THE ROLE OF ROOT-ASSOCIATED FUNGI

IN THE DOMINANCE OF *GAULTHERIA SHALLON*

by

GUOPING XIAO

B.Sc., Hunan Normal University, Changsha, China, 1981
M.Sc., Kunming Institute of Botany, Academia Sinica, Kunming, China, 1986

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Department of Botany

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Field roots of salal (*Gaultheria shallon* Pursh) were examined by light and scanning electron microscopy. Salal formed typical ericoid mycorrhizae characterized by a weft of hyaline hyphae on the surface of the hair roots and crowded hyphal complexes inside the outer layer of cortical cells. Heavy colonization by ericoid mycorrhizal fungi was present in and restricted to the outer of the two layers of root cortical cells. In some cases, a mantle-like structure of hyphae but no Hartig net was observed on the surface of these roots. Neither ecto- nor arbutoid mycorrhizae were observed on salal roots collected in the field. Over 85% of the roots and 90% of the cortical cells within roots were colonized. Four species of root-associated fungi were isolated from field-collected salal roots. Three of them were proven to form ericoid mycorrhizae and one of them formed pseudomycorrhizae in axenic culture. This includes *Oidiodendron griseum* Robak, *Acremonium strictum* W. Gams and two nonsporulating unknown species, which were described in culture.

Twelve known ericoid mycorrhizal fungi were tested for their ability to form mycorrhizae with salal in axenic culture. Five of the fungi developed typical ericoid mycorrhizae. These fungi are *Hymenoscyphus ericae* (Read) Korf & Kernan, *Oidiodendron flavum* Szilvinyi, *O. maius* Barron, *Pseudogymnoascus roseus* Raillo, and *Scytalidium vaccinii* Dalpe, Litten and Sigler.

The ability of the four species of root-associated fungi isolated from salal field roots to use different forms of organic nitrogen was tested in pure culture or in association with salal. The organic forms of nitrogen applied were glutamine (an amino acid), glutathione (a peptide), and bovine serum albumin (BSA, a protein). The fungi tested were *Oidiodendron griseum, Acremonium strictum,* and two nonsporulating unknown fungi. Glutamine was used as readily as ammonium

nitrogen by all four fungi and the plants of salal inoculated by those fungi. *Oidiodendron griseum* on glutathione and *Acremonium strictum* on BSA produced significantly higher yields in pure culture or in association with salal plants. The plants of salal inoculated by all four fungi had higher colonization rate on glutathione or BSA than on ammonium or glutamine. The colonization of salal roots by its root-associated fungi was reduced by application of available nitrogen and simple organic nitrogen, and favored by more complicated organic nitrogen.

In vitro interactions between species of 4 root-associated fungi, *Acremonium strictum*, *Oidiodendron griseum* and 2 unknowns, isolated from field salal roots and 3 ectomycorrhizal fungi of western hemlock (*Tsuga heterophylla* (Raf.) Sarge.), *Pisolithus tinctorius* (Pers.) Coker & Couch, *Rhizopogon semireticulatus* Smith and *Suillus lakei* (Murr.) Smith & Thiers, were examined and characterized on buffered and unbuffered modified Melin Norkrans agar (MMN) at 25^{0}C. Three interaction patterns were revealed, neutral intermingling, deadlock and inhibition. Inhibition, in which the growth of one mycelium was reduced by the other, was the predominant outcome of the pairing of ectomycorrhizal fungi opposing the root-associated fungi of salal. All 3 ectomycorrhizal fungi were inhibited by root-associated fungi of salal, but none of the four root-associated fungi was inhibited by any of the ectomycorrhizal fungi. *Acremonium strictum* was the most aggressive of the root-associated fungi of salal. It inhibited all 3 ectomycorrhizal fungi in 5 out of 6 interspecific pairings on both media. *Oidiodendron griseum* was the second in terms of aggressiveness to the ectomycorrhizal fungi. *Suillus lakei* and *Rhizopogon semireticulatus* were kept in check by 3 of the 4 root-associated fungi on both media.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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献给
Dedication

