900	Ab la	North Cascades
Lupinus	latifolius	+	0	2400	Alpine	North Cascades
Lupinus	latifolius	0	0	2000	Ab la	North Cascades
Lupinus	latifolius	+	0	750	Mt. St. Helens	S. Wa Cascades
Lupinus	latifolius	0	0	1100	Mt. St. Helens	S. Wa Cascades
Lupinus	latifolius	0	+	1676	Pi po/Ab gr	Blue Mtn.
Lupinus	latifolius	0	0	750	Mt. St. Helens	S. Wa Cascades
Lupinus	latifolius	0	0	1768	Ab la	North Cascades
Lupinus	latifolius	0	0	2150	Alpine	North Cascades
Lupinus	laxifloris Dougl.	+	0	1676	Pi po	Blue Mtn.
Lupinus	laxiflorus	+	+	1676	Pi po/Ab gr	Blue Mtn.
Lupinus	lepidus Dougl.	0	0	2963	Shrub steppe	Basin and Range
Lupinus	lepidus	+	+	2585	Shrub steppe	Basin and Range
Lupinus	lepidus	+	+	2347	Shrub steppe	Basin and Range
Lupinus	lepidus	0	+	2512	Alpine	North Cascades
Lupinus	lepidus	0	0	1250	Mt. St. Helens	S. Wa Cascades
Lupinus	lepidus	+	+	1829	Alpine	North Cascades
Lupinus	leucophyllus Dougl.	+	+	1676	Pi po/Ab gr	Blue Mtns
Lupinus	leucophyllus	+	+	2378	Shrub steppe	Basin and Range
Lupinus	micranthus Dougl.	0	0	85	Qu ga	Willamette Valley
Lupinus	micranthus	0	0	85	Qu ga	Willamette Valley
Lupinus	polyphyllus Lindl.	+	0	1768	Ts me	S. Wa Cascades
Lupinus	rivularis Dougl.	0	0	274	Ts he	Coast range
Lupinus	sulphureus Dougl.	0	0	1524	Pi po	Blue Mtns
Lupinus	sulphureus	0	0	1890	Pi po	Blue Mtns
Lupinus	wyethei Wats.	0	0	1524	Ps me	Blue Mtns

Lupinus	wyethei	0	+	1829	Alpine	S. Wa Cascades
Oxytropis	campestris (L.) DC.	+	+	2499	Alpine	North Cascades
Trifolium	repens L.	+	0	1000	Mt. St. Helens	S. Wa Cascades

Root colonization status (composite of 3 to 5 plants per collection), elevation, vegetation zone and physiographic province of native legumes in Oregon and Washington. Roots colonized by intercellular aseptate hyphae staining in trypan blue were considered VA-type; these usually contained vesicles or hyphal coils. Roots colonized by inter- and intracellular septate hyphae were considered septate endomycorrhizal (SE).

1. Forest zones according to Franklin & Dyrness (1973) do not include designations for alpine-subalpine parklands or shrub-steppe zones. Ab am = Abies amabilis zone; Ab gr = Abies grandis zone; Ab la = Abies lasiocarpa zone; Pi po = Pinus pondersa zone; Pi po/Ab gr indicates areas transitional between Pinus ponderosa and Abies grandis forest zones; Ps me = Pseudotsuga menziesia zone; Qu ga = Quercus garryana zone; Ts he = Tsuga heterophylla zone; Ts me = Tsuga mertensiana zone; Mt. St. Helens refers to collections from the devastated zones in Mt. St Helens National Volcanic Monument. .

2. Physiographic provinces follow Franklin and Dyrness (1973).

Table 1.2. Species of papilionoid legumes reported to sometimes occur in nature without mycorrhizae.

Alhagi camelorum Fisch. (Saif & Iffat, 1976)
Alhagi maurorum Med. (Iqbal, Gull & Javed, 1982)
Alhagi pseudoalhagi (Bieb.) Desv. (Selvanov et al., 1966)
Ammodendron conollyi Bunge (Selivanov et al., 1966)
Amphicarpaea edgeworthii Benth. (= Falcata comosa) (Hood, 1964)
Anthyllis vulneraria L. (= A. alpestris, A. polyphylla) (Dominik, 1951, 1961; Dominik, Nespiak & Pachlewski, 1954)
Astragalus alpinus L. (Katenin, 1964, 1972; Strelkova 1956)
Astragalus arenarius L. (Dominik, 1951)
Astragalus glycyphyllos L. (Dominik, 1957, 1961; Dominik & Wojciechowska, 1963)
Astragalus kentrophyta Gray (Lesica & Antibus, 1986)
Astragalus lehmannianus Bunge (Selivanov et al., 1966)
Astragalus parryi Gray (Thomas, 1943)
Astragalus polyacanthus Royle (A. psilocentrus) (Saif & Iffat, 1976)
Astragalus umbellatus Bunge (Strelkova, 1956)
Astragalus unifoliatus Bunge (A. confirmans) (Selivanov et al., 1966)
Astragalus vexilliflexus Sheld. (Lesica & Antibus, 1986)
Baptista tinctoria (L.) Vent. (Hood, 1964)
Caragana frutex (L.) C. Koch (Selivanov et al., 1966)
Cicer arietinum L. (Jones, 1924)
Coronilla varia L. (Dominik, 1961; Rothwell & Vogel, 1982)
Crotalaria maritime (Hood, 1964)
Crotalaria purshii D.C. (Hood, 1964)
Dalbergia malabarica Prain (Mohankumar & Mahadevan, 1987)
Desmodium glutinosum (Muhl.) Wood (D. grandiflorum) (Rothwell & Vogel, 1982)
Desmodium motorium (Houtt.) Merr. (Saif, 1975)
Desmodium nudiflorum (L.) D.C. (Meibomia nudiflora) (Hood. 1964)
Desmodium paniculatum (L.) D.C. (Meibomia paniculata) (Hood, 1964)
Desmodium rotundifolium (Michx.) Torr. & Tray (Rothwell & Vogel, 1982)
Desmodium strictum (Pursh) D.C. (Meibomia stricta) (Hood, 1964)
Erythrina velutina Willd. (Schmidt & Scow, 1986)

Galactia volubilis (L.) Britt. (Hood, 1964)
Genista anglica L. (Höveler, 1892, Mentz, 1906)
Genista germanica L. (Mentz, 1906)
Genista pilosa L. (Höveler, 1892; Mentz, 1906)
Genista tinctoria L. (Mentz, 1906)
Lathyrus japonicus Willd. (L. maritimus) (Dominik, 1951)
Lathyrus niger (L.) Bernh. (Dominik, 1957)
Lathyrus odoratus L. (Lusnikova, 1970)
Lathyrus pratensis L. (Demin, 1971; Truszkowska, 1951)
Lathyrus vernus (L.) Bernh. (Orobus vernus) (Dominik, 1961)
Lespedeza bicolor Turcz. (Rothwell & Vogel, 1982)
Lespedeza cuneata L. (= L. sericea) (Kiernan, Hendrix & Maronek, 1983; Rothwell & Vogel, 1982)
Lespedeza hirsuta (L.) Koch. (Hood, 1964)
Lespedeza violaceae (L.) Pers. (Rothwell & Vogel, 1982)
Lotus corniculatus L. (L. caucasicus) (Dominik, 1961; Dominik, Nespiak & Pachlewski, 1954)
Lupinus albus L. (Thompson & Wildermuth, 1989)
Lupinus diffusus Nutt. (Hood, 1964)
Lupinus latifolius Agardh (Allen, MacMahon & Ianson, 1985)
Lupinus luteus L. (Schlicht, 1889)
Lupinus mutabilis Sweet (Lusnikova, 1970)
Lupinus parviflorus Nutt. (Thomas, 1943)
Lupinus perennis L. (Jones, 1924)
Lupinus polyphyllus Lindl. (Lusnikova, 1970; Pachlewski, 1958)
Lupinus villosus Willd. (Hood, 1964)
Medicago lupulina L. (Meador, 1977; Saif, Ali & Zaidi, 1977)
Medicago sativa L. (Cohen, 1948; Pond, Menge & Jarrell, 1984)
Melilotus incia (L.) All. (Hood, 1964)
Onobrychis sp. (Saif et al., 1977)
Ononis variegata L. (Giovannetti & Nicolson, 1983)
Oxytropis mertensiana Turcz. (Strelkova, 1956)
Oxytropis nigrescens (Pall.) Fisch. (Miller, 1982; Strelkova, 1956)
Oxytropis sericea Nutt. (= O. saximontana) (Thomas, 1943).
Oxytropis sp. (Jones, 1924)
Phaseolus coccineus L. (= P. multiflorus) (Lusnikova, 1970)
Psoralea drupacea Bunge (Saif et al., 1977)
Rhynchosia capitata (L.) D.C. (Saif, 1975)
Rhynchosia minima (L.) D.C. (Saif, 1975)
Rhynchosia tomentosa (L.) H. & A. (Dolicholus tomentosa) (Hood, 1964)

Robinia pseudoacacia L. (Cohen, 1948; Kiernan et al., 1983; Medve, Hoffman & Gaither, 1977; Rothwell & Vogel, 1982)
Sophora sp. (Saif & Iffat, 1976; Shterenberg & Kostyuk, 1955)
Sphenostylis angustifolia Sond. (Cohen, 1948)
Stylosanthes biflora (L.) B.S.P. (Hood, 1964)
Trifolium agrarium L. (Hood, 1964)
Trifolium dasyphyllum Torr. & Gray (Daubenmire, 1941)
Trifolium fragiferum L. (Dominik & Pachlewski, 1956)
Trifolium hybridum L. (Truszkowska, 1951)
Trifolium parryi Gray (Daubenmire, 1941)
Trifolium pratense L. (Truszkowska, 1951)
Trifolium repens L. (Cohen, 1948; Rothwell & Vogel, 1982)
Trigonella foenum-graecum L. (Peyronel, 1924; Strzemska, 1973)
Ulex galii Planch. (Heath & Luckwill, 1938)
Vicia cassubica L. (Dominik & Wojciechowska, 1963)
Vicia cracca L. (Wojciechowska, 1966)
Vicia faba L. (Winter & Birgel, 1953)
Vicia hirsuta (L.) Gray (Wojciechowska, 1966)
Vicia sativa L. (Cohen, 1948)
Vigna mungo (L.) Hepper (= Phaseolus mungo) (Saif et al. 1977)
Vigna vexilata (L.) A. Rich) (Cohen, 1948)
Virgilia capensis (L.) Poir. (= V. oroboides) (Laughton, 1964)

Table 1.3. Species of papilionoid legumes reported to have septate root endophytes.