亲爱的父亲和母亲
To My Parents

肖雨明　　杜连秀
Yuming Xiao and Lianxiu Du

Chapter 1

General introduction

On Northern Vancouver Island between Port McNeill and Port Hardy two distinct forest types have developed on an undulating landscape with elevation less than 300 m. These two forests are an old growth of western red cedar (*Thuja plicata* Donn) and western hemlock (*Tsuga heterophylla* (Raf.) Sarge.) known as CH (Fig. 1.1) with understorey dominated by salal (*Gaultheria shallon* Pursh) which is an evergreen ericaceous plant (Fig. 1.3), and a second growth of western hemlock and amabilis fir (*Abies amabilis* Dougl.) known as HA (Fig. 1.2) with sparse salal. In this area, several thousand hectares of commercial tree plantations on cutblocks are suffering chlorosis and stagnation (Fig. 1.3), a considerable forest regeneration problem (Weetman *et al.* 1989a,b). The growth problem occurs only on the CH clearcuts, not on the adjacent HA clearcuts. To understand this problem, a comprehensive research program, SCHIRP or Salal / Cedar / Hemlock Integrated Research Program, was initiated and a large amount of research has been done (for instance Messier 1991; de Montigny 1992; Fraser 1993; Keenan 1993; Prescott *et al.* 1993a,b,c; Weetman *et al.* 1989a,b, 1990).

The poor performance of the plantations on the CH has been attributed to two factors, low nutrient availability, particularly nitrogen, and severe competition from salal (Messier 1991; Weetman *et al.* 1989a). Striking differences in organic matter content, nutrient availability, and salal cover exist between the two sites (Messier 1991; Keenan 1993; Prescott *et al.* 1993c; Weetman *et al.* 1989a,b). The CH sites have a thick layer (20-60 cm, but mostly >45 cm) of organic matter overlying a Ferro-Humic Podzol soil (Messier 1991) with an average pH ranging

Fig. 1.1 Old growth of western red cedar (*Thuja plicata* Donn) and western hemlock (*Tsuga heterophylla* (Raf.) Sarge.), the CH.

Fig. 1.2 A second growth of western hemlock and amabilis fir (*Abies amabilis* Dougl.), the HA.

Fig. 1.3 Salal (*Gaultheria shallon* Pursh), an evergreen ericaceous plant surrounding a three-year old hemlock seedling showing chlorosis.

from 3.4-4.8 (Prescott *et al.* 1993c), whereas a relatively thinner (10-40 cm) organic matter layer (Messier 1991) with an average pH of 3.2-4.0 (Prescott *et al.* 1993c) covers the same soil on HA sites. There is a higher availability of nitrogen and phosphorus on HA than on CH (Prescott *et al.* 1993c) and foliar analysis showed that the soil of CH is deficient in available nutrients, particularly available nitrogen (Weetman *et al.* 1989a,b). Field investigations have showed that removal of salal from around conifer trees can improve their growth (Messier and Kimmins 1990) and that application of fertilizer can alleviate the nutritional stress of the plantations surrounded by salal (Weetman *et al.* 1989a,b), indicating competition for nutrients from salal. Thus salal has been "accused" of competing for scarce nutrients and decreasing site fertility by immobilizing nutrients in its living tissue (Messier and Kimmins 1990).

To explain the differences between CH and HA, the natural disturbance of windthrow has received attention. There is evidence that HA forests have a history of repeated blowdown disturbance due to their higher elevation, and the most recent one occurred in 1906, while CH forests have existed for several thousand years without the disturbance (Lewis 1982; Keenan 1993). This process has quite different effects on the two ecosystems. Blowdown disturbance can bring mineral soil to the surface of the organic layer and mix them. This could change soil physical properties, and may favor activity of decomposers. This may in turn alter soil chemistry. Consequently, the soil developed on HA, compared to CH, has a thinner organic layer and higher nutrient availability (Weetman *et al.* 1989a,b; Prescott *et al.* 1993c). This soil has favored a fast dense regeneration of western hemlock and amabilis fir, and inhibited the establishment of salal because of shade (Messier 1991).

However, this view regarding the effects of windthrow disturbance on the differences between the two sites has been questioned. Keenan (1993) found that

there was a large accumulation of organic matter in and on both CH and HA forest floor. His experiment to simulate the effects of windthrow by mixing organic and mineral soil horizons showed that mixing had no significant effect on soil nutrient availability in either type. Differences in rates of decomposition of similar substrates between the two sites were not significant and the physical and chemical properties of mineral soils, such as pH, temperature, and bulk density in the two were similar (Messier 1991; Keenan 1993; Prescott *et al.* 1993c). Based on these investigations, Keenan (1993) concluded that the differences, nutrient availability in particular, between the two sites were not due to changes in soil physical properties brought about by windthrow. Instead, the differences in litter quality of the dominant tree and understorey species were the main cause of differences between the two sites. According to Keenan, cedar is a species which is quite tolerant of low nitrogen availability, and utilizes N efficiently by resorbing higher rates of N in foliage prior to senescence. Therefore, its litter quality is quite low in terms of nitrogen availability to decomposers. Cedar is also a species with great longevity that grows to fairly large size, and its wood contains thujaplycins, methylthujate and thujaplicatins that are highly toxic to decomposers (Minore 1983). Since forest floor is the major nutrient source, cedar litter and wood lower the rate at which N is mineralized in the forest floor of the CH and result in low nitrogen availability and accumulation of organic matter due to slow decomposition. On the contrary, hemlock and amabilis fir resorb less nutrients from their leaves before senescence than cedar and their wood does not contain such toxic chemicals. Therefore, decomposition of the organic matter is faster and accumulation of such material is less on HA than on CH.

Cedar seedlings are fewer in quantity and grow more slowly than those of hemlock (Christy and Mack 1984). This might explain why the CH forests are open stands. The openness of the CH forests favors the establishment of salal

whose growth requires a certain amount of light. Salal contributes tannins and polyphenols to the forest floor in litter fall or root exudates (de Montigny 1992) which further reduce nutrient availability. In contrast, the litter of hemlock and amabilis fir contains higher N which creates a forest floor with higher rates of N-mineralization and higher nitrogen availability. This enables hemlock and amabilis fir to grow rapidly and to form dense stands which prevent salal from establishing.

Messier (1991) studied the clearcuts of the two types of forests. His explanation of the differences between the two types of clearcuts is quite different from the others mentioned above. He concluded that the two sites are different in terms of nutrient availability due to salal. After clearcutting and slashburning, salal can quickly recover the CH sites by resprouting from the rhizomes inherited from the old growth. Salal takes up available nutrients and produces large amount of biomass both aboveground and belowground. Because of toxic chemicals such as tannins and phenols, its biomass is resistant to decomposition and immobilizes nutrients, consequently lowering soil fertility. However, this does not happen on HA due to lack of or insignificant amount of rhizomes from the forest.

Salal is widespread in the Pacific Coast of North America from Alaska to California. In British Columbia alone, millions of hectares of forest contain this plant (Gordon F. Weetman, personal communication). Salal is an abundant understorey component in open stands and a dominant noncrop species on sites with partial or no canopy, such as cutovers. It is mainly associated with economically important tree species, including Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and western hemlock.

Salal is relatively shade tolerant with a survival light requirement, measured as photosynthetic photon-flux-density, ranging from 3.9 to 27.2 $\mu mol.m^{-2}.s^{-1}$

(Messier *et al.* 1989) and spreads mainly by vegetative propagation, sprouting from rhizomes. The open CH forests permit light to penetrate the canopy, creating a situation which favors salal growth and resulting in a dense understorey cover of salal aboveground and extensive rhizome network belowground.

Following clearcutting and slashburning of CH forests, salal recovers quickly and densely from its rhizomes inherited from the old growth, achieves complete dominance of the sites and reaches maximum cover of about 60% within four years (Messier and Kimmins 1991). A striking characteristic of ecological interest about salal is that it dominates a site by occupying not only aboveground but also belowground. It produces 2.5 times more belowground biomass than aboveground biomass eight years after harvesting of CH sites (Messier and Kimmins 1991). The rhizomes in this tremendous amount of belowground biomass ramify in the surface organic layer of the soils and give rise to countless fine roots, as thin as 0.7 mm, that form a distinctive ericaceous "hair root" system. The rhizomes and the fine roots equipped with mycorrhizal fungi (Largent *et al.* 1980), intermingle and occupy the whole belowground on a site within the depth of rooting, suggestive of severe belowground competition.

Similar to other ericaceous plants, salal appears to develop best on sites characterized by high disturbance, high organic matter content, low pH, and low nutrient availability. To achieve dominance in this kind of soil, a plant must develop a special strategy to obtain nutrients. Based on a vegetation recovery study, Messier and Kimmins (1991) concluded that "The post-disturbance dominance of salal on these sites following clearcutting and burning appears to be due to: (1) its ability to survive these treatments and rapidly occupy both above-ground and below-ground environments from rhizomes present prior to disturbance, and (2) to resist invasion by other species by preempting resources (nutrients in this case)".