Astragalus alpinus L. (Currah & Van Dyk, 1986)
Desmodium canescens (L.) D.C. (Thomazini, 1974)
Halimodendron halodendron (Pall.) Voss (Shvartsman, 1955)
Indigofera suffruticosa Mill. (Thomazini, 1974)
Lens culinaris Med. (= L. esculenta) (Shterenberg, 1951)
Medicago falcata L. (Demin, 1971)
Medicago sativa L. (Peyronel, 1924)
Melilotus indica (L.) All. (Iqbal et al., 1982)
Melilotus officinalis (L.) Pall. (Peyronel, 1924)
Ornithopus compressus L. (Peyronel, 1924)
Oxytropis foetida (Vill.) D.C. (Peyronel, 1937)
Oxytropis jordalii (Currah & Van Dyk, 1986)
Phaseolus vulgaris L. (Shterenberg, 1951)
Trifolium alpestre L. (Malan, 1938)
Trifolium alpinum L. (Malan, 1938)
Trifolium medium L. (Malan, 1938)
Trifolium pratense L. (Malan, 1938, Peyronel 1924)
Trifolium repens L. (Malan, 1938; Peyronel, 1924; Powell, 1980)
Trifolium thalii Vill. (Malan, 1938)
Trigonella foenum-graecum L. (Peyronel, 1924)
Vicia cracca L. (Malan, 1938)
Vicia sepium L. (Malan, 1938)
Vicia tetrasperma (L.)Schreb. (Iqbal et al., 1982)

Chapter 2. Growth Response of *Lupinus latifolius* to *Phialocephala fortinii* and *Glomus* spp.

By T.E. O'Dell

Summary

In two separate experiments, *Lupinus latifolius* plants inoculated with *Bradyrhizobium* sp. (*Lupinus*) and live or dead *P. fortinii* and *Glomus* spp. grew for sixteen weeks in the greenhouse before harvest. In the first experiment plant growth and fungal colonization was poor. Nonetheless, *Glomus* spp. significantly reduced shoot biomass and *P. fortinii* significantly increased nodule fresh weight of *L. latifolius*. In the second experiment withmuch larger soil volumes available to each plant, *P. fortinii* significantly decreased lupine nodule mass and *Glomus* spp. significantly increased *Trifolium subterraneum* shoot mass in pasteurized, but not in unpasteurized, soil. The results indicate that *L. latifolius* may function wtihout mycorrhizae and that *Glomus* spp. are commensal or parasitic associates of its roots, but *P. fortinii* may be a mutualistic or parasitic associate depending upon experimental conditions.

Introduction

Legumes have world-wide economic significance as crops and as hosts of bacterial N_2 fixation, contributing significant N to agricultural and natural ecosystems. Because they typically form vesicular-arbuscular mycorrhizae (VAM), most legumes can

be considered tripartite symbioses between plant, nodulating bacteria and VAM fungus.

Asai (1944) was the first to document the importance of VAM to legume growth. Many agricultural legumes grow better and fix more N when colonized by VAM fungi (Asai, 1944; Bethlenfalvay & Yoder, 1981; Crush, 1974). Such growth increases are due to enhanced P nutrition, increased drought tolerance, and other, poorly understood mechanisms (Ames & Bethelanfalvay, 1987; Bethlenfalvay et al., 1987).

Septate fungal endophytes of roots (SE), with unknown functions, have been reported from many host plants (Peyronel, 1923; Melin, 1923; Currah & Van Dyk, 1986). Some SE isolates from Lupinus sp., orchids, Ericaceae, and Rosaceae form Phialocephala fortinii Wang & Wilcox anamorphs in culture (Chapter 1; Currah, Sigler & Hambleton, 1987; Stoyke & Currah, 1991). P fortinii, one of the taxa collectively referred to as Mycelium radicis atrovirens, is also a "pseudomycorrhizal" associate of Pinaceae (Richard & Fortin, 1973; Wang & Wilcox, 1985). Thus, P. fortinii can associate with roots of plants from many recognized categories of mycorrhizal type (orchid, ericoid, VA, and ectomycorrhizal). Effects of SE on host growth have rarely been investigated, but an SE isolate from a Carex sp. enhanced growth and P nutrition of its host in typical mycorrhizal fashion (Haselwandter & Read, 1982).

The legume genus Lupinus is widespread in the Pacific Northwest. Roots of Lupinus species from field and forest habitats were colonized by either or both VAM or SE fungi in about half the collections examined (Chapter 1). One SE isolate from Lupinus latifolius Agardh. formed P. fortinii conidiophores when cultured (chapters 1 & 3). To further characterize interactions between lupine and root-colonizing fungi, we investigated the effects of P. fortinii and VAM (Glomus spp.) fungal associates on growth and nodulation of L. latifolius.

Methods

Plants

Seed of Lupinus latifolius was collected from a natural population in the Gifford-Pinchot National Forest, Lewis County, Washington. A voucher collection from this population is in the Oregon State University herbarium. Seed was kept in a freezer until use. For germination, lupine seeds were wetted in Tween 80 (J.T. Baker Co.), rinsed with distilled water, scarified in concentrated HCl for one half hour, disinfected in 5% H_2O_2 overnight and germinated on 1% malt extract agar. Seed of Trifolium subterraneum cv. Mt. Barker (subterranean clover) was purchased from Cenex Co. (Tangent OR.). Clover seed was not scarified in HCl, but was otherwise treated the same as lupine.

Fungi

A. VAM fungi.

Inoculum of Glomus intraradices, G. etunicatum, and G.deserticola was purchased from Native Plants Inc. (Salt Lake City, UT). A mixture of these species was used to increase the chance of at least one benefiting the host.

B. Phialocephala fortinii

Isolation of P. fortinii is described in Chapter 3. Cultures of P. fortinii were maintained on MEA and MMN agar slants.

Inoculum of P fortinii was prepared by growing fungal cultures for one month on petri dishes of MMN agar. Agar plugs were transferred to MMN liquid culture with glass shards and grown for three weeks with shaking. Mycelial slurries from liquid cultures were added to sterile peat-vermiculite containing MMN and grown for two months (Molina and Palmer, 1982). Colonized peat-vermiculite MMN was rinsed in cold tap water for one hour before mixing with other inocula.

Bradyrhizobium sp. (Lupinus)

Bacterial cultures were obtained by the same method as the fungi. Slow-growing bacterial colonies were streaked to purity on YEM and single colonies subcultured on YEM agar slants and frozen for later use. Those isolates tested formed nodules, able to reduce acetylene, on roots of Lupinus latifolius and accordingly can be designated Bradyrhizobium sp. (Lupinus). An isolate of Rhizobium leguminosarum bv. trifolii was provided by P.J. Bottomley (Oregon State University, Microbiology). Uninoculated controls did not nodulate in pure culture synthesis tubes. Inoculum of B. sp. (Lupinus) was grown in YEM broth shaker culture at 25°C for seven to ten days.

Growing conditions and inoculations

Experiment 1

The first experiment to test effects of VAM and SE fungi individually and in combination, was conducted with plants grown

in 150 ml containers (Leach tubes) filled with pasteurized potting soil (Willamette River sandy loam: peat: sand: pumice, 1:1:1:2). Inoculants were: (1) 2 ml per tube Phialocephala fortinii in peat-vermiculite MMN; or (2) sterile, washed peat-vermiculite MMN; (3) 3 ml per tube live inoculum (spores and root fragments) of Glomus spp. or (4) killed inoculum plus filtrate (8μ) from VAM inoculum. Each tube was planted with one L. latifolius seedling. All plants received Bradyrhizobium sp (Lupinus). The mix of Glomus spp. and Phialocephala fortinii were applied as treatments in a two by two complete factorial design. An experimental unit (replicate) consisted of one container with a lupine seedling and appropriate inoculum. Containers were completely randomized in racks on the greenhouse bench, with 20 replicates per treatment. Another group of twenty plants received controls for fungi and sterile, distilled water in place of Bradyrhizobium sp. (Lupinus). Plants were grown in a greenhouse with supplemental lighting for sixteen weeks.

Experiment 2

Poor plant growth and low colonization of roots by inoculated fungal endophytes in experiment 1 suggested that 150 ml containers did not provide adequate soil volume for lupine seedlings and that greenhouse potting soil was not satisfactory for development of the fungi. Accordingly, experiment 2 was conducted with plants grown in field soil mixed 1:1 with Geolite (an expanded clay material) in one-gallon pots. The soil,

collected from mapping unit R-7, Prineville Ranger District, Ochoco National Forest, Oregon, is classified as a moderately well-drained dark brown sandy loam, ash-derived over rhyolite tuff. Half of the soil was pasteurized for one hour at 70°C before filling pots.

Inoculants were the same as in experiment one, except that the VAM mixture contained only G. intraradices and G. etunicatum, and the control for SE inoculation consisted of autoclaved P. fortinii inoculum in place of sterile media. Each pot received 100 ml total inoculum mixture: 20 ml VA; 25 ml P. fortinii; 55 ml sterile quartz sand. The design was a three-way complete factorial of VA by SE (P. fortinii) by soil pasteurization. There were ten replicates of L. latifolius per treatment combination. Five replicates per treatment of subterranean clover (Trifolium subterraneum) were also planted to confirm that soil conditions were such that a VAM-dependant host would respond to inoculation. Pots were completely randomized on the greenhouse bench and rotated to different positions twice during the experiment to reduce local environmental effects. Plants were grown in a greenhouse with supplemental lighting for sixteen weeks.

Fungal colonization

A 50 cm subsample of each root system was cleared for 12 hours in 5 % KOH at 70 °C, acidified in 1% HCl and stained for 12 hours in 0.1 % trypan blue at 70 °C. The roots were examined at

20-1000x magnification and colonization scored as present if dark, septate intracellular hyphae were observed.

Statistical analyses

Exploratory analyses (plots of residuals vs. predicted values, and normal probability plots of residuals) revealed non-normal distributions of most variables. Analysis of variance (ANOVA) was conducted on data logarithmically transformed to approximate a normal distribution (Sabin & Stafford, 1990). Tests of overall significance of treatments was by ANOVA. Results presented are medians back-transformed from (transformed) data on which differences were tested. We assessed differences between means by Fisher's protected least significant difference (FPLSD; Petersen, 1985). Statistical analyses were conducted on Macintosh computers using Data Desk 3.0 and Statview 512+ software.

Results

Experiment 1

Inoculation with Glomus spp. significantly ($p \leq 0.05$, FPLSD) reduced shoot mass of four month-old L. latifolius plants (Table 2.2). Nodule fresh weight was significantly increased by P. fortini (Table 2.2). Emergence and survival were reduced in L. latifolius seedlings which did not receive inoculum of Bradyrhizobium sp. (Lupinus) (25 % vs. 66% survival).

Experiment 2

The most striking difference from the previous experiment was that lupine plants were an order of magnitude larger at the conclusion of experiment 2 (0.37 g vs. 2.7 g). Nodule dry weight was significantly decreased by P. fortinii. There was significant interaction between effects of VA inoculation and soil pasteurization on clover biomass. Biomass of clover in pasteurized soil was increased by VAM inoculation ($p \leq 0.01$ FPLSD); in unpasteurized soil VAM inoculation had no significant effect. In the absence of VA inoculation clover produced significantly more biomass in unpasteurized than pasteurized soil.

No VAM colonization was observed on either host. Either subsampling of root systems was inadequate, or colonization did not occur. Given the positive response of clover to inoculation with Glomus spp., the former explanation seems likely. In contrast, SE colonization was observed and, though present on some controls (pasteurized soil + killed P. fortinii), was significantly more frequent on plants inoculated with live P. fortinii.

Discussion

Poor plant growth and colonization led us to conduct the second experiment with a larger soil volume ad soil colloected from a natural lupine site. The increased biomass of clover with

VAM inoculation in experiment two indicates that conditions were adequate for testing mycorrhizal response. Thus the interaction between L. latifolius and the fungi tested was commensal or parasitic under these conditions. The lack of response of L. latifolius to VAM fungi in experiment 2 is consistent with other reports for the genus Lupinus (Snyder, 1984), the negative response observed in experiment one is similar to the response of some other non-mycorrhizal plant species (e.g. Salsola kali) to inoculation with VAM fungi (Allen, Allen & Friese, 1989).

The lack of response of L. latifolius to VAM fungi may be explained by the unusual P-solubilizing ability of some Lupinus species (Gardner & Boundy, 1985). Since enhanced P uptake is the most frequently reported benefit of VAM colonization, plants with other mechanisms for obtaining P are less likely to benefit from VAM. Lupinus species are typically colonized at low levels by VAM fungi (Bedmar & Ocampo, 1986; Chapter 1; Trinick, 1977). However, since colonization intensity is a poor predictor of growth response, this does not preclude some benefit to Lupinus species under some conditions.

Mycotrophic interactions with higher plants include a spectrum from virulent pathogen to obligate mutualist. Our investigation of L. latifolius characterize it as non- or facultatively mycorrhizal. Glomus spp. appear to be parasitic or commensal endophytes of L. latifolius roots. This is in strong

contrast to many legume species, which are among the most positively responsive of obligate VAM mycotrophs. Phialocephala fortinii increased nodule fresh weight in experiment 1 suggesting that it is a mutualist under some conditions.

Fungi with unknown functions which inhabit leaves of higher plants are referred to as endophytes. These associations are extremely widespread; so ubiquitous that Hawksworth (1991) proposes to call them "mycophyllas", recognizing that they may be equally widespread to mycorrhizae. Carol (1988) discusses the range of interaction of such fungi: from latent pathogen to mutualistic symbiont. He introduces the concept of inducible mutualism to describe those associations where benefit is conferred to host only under particular conditions. P. fortinii appears to be more an endophyte than a mycorrhizal fungus.

The ability of L. latifolius to thrive in the absence of mycorrhizae is adaptive to primary successional habitats. If it fixes significant N or solubilizes P under these conditions, it may be well-suited to restoration of severely disturbed habitats where these nutrients are often limiting.

Acknowledgments

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Table 2.1. P-values from analysis of variance of selected response variables

Experiment 1	Nodule mass		Shoot mass	
VA	.69		**.01**	
SE	**.02**		.82	
VAxSE	.93		.25	
Experiment 2	clover	lupine	clover	lupine
VA	nd	.65	.01	.12
SE	nd	**.04**	.38	.36
VAxSE	nd	.57	.22	.44
Soil	nd	.74	.35	.32
SoilxVA	nd	.42	**.01**	.34
SoilxSE	nd	.11	.17	.29
SoilxVAxSE	nd	.66	.06	.73

Table 2.2 Nodule and shoot mass of L. latifolius

	Nodule mass[1]		Shoot mass	
Experiment 1	-	+	-	+
VA	0.314a[2]	0.304a	0.406b	0.332 a
SE	0.273a	0.358b	0.372a	0.373 a
Experiment 2				
Lupine				
VA	0.172a	0.141a	2.74a	2.16a
SE	0.172a	0.143b	2.611a	2.31a

1 Nodule mass in experiment one is fresh weight; in experiment two it is dry weight.

2. Medians of a given response variable in the same row not sharing a common letter are significantly different ($P \leq 0.05$, FPLSD).

Table 2.3. Average shoot weight of Clover, experiment 2.

Clover	VA-	VA+
pasteurized	0.066a	0.408 c
unpasteurized	0.185b	0.208 b

Means not sharing a common letter are significantly different (P≤0.05, FPLSD).

Chapter 3. Root Colonization of Lupinus latifolius and Pinus contorta by Phialocephala fortinii

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Summary

Root colonization patterns of Phialocephala fortinii inoculated on Lupinus latifolius (broad-leafed lupine), a nitrogen-fixing legume, and Pinus contorta (lodgepole pine) were studied. The fungus colonized root epidermal and cortical cells in the root hair zone on ultimate lateral pine roots, as well as cortical and epidermal cells of primary roots of both hosts. Fungal colonization was inter- and intracellular with sclerotia forming in cells of both hosts. Labyrinthine tissue, a type of fungal differentiation that occurs in the Hartig net of ectomycorrhizae, formed sporadically on pine roots. Similar colonization has been observed on conifers and many other plants, but this report is the

first showing that a single fungus can form such structures on both pine and lupine.

Keywords: Lupinus latifolius, Pinus contorta, Phialocephala fortinii, Mycelium radicis atrovirens, septate root endophytes.

Introduction

Fungi colonizing roots include well-known pathogens and mutualists as well as frequently observed types with unknown functions. Researchers call intracellular fungal colonization that does not fit recognized categories of mycorrhizae or pathogen "neutral" (Smith & Smith, 1990), "endophytic" (Currah, Siegler & Hambleton, 1988; Stoyke & Currah, 1991), "pseudomycorrhizal" (Melin, 1923; Kowalski, 1973; Wilcox & Wang, 1987), "weakly pathogenic" (Egger & Paden, 1986; Wilcox & Wang, 1987), or "dark-septate" (Haselwandter & Read, 1980; Currah & Van Dyk, 1986). Such colonization occurs on roots of Lupinus spp., other legumes, conifers, and many vascular plants (Melin, 1923; Peyronel, 1924; Thomas, 1943; Haselwandter & Read, 1982; Currah & Van Dyk, 1986; Cazares, 1992; Fischer, 1992; Chapter 1), In most cases the fungi responsible have not been cultured or identified. We will herein refer to the more or less symptomless, intracellular fungal colonization of roots collectively as septate endophytes (SE); some are hyaline, hence "dark-septate" is inappropriate. SE are reported to increase growth of host plants by Haselwandter & Read (1982) and Wilcox & Wang (1987), but for the most part growth response is unknown. Although they are

widespread and of potential ecological importance, SE are generally poorly documented (Harley & Smith, 1983, p. 359; Smith & Smith, 1990). The structure of the root-fungal interface can evidence the nature of a symbiosis (Bracker & Littlefield, 1973; Smith & Smith, 1990). For example, many root pathogens degrade host tissues and colonize the vascular cylinder while most types of mycorrhizae form complex, long-lived structures presumably involved in nutrient exchange (e.g. arbuscules; labyrinthine tissues).