However, if the nutrients locked in the organic matter on the CH sites are not available to western red cedar and western hemlock, they should not be accessible to salal either. If the CH soils are low in nutrient availability or nutrient deficient (Weetman *et al.* 1989a,b) due to lack of disturbance (de Montigny 1992) or low litter quality (Keenan 1993) or immobilization of nutrients in living tissue by salal (Messier 1991), why does salal grow so well on such adverse soil? The contradiction of good growth of salal on the nutrient deficient CH sites suggests that salal growth is not limited by the nutrient availability on such sites, and that there must be other factors which contribute to the growth of salal in such adverse soils.

The question

The chlorosis and poor growth of plantations of western hemlock on CH, and the alleviation of the nutritional stress by nitrogen fertilization certainly indicate that the soil is deficient in available nitrogen. The vigorous growth and dominance of salal would also suggest that this plant gets a good supply of nitrogen. This might be confirmed by the fact that salal has not responded much to nitrogen fertilization (Sabhasri 1961; Weetman *et al.* 1989a,b). The question here is where salal gets enough nitrogen if the soil is deficient in available nitrogen. The logical answer would be the thick layer of organic matter since it is the only nitrogen-rich matter on the CH sites. Again, the question is how salal gets nitrogen from the organic matter which is generally unavailable to green plants.

The hypothesis

Rayner *et al.* (1911) first suggested that mycorrhiza might be an important factor in accounting for the dominance of heather (*Calluna vulgaris* (L.) Hull), an ericaceous plant native to northern Europe. Research has since repeatedly

demonstrated that it is an ericoid mycorrhizal fungus, *Hymenoscyphus ericae* (Read) Korf & Kernan, that plays a crucial role in the dominance of this plant in the edaphically stressed environments by, amongst other things, providing the host with access to various sources of nutrients unavailable to uncolonized plants (Read 1987a). In addition, it was found that the mycorrhizal roots of *C. vulgaris* secreted fungitoxin which plays a crucial role in maintaining the dominance of the heather even though it is not known whether the fungitoxin is secreted by the mycorrhizal roots or by the fungal symbiont (Handley 1963). Recent reports have shown that other root-associated fungi such as rhizosphere fungus *Penicillium bilaji* (Kucey and Leggett 1989) and pseudomycorrhizal fungi (Haselwandter and Read 1982; Wilcox and Wang 1987) can increase the growth of their host plants. Based on the investigation of *Calluna* mycorrhizae, other root-associated fungi and the related work that has been done so far on CH sites, my hypotheses are that the root-associated fungi, ericoid mycorrhizal fungi in particular, of salal might be able to utilize organic nitrogen and provide salal with access to those organic nitrogen sources so that it can recover quickly from disturbance and dominate the highly nutrient-stressed sites. Furthermore, the root-associated fungi of salal might be antagonistic to the ectomycorrhizal fungi of western hemlock which are critical to the growth of western hemlock.

Objectives

Although it is known that salal forms ericoid mycorrhizae (Largent *et al.* 1980), and that the mycorrhizal fungus of *Calluna vulgaris* can use organic nitrogen, there has been no research on the root-associated fungi of salal, including ericoid mycorrhizal fungi, and their roles in the dominance of this plant. This thesis attempted to test the above hypotheses and broaden our knowledge of ericoid mycorrhizae. The objectives were:

1. To determine the root-fungal association status of salal, including root morphology, association types, and extent of colonization in the field.
2. To identify the root-fungal associations of salal, including isolation of the root-associated fungi from field salal roots, association synthesis, and morphological identification of the fungi.
3. To test if salal forms ericoid mycorrhizae with all the known ericoid mycorrhizal fungi.
4. To test the ability of the root-associated fungi of salal and the plants of salal inoculated with the fungi to utilize different organic nitrogen sources.
5. To test the antagonism of salal root-associated fungi to ectomycorrhizal fungi of western hemlock.