Phialocephala fortinii Wang & Wilcox is one fungal taxon known to form SE colonization's on several hosts. The type specimen of P. fortinii is an isolate from Pinus sylvestris roots. It is one of several fungal taxa known from many coniferous hosts referred to collectively as Mycelium radicis atrovirens Melin (Wang & Wilcox, 1985). In dual culture studies P. fortinii killed seedlings of Pinus resinosa and Picea rubens within seven months at pH 5.7; at pH 3.0 the seedlings survived but were stunted, with fungal colonization of stelar tissues and some Hartig-net formation (Wilcox & Wang, 1987). The association was called pseudomycorrhizal, meaning "weak pathogens with some morphological traits of ectomycorrhizae" (Wilcox & Wang, 1987). P. fortinii was isolated from Arctostaphylos uva-ursi, Cassiope mertensiana, Luetkea pectinata and Vaccinium scoparium by Stoyke & Currah (1991) who also studied its colonization of Menziesia ferruginea roots in dual culture. Stoyke & Currah (1991) described P. fortinii as an endophyte of these hosts,

forming extensive hyphal wefts on the root surface and "intracortical sclerotia of compact, darkly pigmented and irregularly lobed, thick-walled hyphae". Though P. fortinii was frequently isolated from ericaceous hosts, Stoyke & Currah (1991) clearly distinguish its "endophytic" colonization from ericoid mycorrhizae by its formation of intracellular sclerotia rather than hyphal coils. P. fortinii has also been isolated from several orchid species (Currah et al., 1987; Currah, Hambleton & Smreciu, 1988) but colonization of these hosts has not been characterized.

In a survey of field-collected lupine roots, O'Dell & Trappe (Chapter 1) found septate endophytes in 7 of 12 species and 19 of 44 collections examined. Some of these lupines grew in conifer forests. A fungal isolate from roots of Lupinus latifolius Agardh. growing under Pinus contorta Dougl. produced Phialocephala fortinii conidiophores in culture, the first report of its occurrence on a legume. We examined the structures formed by P. fortinii inoculated on lupine and pine under controlled conditions to characterize structures formed by their interactions.

Methods

Plant Material

Seed of Lupinus latifolius was collected from a naturally occurring population in the Gifford-Pinchot National Forest, Lewis County, Washington. A voucher collection from this

population is in the Oregon State University herbarium. Seed was stored at 0°C until use. Seed of Pinus contorta was obtained from the USDA Forest Service. Pine seeds were wetted in Tween 80 (J.T. Baker Co.), rinsed with distilled water, disinfected in 5% H_2O_2 overnight and germinated on 1% malt extract agar. Lupine seed was treated similarly with the addition of a 30 min. scarification in concentrated HCl after wetting. Seeds lacking contaminating bacteria and fungi had seed coats removed and were transferred to growth pouches (Northrup King, Minneapolis, MN) containing 10 ml of sterile, distilled water one week after germination.

Fungi

Lupine seedlings from a population in the Ochoco National Forest, Wheeler Co., Oregon known from previous sampling to be colonized by SE were excavated along with field soil and grown in a greenhouse for three months before isolating root colonizing fungi and nodulating bacteria. Nodules and root segments were washed in Tween 80, surface sterilized in 0.1 % $HgCl_2$ and crushed on yeast extract-mannitol agar (YEM) (10.0 g mannitol, 0.4 g yeast extract, 0.1 g NaCl, 0.2 g $MgSO_4$, 0.5 g K_2HPO_4 per liter). Slow-growing fungal colonies were subcultured on to YEM, MMN (modified Melin-Nokrans media; Molina & Palmer, 1982) or 0.5% malt extract agar (MEA). Fungal cultures were maintained on MEA or MMN agar slants. Inoculum of P. fortinii was prepared by growing fungal cultures for one month on petri dishes of MMN

agar, collecting 10 mm diameter plugs from the growing margin of the fungal colony, and placing these colonized plugs on water agar petri plates for one week before transferring them to growth pouches containing lupine or pine seedlings.

Bacteria

Bacterial cultures were obtained by the same method as the fungi. Slow-growing bacterial colonies were streaked to purity on YEM and single colonies subcultured on YEM agar slants and frozen for later use. Those isolates tested formed nodules, able to reduce acetylene, on roots of Lupinus latifolius and accordingly can be designated Bradyrhizobium sp. (Lupinus). Lupinus seedlings were inoculated by pipeting about 0.5 ml of a 10^3 dilution (10^5 colony-forming units) of a broth culture directly on the roots of seedlings after one week in the growth pouch.

Inoculation & Growing Conditions

Axenic Culture

Pure culture synthesis tubes containing one 13 by 18 cm piece of Whatman No. 3 filter paper and 50 ml modified Murashige and Skoog woody plant media (minus N, minus carbohydrate, one-fourth strength P) were autoclaved, planted with one seedling of L. latifolius, and inoculated with 10^5 colony-forming units Bradyrhizobium sp. (Lupinus) in YEM. Controls for bacterial inoculation received sterile YEM. A single agar plug colonized by one of three fungal species, or uncolonized for controls, was

placed below the radicle of each seedling. Plants were grown under artificial light in a 20°C water bath for eight weeks before clearing and staining to assess fungal colonization.

Growth Pouch

Ten to 15 seedlings of each species were grown at 20°C in growth pouches under fluorescent light with 9/15 hour day/night cycle, watered with distilled water as necessary, and fertilized with five ml of half-strength modified Melin-Nokrans nutrient solution (Marx & Bryan, 1975) monthly during the course of the study. Lupines were inoculated with Bradyrhizobium sp. (Lupinus) after one week; both plant species were inoculated with P. fortinii after four to five weeks in the growth pouch (Figures 3.1 & 3.4), or were left uninoculated as controls. Lupine roots were harvested 12 weeks after inoculation; pine roots were harvested 24 weeks after inoculation.

External Morphology, Light Microscopy & Clearing of Roots

The external morphology of root systems was examined periodically with a dissecting microscope to monitor fungal colonization. Colonized root segments of five individuals of each species were processed for microscopy. Root segments for sectioning were cut with a razor blade, placed in 2.5% gluteraldehyde in 0.1M HEPES buffer for four hours, then rinsed four times in cold buffer, dehydrated in a graded ethanol series over three days, infiltrated with LR White acrylic resin (London

Resin Company) and embedded in gelatin capsules. Embedded roots were sectioned with glass knives on a microtome and stained with 0.1% Toluidene blue O in 1.0% sodium borate.

For clearing, roots were placed in 3.0 % KOH overnight in a 70 °C water bath, then rinsed in distilled water and mounted in polyvinyl alcohol.

Photography

Photomicrographs were recorded on Kodak TMAX 100 film with a green filter to enhance contrast and developed in TMAX developer (Kodak Co.) following manufacturers' instructions.

Results

Isolations of fungi

About fifteen isolates, including at least three taxa (judging from colony morphology), were obtained. One such culture formed colonization resembling that of field-collected roots when inoculated on to lupine roots in axenic culture. This fungal culture formed Phialocephala fortinii Wang & Wilcox conidiophores after 15-18 month's storage at 5 °C (Figures 3.2 & 3.5) and is the subject of this study. The other isolates tested were sterile and failed to colonize lupine intracellularly under these conditions.

Growth pouch

In growth pouches root branching differed considerably between the two plant species. Lupinus latifolius grew with a tap root, few adventitious and lateral roots emerged, forming a loosely racemose root system (Figure 3.1). All lupine plants inoculated with Bradyrhizobium sp. (Lupinus) formed nodules that were absent those not inoculated (Figures 3.1, 3.8 & 3.9). Older lupine roots had a zone of cavitation (collapsed cells) within the cortex (Figures 3.13 & 3.14). Pinus contorta had a tap root with numerous first order laterals, second and third order branching occurred but dichotomy was rare (Figures 3.4 & 3.6).

P. fortinii grew evenly on to the pouch from agar plugs. Occasionally the fungus grew within fibers of the pouch forming sclerotia-like irregularly swollen cells similar to those described in roots (see below). Loose wefts of hyphae grew along the roots surface with some branching, surface patches of sclerotium and sporadic inter and intracellular colonization of the cortex. The haphazard cortical colonization resulted in localized areas of greater colonization and sclerotium development scattered along primary and secondary roots of both hosts (Figure 3.3).

Primary and secondary roots and nodules of L. latifolius were colonized by inter- and intracellular hyphae of P. fortinii (Figures 3.3 & 3.7-3.9). Colonization was restricted to the

epidermis and outer cortex of roots and nodules (Figures 3.7-3.9, 3.14 & 3.15). Root hairs and other epidermal cells contained both cylindrical hyphae and sclerotia. The sclerotia were composed of thick walled, irregularly lobed and compacted cells which sometimes formed sheets several cells thick. Similar intercellular sheets of sclerotia were observed in the cavitation zone. Cortical colonization was similar to epidermal and restricted from the endodermis and vascular cylinder.

Colonization of Pinus contorta occurred on first order and second order lateral roots and resembled that of L. latifolius. Sclerotia formed inter- and intracellularly in epidermal and cortical cells (Figures 3.10, 3.16 & 3.17); root hairs were colonized (Figure 3.12); and the fungus did not penetrate the vascular tissues. Patches of Hartig net (labyrinthine fungal tissue) occurred on primary roots of pine (Figure 3.11) but were not observed on lupine. Colonization of lateral pine roots tended to occur in the proximal portion of the roots. The basal portion of these roots often had a sporadic mantle and fungal colonization of wounds produced by the emergence of higher order lateral roots was consistently observed (Figure 3.18).

Discussion

Colonization of L. latifolius and Pinus contorta by Phialocephala fortinii was strikingly similar. All the structures occurring on lupine, plus some additions, were present on pine. Intercellular hyphae and intracellular sclerotia commonly

occurred on primary and secondary roots of both hosts. P. fortinii formed labyrinthine tissue (Hartig net) on pine, on which it also colonized proximal portions of ultimate lateral roots. The development of a cavitation zone on L. latifolius roots could indicate a hypersensitive reaction, however no other degradation of these roots was observed. Since control roots (Figure 3.13) as well as those colonized by P. fortinii exhibited this feature it is not caused by P. fortinii, but is either normal secondary root development of L. latifolius or an artifact of growing conditions. One possible cause is the dryness of the growth pouches, required to reduce bacterial contamination of lupine seed. Drought causes cavitation in roots of Agave sp. (North & Nobel, 1991).