Literature

In the rhizosphere of a plant, there are certain species of fungi present quite consistently on the root surfaces (rhizosphere fungi) or in the tissues or cells of the roots of many species of plants. Fungi colonizing root tissues or cells include pathogens, mutualistic symbionts such as mycorrhizal fungi and the fungi whose colonization does not fit any recognized categories of mycorrhizal or pathogenic colonization either structurally or functionally. In the literature, this group of fungi have been given a variety of names for example endophytes (Currah, *et al.* 1987; Stoyke and Currah 1991) and pseudomycorrhizal fungi (Melin 1923; Kowalski 1973; Wilcox and Wang 1987). Some colonization of plant roots by this group of fungi have been found to be beneficial to the plants (Haselwandter and Read 1982; Wilcox and Wang 1987). In this thesis, all the fungi either consistently present on the root surfaces or colonizing roots of plants are considered as root-associated fungi. Since ericoid mycorrhizal fungi, to date, have been the major root associates of ericaceous plants, this literature review will only cover ericoid mycorrhizae.

Mycorrhizae are mutualistic symbiotic associations between fungi and plant roots. Commonly accepted types of mycorrhizae are ectomycorrhizae, ectendomycorrhizae, vesicular-arbuscular, ericoid, arbutoid, monotropoid and orchid mycorrhizae. Ericoid mycorrhizae occur only in most Ericaceae, Epacridaceae and Empetraceae in the order Ericales. Ericoid mycorrhiza is characterized by a light weft of hyphae on the root surface and extensive and dense hyphae in the root cortical cells.

Several thousand plant species have now been examined for mycorrhizal

formation and it is found in all categories of higher plants (Harley and Smith 1983). In the British flora, 80% of the species of angiosperms, 100% of the gymnosperms and 70% of the pteridophytes are potentially mycorrhizal (Harley 1989). The fungi involved in mycorrhiza formation include zygomycetes, ascomycetes, basidiomycetes and deuteromycetes. About 5-6 genera and more than 100 species of zygomycetes are mycorrhizal (Harley 1989). Most of them form vesicular-arbuscular mycorrhizae. Some of them form ectomycorrhizae. Several hundred species of ascomycetes form ectomycorrhizae (Harley 1989). A few ascomycetes form ericoid mycorrhizae. More than 5000 species of basidiomycetes distributed in hymenomycetes and gasteromycetes are involved in mycorrhiza of angiosperms and gymnosperms (Harley 1989). Deuteromycetes such as *Cenococcum graniforme* Fr. form ectomycorrhizae and some form ericoid mycorrhizae

Ericoid Mycorrhiza Research

1. The fungi and patterns of colonization

Ericoid mycorrhiza research started in 1907 (Ternetz, cited by Bain 1937), although this kind of mycorrhiza was not defined until later (Harley 1969), with ericaceous plants such as *Calluna vulgaris* (L.) Hull in Europe. Ericaceous plants can form heathlands where other plant species including trees are excluded because of the harshness of the environment. The soils of the heathlands are often nutrient deficient, particularly in nitrogen (Mooney 1983). Because of cold weather, mineralization is inhibited so that organic matter accumulates. Organic acids occur and therefore reduce soil pH. This increases leaching, a process of loss of base cations, further reducing soil pH. In this kind of situation, metallic cations are more available and make the soil toxic to plants. To survive, a plant must develop a special strategy to overcome these stresses. Early researchers were

trying to understand the basis of the success of ericaceous plants and found that fungi were involved in the ericaceous plants in the form of mutualistic association.

These early studies of ericoid mycorrhiza centered mainly on species of fungi and the patterns of fungal colonization (Rayner 1913, 1915, 1922; Dufrenoy 1917; Christoph 1921; Doak 1928; Rayner and Levisohn 1940). Ternetz (1907), working on a number of ericaceous plants, isolated a fungus, *Phoma radicis,* from the roots and identified it as the mycorrhizal associate. Because of the difficulty of obtaining sterile seedlings for inoculation tests, she assumed that the mycorrhizal fungus must be present in the seed. In 1913, Rayner isolated a fungus from the seeds of *Calluna vulgaris*. She assumed that her isolate from the seed was the same fungus studied by Ternetz because she believed that infection was systemic even though she failed to isolate the fungus from the roots of ericaceous species (Rayner 1913, 1915, 1922, 1927, 1929a, 1929b, Rayner and Levisohn 1940). It is she who first suggested that mycorrhizal colonization might influence the natural distribution of *Calluna vulgaris* (Rayner *et al.* 1911). During that time, other workers shared this belief of systemic infection (Dufrenoy 1917; Addoms and Mounce 1931, 1932; Lewis 1924).

However, there was controversy among the early workers on ericoid mycorrhizal fungi. Many others isolated a similar, sterile, septate and dark fungus not related to *Phoma radicis* from the roots of a variety of ericaceous plants which was demonstrated to produce ericaceous mycorrhizae and structures they called "hyphal complexes", "Knot", and "Hyphenknauel" (Christoph 1921; Doak 1928; Bain 1937; Freisleben 1933, 1934). Christoph (1921) first isolated a fungus from the roots of *Calluna vulgaris* which produced typical mycorrhiza in back-inoculation to *Calluna* seedlings by growing the plants in sterilized soil and then in open pots. Uninoculated seedlings were mycorrhiza-free. Afterward, Doak (1928) briefly reported his isolation of a mycorrhizal fungus from roots of

Vaccinium corymbosum and *V. pennsylvanicum*. This fungus produced mycorrhiza in sterile ericaceous seedlings. Freisleben (1933, 1934), working with *Vaccinium myrtillus*, *V. vitis-idaea* and *V. uliginosum*, isolated an ericaceous endophyte. In back-inoculation experiments, he proved that this fungus could produce the "Knot" type of mycorrhiza with the same hosts under totally controlled conditions in which the plants were grown in a presterilized peat-sand mixture in flasks and handled aseptically throughout the experiment. He called this fungus *Mycelium radicis myrtilli*, meaning a sterile mycelium from the roots of *Vaccinium myrtillus*.

An important contribution to ericaceous mycorrhizal research occurred in 1937. In a carefully designed experiment, Bain (1937) successfully isolated a fungus from roots of ericaceous species, *Vaccinium macrocarpon*, *V. canadense*, *Chamaedaphne calyculata* and *Ledum groenlandicum*. In his isolation, selected roots were cleaned in sterilized water. Epidermis layers were detached from the roots, the pieces of epidermis containing mycorrhizal cells located under a compound microscope were transferred to a film of nutrient agar on a cover glass, covered with a small drop of clear water agar, and then inverted over a Van Tieghem cell with a drop of sterile water in the bottom. The entire piece was then transferred to a test tube. In subsequent pure culture, this isolate was septate, extremely slow-growing, and dark in color. For resynthesizing, seeds were surface-sterilized in calcium hypochlorite solution for 3 minutes or longer and germinated in test tubes containing 1% water agar. The seedling cultures of *Vaccinium macrocarpon* Ait. were inoculated with the isolate aseptically. All isolates produced the "hyphal complex form of mycorrhiza". The isolates also produced mycorrhiza in cranberry (*Vaccinium macrocarpon* Ait.) seedlings in an inoculated artificial soil composed of ground cork and sand. Due to the absence of

the perfect stage, the taxonomic position of the isolate was uncertain, i.e. it was a sterile mycelium.

Harley (1950) pointed out that in the families in the Ericales, at least two kinds of anatomically different mycorrhizae (ericoid and arbutoid) exist and suggested that a structural distinction could be made between most Ericaceae, Epacridaceae and Empetraceae, and *Arbutus* and Pyrolaceae. Brook (1952) working with *Pernettya macrostigma* clearly described the infection patterns of mycorrhiza in ericaceous plants as follows:

"In early stage of mycorrhiza formation, a network of fine hyaline hyphae spreads over the root surface with branches penetrating cortical cells. Each infected cell becomes filled by densely coiled and branching knots. Infection is through the outer cell wall, rarely from an adjacent cell, and intercellular hyphae are infrequent. Short collars of cell-wall material surround a few of the penetrating hyphae."

Other researchers (Burgeff 1961; McNabb 1961) working on ericaceous plants, isolated similar sterile, slow-growing dark endophytes which they showed to be mycorrhizal with members of the Ericaceae. Harley (1969) reviewed all of the work done and first used the term "ericoid mycorrhiza" for the mycorrhiza association occurring in a majority of Ericales. However, taxonomic position of the ericoid mycorrhizal fungus was still a puzzle.