No adverse reaction of either host to P. fortinii was observed, nor was there extensive degradation of host tissue or colonization of vascular tissues. After four months in growth pouch and extensive colonization by P. fortinii the plants and root systems of both species appeared healthy. The formation of similar fungal structures in roots of both hosts without significant adverse reaction indicates a commensal or mutualistic association.

The colonization of Pinus contorta by Phialocephala fortinii here described is virtually identical to P. fortinii colonization of Cassiope mertensiana, Luetkea pectinata and Menziesia ferruginea (Stoyke & Currah, 1991). It is also similar to the colonization of Pinus contorta by Geopyxis carbonaria and Trichophaea

hemisphaerioides, both of which form complex intracellular structures (sclerotia?) in the root cortex but fail to penetrate the vascular cylinder (Egger & Paden, 1986). Another striking similarity is the formation of a rudimentary Hartig net by G. carbonaria on roots of P. contorta.

Colonization of proximal portions of lateral roots of pine by P. fortinii is quite different from the colonization of distal portions of roots typical of ectomycorrhizal fungi on compatible hosts (e.g. Massicotte, Peterson & Ashford, 1987). P. fortinii therefore appears to occupy a rhizoplane niche distinct from that used by ectomycorrhizal fungi and likely functions differently as well. Simultaneous colonization of root systems by P. fortinii and typical ectomycorrhizal fungi may occur, as evidenced by the isolation of P. fortinii and dark sterile fungi (many of which may be P. fortinii (Stoyke & Currah, 1991)) from ectomycorrhizae (Levisohn, 1954; Trappe, 1962; Summerbell, 1982; Wang & Wilcox, 1985).

The separation of colonized outer cortex from the inner cortex by a cavitation zone makes direct physiological interaction between L. latifolius and P. fortinii unlikely. This data, together with lack of growth response of L. latifolius to inoculation with P. fortinii (Chapter 2) indicate that P. fortinii is a commensal saprotroph of L. latifolius roots under these conditions (growth pouch and greenhouse). This does not preclude a beneficial association with other hosts, or with Lupinus under

other conditions. Stoyke & Currah (1991) speculate that a dark septate fungus found by Haselwandter & Read (1982) to increase growth and phosphorus concentration of two Carex species was P. fortinii. Carol (1986) introduced the concept of inducible mutualism to describe a plant-fungal association where benefit is conferred only under particular conditions such as in the presence of a grazing animal or fungal pathogen. P. fortinii may compete with pathogens by colonizing cortical tissue before sloughing or it may produce compounds antagonistic to pathogens or grazers, in either case it would be an inducible mutualist. Keah & Brown (1990) showed that Psilocybe semilanceata (Fr. :Secr.) Kumm., an SE of roots of some grasses, inhibits growth of some pathogenic fungi in culture.

Though P. fortinii has been isolated from roots of Pinaceae, Rosaceae, Orchidaceae, and Leguminosae, few studies have been made of its colonization of roots and effects on host growth (Wang & Wilcox, 1985; Currah, Siegler & Hambleton, 1987; Stoke & Currah, 1991). The wide range of hosts and habitats where P. fortinii occurs indicates the potential for significant ecological functions awaiting discovery.

Gallaud (1905) first described SE colonization on Allium sphaerocephalum L. and Ruscus aculeatus L. Peyronel (1922) documented it on Triticum aestivum L. and then reported SE on 135 species of angiosperms (Peyronel, 1924). Although convinced by his observations of fungal cultures and field collected roots

that several different fungal taxa were represented, for the sake of simplicity Peyronel referred to all of them as "the Rhizoctonia". Colonization by "the Rhizoctonia" involved typical simple and branched hyphae that sometimes produced

> "... more short, branched, clavate...barrel-shaped segments, morphologically similar to conidia of Oidium or better yet, Monilia"

(Peyronel, 1924). These swollen hyphae sometimes aggregate and coil into bunches of thick-walled cells which Peyronel called "stromatic nodules". Similar structures were observed on roots of Pinaceae by Melin (1924), who called them "pseudomycorrhizae" to indicate their (Melin judged) parasitic rather than mutualistic behavior. Melin called pseudomycorrhizal fungi Mycelium radicis atrovirens (M.r.a.), a name that has since been applied to many sterile dark fungi isolated from ectomycorrhizae.

Strains of M.r.a. vary in the structures that they form on ectomycorrhizal hosts. Some form classic ectomycorrhizae, others "pseudomycorrhizae" , still others are characterized as pathogenic (Kowalski, 1973; Wilcox & Wang, 1987). The M.r.a. complex includes at least two form-species based on conidial morphology. Some isolates of M.r.a. were discovered by Richard & Fortin (1973) to form Phialocephala dimorphospora Kendrick conidiophores after extended exposure to low temperature. Wang & Wilcox (1985) described P. fortinii from an isolate obtained

from roots of Picea abies. Phialocephala dimorphospora formed "pseudomycorrhizae" on roots of Pinus resinosa Ait. and increased host growth at low pH (3.5), whereas Phialocephala fortinii was pseudomycorrhizal or pathogenic (based on degradation of host tissues and colonization of vascular tissues) on the same host (Wilcox & Wang, 1987). Since most experiments regarding M.r.a. have used unidentified isolates, it is hardly surprising that confusion remains as to whether these organisms are parasitic, commensal or mutualistic.

Peyronel (1923, 1924) is often cited as the first to document the widespread occurrence of septate hyphae on plant roots (cf. Rayner, 1927; Gadd, 1929; Burges, 1936; Harley, 1950). So frequently did this occur with VAM that he coined the phrase "dual association" for this phenomenon. Peyronel felt that "the Rhizoctonia" facilitated VAM colonization of some hosts and hypothesized that it might benefit some hosts but parasitize others depending on the precise fungus involved:

> "Sembra, insomma, predominare in questo fungo la vita saprofitaria od emiparassitaria su quella simbiotica propriamente detta. Direi quasi che il suo parassitismo e troppo attenuato per raggiungere, nel maggior numero dei casi, una vera e propria simbiosi." ("It appears, in short, the saprophytic or hemiparasitic life predominates in this fungus over the proper symbiotic one. It can be said that its parasitism is too much mitigated, in the greater number of cases, to overtake a regular symbiosis.").

This statement is somewhat ambiguous, but Rayner (1927) ignored the second sentence in her interpretation "...it ["the

Rhizoctonia] behaves rather as a quasi-parasite or saprophyte than as a true symbiont.".

Acknowledgments

The research was funded by the Indo-US Science and Technology Initiative and National Science Foundation Grant BSR 8717427. Sheri Shenk and Al Soeldner provided helpful guidance with microtechnique. The USDA Forest Service Pacific Northwest Research Station provided facilities for this research. Dr. Peter Bottomley provided facilities for bacteria isolations.

Figure 3.1. Lupinus latifolius seedlings in growth pouch with agar plug (arrow) of Phialocephala fortinii.

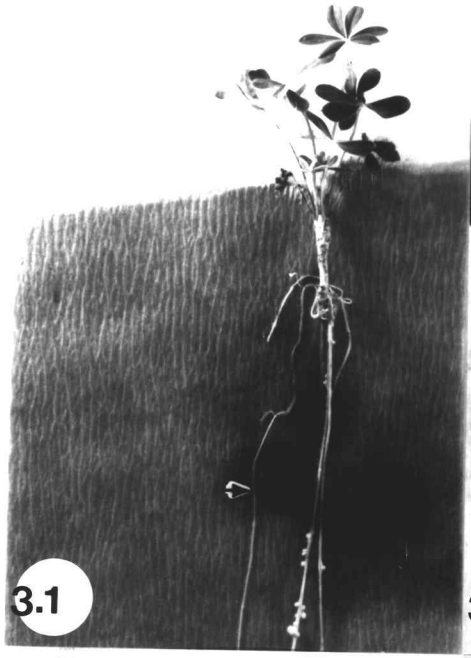
Figure 3.2. Conidiophore of P. fortinii in pure culture. Bar = 10 µm.

Figure 3.3. Root of L. latifolius colonized by P. fortinii.

Figure 3.4. Pinus contorta seedlings in growth pouch with agar plug (arrow) of P. fortinii.

Figure 3.5. P. fortinii in pure culture with characteristic conidiophore and hyphal coils.