Pearson and Read (1973a), using direct plating and macerating methods similar to Bain's (1937), successfully isolated an ericoid mycorrhizal fungus from the roots of *Calluna vulgaris* and confirmed the formation of ericoid mycorrhiza with aseptic seedlings of *Calluna vulgaris* and of *Erica* spp. using a combination of sterilized soil and dilute water agar. The fungus was named *Pezizella ericae* Read (Read 1974) based on characteristics of its perfect stage obtained after aseptically inoculating seedlings of *Calluna vulgaris* growing on soil sterilized by gamma

irradiation. This species has since been transferred to *Hymenoscyphus* under the name *Hymenoscyphus ericae* because of the lack of gelatinized excipular cells (Kernan and Finocchio 1983). This fungus formed ericoid mycorrhizae with a wide range of ericaceous species including *Calluna vulgaris*, *Vaccinium myrtillus*, *Erica cinerea* L., *Rhododendron ponticum* L. and *Gaultheria procumbens* L. (Pearson and Read 1973a; Bonfante-Fasolo *et al.* 1984). The successful isolation of the fungus, confirmation of formation of ericoid mycorrhiza and the determination of its taxonomic position finally ended the past confusion about whether the ericaceous endophyte was a species of *Phoma*, and whether ericaceous endophytes were systemic.

In a bidirectional nutrient transfer experiment in the field, *Clavaria argillacea* Pers. : Fr. was suggested to form ericoid mycorrhiza (Englander and Hull 1980). ^{32}P was applied to *Clavaria* fruiting bodies developing beneath *Rhododendron* plants and the plants were exposed to $^{14}CO_2$. With time, the fruiting bodies of *Clavaria* had high levels of ^{14}C radioactivity and the roots of experimental plants exhibited elevated levels of ^{32}P radioactivity. The results indicated that the fungus gained carbon from the plants and provided the plants with phosphorus reciprocally so that a mycorrhizal relationship was suspected to exist between the plant and the fungus. But isolation of the fungus *Clavaria argillacea* from the *Rhododendron* plants and inoculation of the potential mycorrhizal fungus to the *Rhododendron* plants have failed (Read 1983).

Later, other ericoid mycorrhizal fungi were found and confirmed to form ericoid mycorrhizae with their ericaceous hosts. Couture *et al.* (1983) with isolates of fungi from naturally-colonized roots of *Vaccinium angustifolium* L. and *V. corymbosum* L., inoculated seedlings of *V. angustifolium* aseptically growing on sterilized fine silica sand. Their isolates could successfully colonize the roots of

V. angustifolium and *V. corymbosum*, and formed structures typical of the ericoid mycorrhizae formed by *Hymenoscyphus ericae*. But the isolates are quite different from *H. ericae* in their cultural characteristics as well as the presence of thick-walled guttulate cells in the substrate. They identified this isolate as *Oidiodendron griseum* Robak. It has been pointed out that this species can survive in a variety of quite different habitats such as soils, pine needles, conifer humus and rhizosphere of plants (Dalpe 1986).

In an axenic synthesis of ericoid mycorrhiza experiment, another two pre-identified *Oidiodendron* species, *O. cerealis* (Thum.) Barron and *O. rhodogenum* Robak were proved to be able to colonize *Vaccinium angustifolium* and produced typical patterns of ericoid mycorrhizae (Dalpe 1986). Dalpe (1989), using axenic synthesis, found that some ascomycetes in the Onygenales, could form ericoid mycorrhizae with *Vaccinium angustifolium*. They are *Myxotrichum setosum* (Eidam) Orr & Kuehn, *Gymnascella dankalienses* (Castellani) Currah and *Pseudogymnoascus roseus* Raillo. Taxonomically, *Myxotrichum setosum* with an *Oidiodendron* anamorph and *Pseudogymnoascus roseus* with a *Geomyces* anamorph have been placed in the family Myxotrichaceae, and *Gymnascella dankalienses* has been included in the family Gymnoascaceae (Currah 1986). Douglas *et al.* (1989) proved *Oidiodendron maius* Barron to be mycorrhizal with *Rhododendron* cv. 'Pink Pearl'. In the same year, *Scytalidium vaccinii* Dalpe, Litten & Sigler was found to be an ericoid mycorrhizal fungus (Dalpe 1989). Recently, *O. chlamydosporicum* Morrall, *O. citrinum* Barron, *O. flavum* Szilvinyi, *O. periconioides* Morrall, *O. scytaloides* and *Oidiodendron cerealis* (Thum.) Barron formed ericoid mycorrhizae with *Vaccinium angustifolium* (Dalpe 1991). *Oidiodendron cerealis* (Thum.) Barron has been transferred to *Stephanosporium cerealis* (Thum.) Swart (Swart 1965).