Figure 3.6. P. contorta roots on growth pouch with agar plugs colonized by P. fortinii.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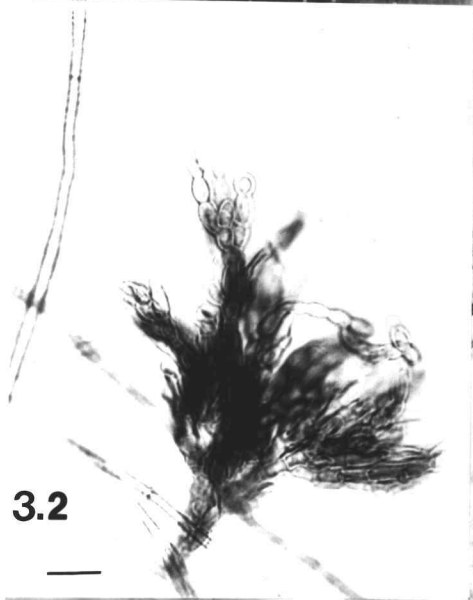
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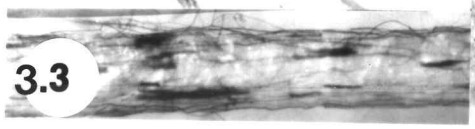
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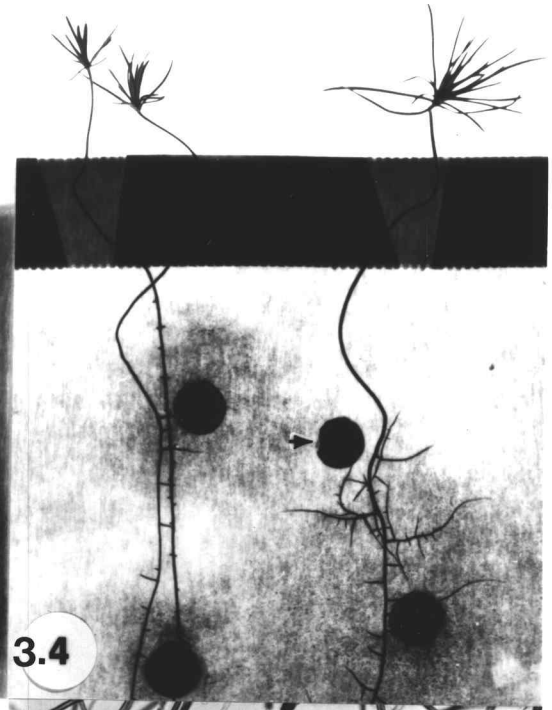
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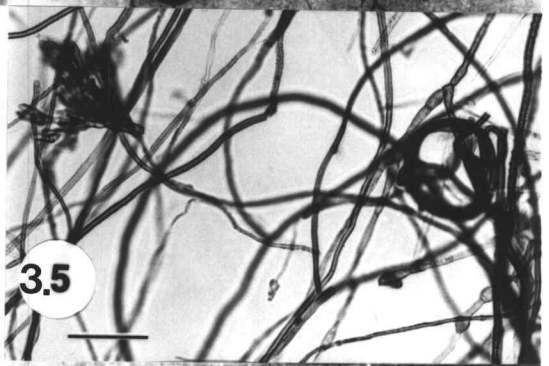
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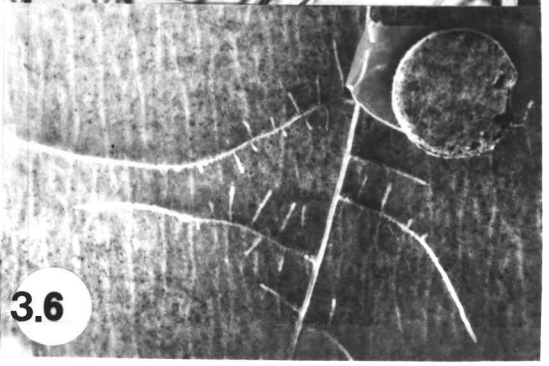
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Figure 3.7. Cleared root of L. latifolius showing colonization by P. fortinii. Intracellular sclerotia in epidermal cells of primary root (arrow) and extraradical hyphae (double arrow) bar = 50 μm.

Figures 3.8 and 3.9. Cleared root nodule of L. latifolius colonized by P. fortinii. Figure 8 (inset) nodule and primary root with associated hyphae. Figure 9. Intracellular sclerotia in epidermal cells of root nodule (arrow) bar = 100 μm.

Figure 3.10. Cleared root of P. contorta colonized by P. fortinii. Intracellular sclerotia in epidermal cells of primary root (arrow) and intercellular hyphae (double arrow). Bar = 100 μm.

Figure 3.11. Cleared primary root of P. contorta showing labyrinthine tissue (arrow) of P. fortinii. Bar = 10 μm.

Figure 3.12. Transverse section of P. contorta root showing colonization of root hairs by P. fortinii. Bar = 30 μm.

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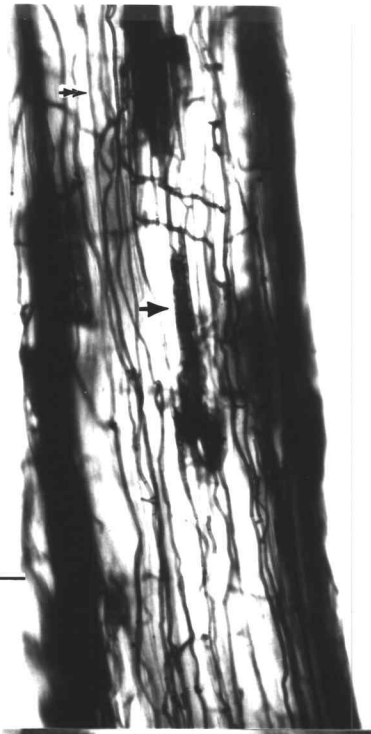

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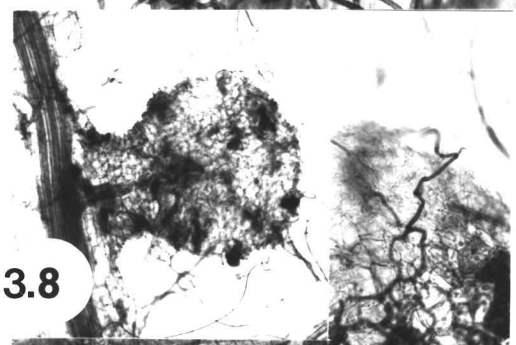

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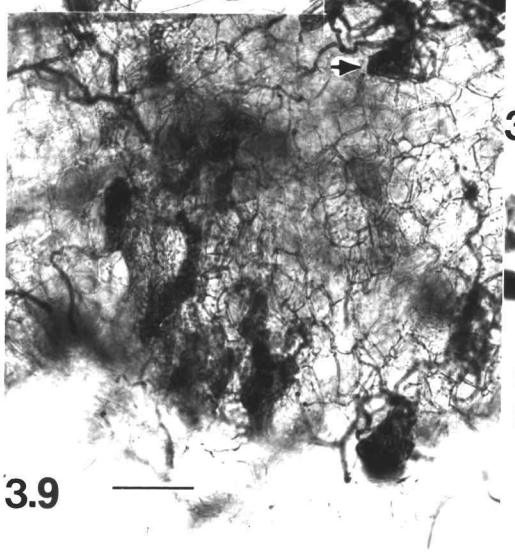

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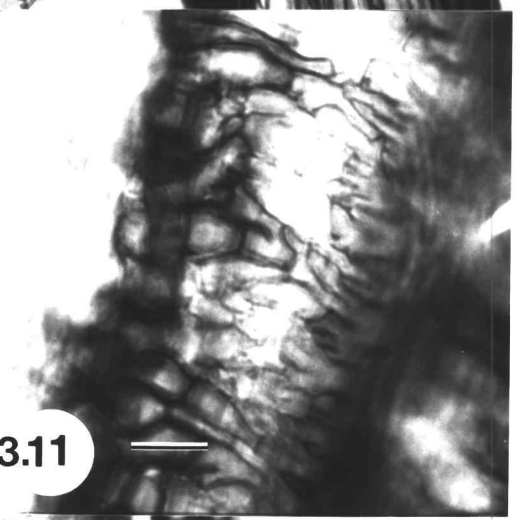

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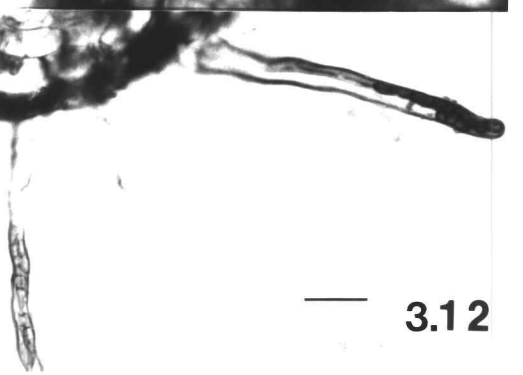

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Figure 3.13. Transverse section of uncolonized L. latifolius root, cavitation zone (arrow) within cortex. Bar = 20 μm.

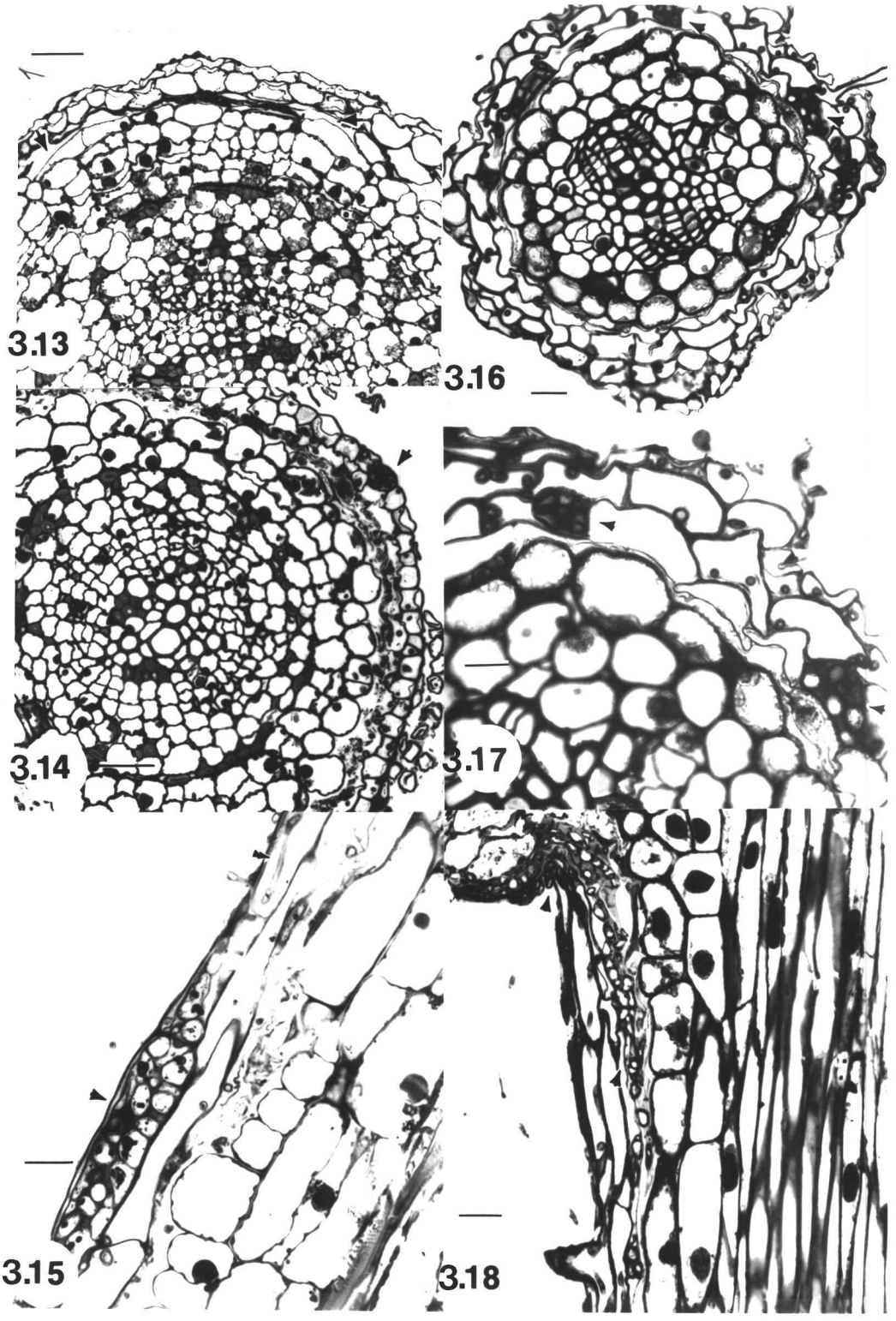
Figure 3.14. Transverse section of L. latifolius root colonized by P. fortinii. Sclerotium (arrow) and cylindrical hyphae (double arrow) in cells of cortex and in cavitation zone. Bar = 20 μm.

Figure 3.15. Longitudinal section of L. latifolius root. sclerotium (arrow) and linear intracellular hypha (double arrow) of P. fortinii. Bar = 10 μm.

Figure 3.16. Transverse section of P. contorta short root colonized by P. fortinii. Sclerotia (arrows). Bar = 20 μm.

Figure 3.17. Transverse section of P. contorta short root colonized by P. fortinii. Sclerotia (arrows). Bar = 10 μm.

Figure 3.18. Longitudinal section of P. contorta short root colonized by P. fortinii with basipetal colonization of wound caused by secondary root emergence. Bar = 10 μm.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Chapter 4. Natural ^{15}N Abundance Estimates of Nitrogen Fixation by Three Legume Species in Conifer Plantations of the Wenatchee National Forest

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Summary

Nitrogen fixation (natural ^{15}N abundance), root morphology and mycorrhizal colonization of three legume species seeded in clearcut forest sites were compared. Medicago lupinula derived significantly more N from atmospheric sources than the other two legumes on a percentage basis, but among species differences in N from atmosphere per hectare were not significant. Lupinus albicaulis cv. Hederma had significantly more coarse roots and less vesicular-arbuscular mycorrhizal colonization than M. lupinula or Trifolium hybridum.

Introduction

Recognition of the potential uses of legumes in forestry is growing. As hosts of symbiotic diazotrophic (N_2-fixing) bacteria, legumes can provide substantial quantities of nitrogen to conifer plantations, thereby increasing early survival and growth of trees

(Gadgil, 1971; Smethurst & Turvey, 1986). Legumes also serve as forage for cattle and wildlife.

While some legumes establish naturally in conifer stands of the Pacific Northwest, N inputs from such sources are small compared to pasture or cropping systems (Hendrickson & Burgess, 1989). Planting legumes in conifer plantations could increase legume cover and consequent N fixation and forage production.

Legumes have received relatively little attention in forestry compared to agricultural research, and actual N inputs from fixation by legumes have never been estimated in an operational forestry context. Legumes growing in soil have access to soil and atmospheric N. Measurements of total N include both of these sources, but natural abundance of ^{15}N can distinguish between them, allowing estimates of quantities of N from fixation (Shearer & Kohl, 1986).

In addition to their nitrogen-fixing bacteria, many legumes host fungal root symbionts and so can be considered members of a tripartite symbiosis (Barea & Azcon-Aguillar, 1983). In most cases the fungi involved form vesicular-arbuscular mycorrhizae (VAM) which provide nutrients and water to the host plant. Legumes often produce more biomass and fix more N when VA mycorrhizal (Ames & Bethlenfalvay, 1987; Crush, 1974; Daft & El-Giahmi, 1976).

Unlike rhizobia, inoculum of VAM fungi is laborious to produce and not commercially available at present. If VAM inoculum is reduced by post-harvest treatment of plantations (e.g. broadcast burning) it could limit establishment of some legume species. Lupinus spp. are less frequently colonized by, and presumably less dependent on, VAM than many legumes and might therefore be expected to establish more reliably on a range of sites than more VAM-dependent legume species. This study compared N-fixation and mycorrhizal colonization of a lupine and two other legume species seeded into broadcast-burned clearcuts in Wenatchee National Forest.

Methods

Sites

Four legume species trial blocks were established in the Wenatchee National Forest between 14 May and 14 June, 1990. The sites (blocks) ranged from 500 to 1500 meters elevation in the Pseudotsuga menziesii zone (Franklin & Dyrness, 1974). Each site was planted with ten species, eight legumes and two non-legumes, in a completely randomized block. Inocula of appropriate rhizobia were applied to legume seed according to manufacturers instructions prior to seeding. On each block seed of each species was broadcast by hand into a 53.5 m^2 area at a rate of 85 to 515 seeds per m^2 (0.9 to 4.6 kg per ha). Smaller seeded species such as black medic were seeded at the higher

rate while larger seeded species such as hederma lupine were seeded at the lower rate.

Plants were collected for root analyses in July, 1991. Final harvest for forage productivity and nitrogen and phosphorus content was in October, 1991. The three most productive legumes (Table 4.1), Lupinus albicaulis Dougl. cv. Hederma (hederma lupine), Medicago lupinula L. (black medic), and Trifolium hybridum L. (alsike clover), were selected for N analyses; blue wildrye (Elymus glaucus) was the non-nitrogen fixing reference species.

Collecting and processing plants

Three to five individuals of each species were randomly chosen for harvest at each site. Plants were carefully excavated to obtain as much of the root system as possible and stored at five °C until processed. Roots were washed, weighed fresh and total root length and length of roots greater than one mm diameter were estimated by the grid intersect method (Tennant, 1975) using a grid of one cm squares. A subsample of approximately ten percent of total root weight was removed and placed in a capsule to clear and stain for fungal colonization. The remaining roots and shoots were dried and weighed. All tissues were dried for at least 48 hours in a 70 °C oven.

Tissue analyses

Approximate 1.5 g of leaf tissue was removed from each sample and ground in a Wiley mill to pass through a 40 mesh screen. The ground tissue was analyzed for total (Kjeldahl) N and P (Nelson & Sommers, 1972).

^{15}N

Forty milligram samples of plant tissue were analyzed by Boston University Stable Isotope Laboratory using a Finnigan-MAT Delta-S mass spectrometer with a Heraeus CN analyzer and Finnigan trapping box. Values were reported as $\partial^{15}N$, which equals:

$(^{15}N/^{14}N$ sample - $^{15}N/^{14}N$ standard)÷$^{15}N/^{14}N$ standard * 1000 $\partial^{15}N$ per mil.

Atmospheric N_2 was the standard.

Fraction of N derived from atmospheric N_2 (fNdfa) was calculated by the formula:

$fNdfa = (\partial^{15}N_o - \partial^{15}N_t) \div (\partial^{15}N_o - \partial^{15}N_a)$ (Shearer & Kohl, 1989).

Where $\partial^{15}N_o$ is the ∂ value of a neighboring, non-N-fixing plant, in this case blue wildrye from the same block, $\partial^{15}N_t$ is the ∂ value of the legume growing in the field (i.e. with access to soil, as well as atmospheric N), and $\partial^{15}N_a$ is the ∂ value of the legume grown with atmospheric N as the sole source of nitrogen. We

used the lowest published values of $\partial^{15}N_a$ for each genus: -1.82 $\partial^{15}N$ for Lupinus (Shearer & Kohl, 1989), -1.4 $\partial^{15}N$ for Trifolium (Ledgard, 1989), and -0.92 $\partial^{15}N$ for Medicago (Yoneyama et al., 1986). Aboveground N from fixation per hectare was calculated as nitrogen content (%N) * fNdfa* biomass per hectare.

Fungal colonization

Two types of fungal colonization were distinguished: vesicular-arbuscular mycorrhizae (VAM) were distinguished by the presence of vesicles or arbuscules or both; septate endophytic fungi were distinguished by the presence of septate intracellular hyphae. A subsample of each root system was cleared for 12 hours in 5.0 % KOH at 70 ° C, acidified in 1.0 % HCl and stained for 12 hours in 0.1 % trypan blue at 70 ° C (Phillips & Hayman, 1970). Roots were examined at 20 to 50 x magnification, fungal colonization estimated by category (0= not observed; 1= <5% of root length colonized; 2= 5-25%; 3= 26-50%; 4= 51-75%; 5= >75%) and confirmed by examination at higher (100-1000x) magnification. Frequency of colonization is reported as the percentage of subsamples examined having any colonization.

Statistical analyses

Data for each species were averaged by block. Preliminary analyses revealed unequal variances and non-normal distribution of residuals which were reduced by logit (Z = log(P/1-P)) transforming percentage data (Sabin & Stafford, 1990). Response

variables were subjected to analysis of variance and, when appropriate, differences between adjacent ranked means tested by Fishers Protected least significant difference (FPLSD; Petersen, 1985). Results presented are medians back-transformed from means on which tests for differences were conducted. Analyses were conducted on Macintosh computers using Data Desk 3.0 and Statview 512+ software.

Results

Root morphology

Roots of hederma lupine had a significantly larger proportion of coarse roots (> 1mm diameter) than the other three species. Almost half of lupine roots were coarse, while rye, medic and clover roots had less than 5% coarse roots (Table 4.2).

Nitrogen and phosphorus content

The three legumes contained significantly more N and less P than rye. In the first year of growth, black medic derived significantly more N from fixation than hederma lupine (60 vs. 34 %), but neither of these species differed significantly from alsike clover. Estimated aboveground plant tissue N derived from fixation by species and site ranged from 6.1 to 39.9 kg per hectare (average = 22.0 kg/Ha) and did not vary significantly by species or block.

VAM & SE colonization

Blue wildrye and hederma lupine were colonized significantly less by VAM than were alsike clover and black medic (Table 4.2). Colonization of the former species was both less intense (proportion of root system) and less frequent (proportion of plants sampled) than the latter two. VAM colonization did not vary significantly by block.

SE colonization differed significantly by block but not by species.

Discussion

Nitrogen derived from fixation by species and block (replicate) ranged from 6.1 to 39.9 kg per hectare, averaging 22.0 kg/Ha. Although this amount of nitrogen is lower than that obtained in some studies of field crops, it is more than ten times the estimated 1.9 kg/Ha from Lupinus arcticus in a logged Pinus contorta stand (Hendrickson & Burgess, 1989), verifying the potential to increase N inputs from biological sources through management practices. Although species differed in fNdfa, this did not translate into significant differences in amounts of N fixed because of the variation in biomass production.

The value of $\partial^{15}N_a$ (reference legume dependent on atmospheric N) varies with the age of the plant (Awonaike, Kumarasinghe & Danso, 1991; Evans et al., 1987; Shearer & Kohl,

1989) and bacterial strain (Bergersen et al., 1986; Ledgard, 1989). Although the legumes planted in this study were inoculated, they are likely nodulated by indigenous as well as the inoculated strains of rhizobia. Rather than attempting to grow the three species in liquid media for over a year with a realistic mix of nodulating bacteria, we relied on published values of $\partial^{15}N_a$. Using the lowest (most negative) value of $\partial^{15}N_a$ gives a conservative (low) estimate of fNdfa.

Among species variation in VAM colonization was substantial and, as predicted, hederma lupine was the least colonized of the legumes. However, VAM propagules were apparently available at all sites, since neither frequency nor intensity of VAM was affected by site. No relationships between VAM colonization and nodulation or N-fixation were detected. Apparently other factors are more important determinants of productivity and N fixation by these plants.

It is interesting to note that the legume with the coarsest root system was the least mycorrhizal. Hederma lupine was colonized in only nine percent of collections examined, and at very low levels (rarely more than five percent of root length) in contrast to 35 to 50 percent of black medic and alsike clover collections colonized, with an average of about 25 percent of root length colonized. Baylis (1975) describes two strategies for phosphorus aquisition by plant roots: (1) non-mycorrhizal plants with extensive fine roots and abundant root hairs, and (2)

mycorrhizal hyphae as a functional extension of the root system. Although lupines lack abundant fine roots and abundant, long root hairs, they are apparently non- or facultatively mycorrhizal and illustrate a third strategy for phosphorus uptake. L. albus roots produce large quantities of citrate ions (Gardner & Boundy, 1983) which increase phosphorus availability; this mechanism may occur in other species of Lupinus.

Acknowledgments

The USDA Forest Service Soil Microbiology Team, PNW Experiment Station provided facilities for this research. This study was funded in part by the U.S.- Indo STI.

Table 4.1. Average aboveground biomass of legume species[1]

	Alsike clover	Black medic	Hederma lupine
Average biomass (kg ha^{-1})	1503	2256	2631

[1]Java-Sharpe and Everett, unpublished data.

Table 4.2. P-values from analysis of variance for selected response variables.

Variable	Block	Species
Percent coarse roots	0.69	0.005
VAM colonization	0.35	0.001
VAM frequency	0.25	0.002
SE category	0.001	0.52
SE frequency	0.001	0.35
Shoot % N	0.92	0.004
Shoot % P	0.30	0.007
fNdfa[1]	0.005	0.065
Kg Ndfa[2]/Ha	0.52	0.72

[1]fraction of N derived from atmosphere

[2]N derived from atmosphere

Table 4.3. Medians by species of selected response variables.[1]

	Alsike clover	Black medic	Blue wildrye	Hederma lupine
Percent coarse roots	3.4a	3.2a	0.0a	46.1b
VAM colonization	1.98a	1.94a	0.10b	0.18b
VAM frequency	0.48a	0.35a	0.07b	0.09b
SE catagory	0.46a	1.8a	0.0a	0.09a
SE frequency	0.16a	0.22a	0.04a	0.16a
Shoot % N	2.56b	2.73ab	1.19c	3.23a
Shoot % P	0.31a	0.27a	0.46b	0.30a
fNdfa[2]	0.51ab	0.60a	nd	0.36b
Kg Nfda[3]/Ha	18.8a	25.9a	nd	21.3a

[1]values within rows not sharing a common letter are significantly different ($p \leq 0.05$, FPLSD).

[2]fraction of N derived from atmosphere

[3]N derived from atmosphere

Chapter 5. General Summary

By Thomas E. O'Dell

What was Previously Known

Vesicular-arbuscular mycorrhizae (VAM) and Legumes

Legumes are generally colonized by VAM fungi when growing in field soil (Barea & Azcon-Aguillar, 1983). Many experiments have documented increased growth, nodulation and N-fixation as a result of VAM formation by legumes. Because of the nutrient demands of N-fixation, increased phosphorus from VAM particularly benefits most nodulated legumes. One apparent exception to the mycorrhiza-dependent legumes are lupines. Observations of low VAM levels in lupine roots led to the conclusion that they were nonmycorrhizal (Trinick, 1977). Few experiments have been conducted to determine whether lupines respond to VAM inoculation despite low levels of colonization.

Septate Endophytic Fungi of Roots

Fungi colonizing roots in soil include other, apparently non-pathogenic types in addition to commonly accepted categories of mycorrhizae. Although observed for decades and documented from a great variety of plant species, morphology of colonizations or plant growth response of these septate endophytic fungi have been studied only rarely (Currah et al., 1986; Haselwandter & Read, 1982; Peyronel, 1923; Stoyke and Currah, 1990).

Legume N fixation in Forestry Applications

A number of legumes, including several species of lupines, have been shown to increase early survival and N content of conifer seedlings when seeded into plantations (Gadgil, 1971). In addition lupines can increase P content of radiata pine (Smethurst & Turvey, 1986). We know of no attempts to quantify N inputs from fixation by legumes in an operational forestry context.

What was Discovered by this Research

VAM in Lupines

1. VAM-type colonizations occur in about half of the populations sampled, but colonization levels appear to be low. fungal colonizations were never observed in roots of some species.

2. Growth of Lupinus latifolius in the greenhouse was reduced or unaffected by inoculation with VAM fungi, even under conditions where growth of subterranean clover was increased.

SE in Lupines

3. Septate endophytic fungi occurred in roots of lupine almost as frequently as VAM-type colonizations. This is apparently the first report of SE in Lupinus roots.

4. One of the septate endophytes of lupines is Phialocephala fortinii, which is becoming recognized as a root endophyte of many plant species.

5. Growth of Lupinus latifolius in the greenhouse was unaffected by inoculation with P. fortinii; nodulation was increased in one experiment and deceased in another.

6. Colonization of L. latifolius and Pinus contorta roots by P. fortinii was not obviously pathogenic and resembled colonization by this fungus of conifers and other plants described elsewhere (Stoyke & Currah, 1990; Wilcox & Wang, 1987).

N-Fixation

7. An average of 22 kg-ha^{-1} N was fixed during fifteen months by Lupinus albicaulis cv. hederma and two other legumes seeded into conifer plantations in the Wenatchee National Forest, Washington.

What it All Means (Conclusions and Speculations)

VAM and Lupines

VAM are mild parasites or commensal associates of L. latifolius roots under the conditions tested. VAM may benefit L. latifolius under some condition or stress not encountered in these experiments (e.g. drought). Other lupine species may benefit from mycorrhizal colonization (e.g. L. texensis [T. Wood, unpublished data]).

Phialocephala fortinii and Lupines

P. fortinii appears to be a weak parasite or commensal associate of L. latifolius roots under some conditions, possibly a

mutualist under others. P. fortinii may be an inducible mutualist of L. latifolius, requiring particular conditions before benefitting the host. Resistance to disease and grazing are two examples of inducible mutualism commonly exhibited by leaf endophytic fungi.

Even if P. fortinii does not directly benefit L. latifolius, it may affect soil processes with positive effects for the plant. Presumably P. fortinii ingests carbon leaking from L. latifolius roots. The fungus may bind soil particles or leak carbon used by bacteria to bind soil into aggregates. Soil aggregation is important for water holding capacity and aeration and sometimes correlates with plant available nutrients (Tisdall & Oades, 1982). Thus a fungus appearing to be a parasite of a particular plant may actually be of indirect benefit.

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