There are, to date, 13 species of fungi which have been proven to form ericoid mycorrhizae with ericaceous plants in axenic culture (Table 4.1). Using molecular techniques, Egger and Sigler (1993) determined that *Scytalidium vaccinii* is the arthrconidial anamorph of *Hymenoscyphus ericae*.

2 Effects of ericoid mycorrhizae on plant growth

Brook (1952) is the earliest worker to show that ericoid mycorrhizae could improve plant nutrient status and stimulate the growth of the hosts. He used a mixture of quartz sand and clay as soil in which clay was washed with acid and sterilized with Formalin, and sand sterilized by autoclaving. Sterile seedlings of *Pernettya macrostigma* were grown in the soil and inoculated with a washed piece of mycorrhizal root. He found that some of the treated seedlings had significantly higher dry weight than non-mycorrhizal seedlings. Later, an experiment comparing mycorrhizal and non-mycorrhizal plants of *Pernettya macrostigma* confirmed Brook's finding that ericoid mycorrhiza formation could improve growth of the hosts (Morrison 1957). More recent work on this has been done by Dr. D. J. Read and his colleagues at University of Sheffield. All their work focuses on why *Calluna vulgaris* is so successful in colonizing heathland and excluding non-ericaceous plants. Heathlands occur widely in northwest Europe and are dominated by the ericaceous plants *Calluna vulgaris*. The soil is characterized by low pH, high organic content and low nutrient status. Other plant species are excluded from the acid mor-humus soil because of the acidic, extremely nutrient-poor soil (Read 1984). In this kind of soil, surviving requires success in obtaining nutrients. Studies have shown that ericaceous plants, with their mycorrhizal associations, are highly tolerant of the adverse soil conditions in heathlands (Read 1984). The ericoid mycorrhizal fungi absorb phosphate ammonium and particularly provide their host plants with access to soluble and

insoluble organic nitrogen compounds. The fungi protect their hosts from toxic metals which are soluble in the very acid soil solution (Harley 1989).

Nitrogen nutrition

In order to test the effects of mycorrhizal colonization, Read and Stribley (1973) designed an experiment to compare the growth of mycorrhizal and non-mycorrhizal plants. They used sterilized soil from heathland with 0.5% water agar as the medium. Sterile seedlings of *Calluna vulgaris* and *Vaccinium macrocarpon* obtained by aseptic germination were transferred to the medium. To produce mycorrhizal seedlings, some of the seedlings were inoculated with an isolate of *Hymenoscyphus ericae*. When harvesting, comparison showed that the dry weight yield of the mycorrhizal plants was significantly greater than that of the non-mycorrhizal plants. To their surprise, there was a very significant increase in the nitrogen content of mycorrhizal plants of both species and a less significant increase of phosphorus level, which is quite different from the situations occurring in other kinds of mycorrhizae (Harley and Smith 1983). This was confirmed in another similar study about the effects of mycorrhizal colonization on growth of hosts (Stribley *et al.* 1975). Mycorrhizal colonization increased the whole plant dry weight, and nitrogen content. All the results seemed to suggest that the increase in growth of mycorrhizal plants may be mainly due to enhanced uptake of nitrogen from nitrogen poor soil instead of phosphorus or other nutrients by the ericaceous mycorrhizal plants. It was suggested that ericoid mycorrhizal plants benefit from a root system extended by the extramatrical mycelium of the endophyte and that the enhanced nitrogen status of mycorrhizal plants may be a result of increased rates of absorption of ammonium because in this kind of heathland soil, ammonium is the sole source of mineral nitrogen available to plants (Stribley and Read 1974).

Stribley and Read (1976) aseptically grew mycorrhizal and non-mycorrhizal plants of *Vaccinium macrocarpon* in a controlled environment for 12 weeks on sand. The mycorrhizal plants were obtained by inoculation of sand culture with 5 mg fresh weight of macerated mycelium of ericoid mycorrhizal endophyte. They supplied five different concentrations of ammonium nitrogen from 1.0 to 56.0 ppm to the plants. All plants received a full complement of other mineral elements essential to growth. They found that non-mycorrhizal plants showed a significant increase in whole plant dry weight and nitrogen content with increasing concentrations of ammonium nitrogen. Mycorrhizal plants had higher dry weight and nitrogen content only at intermediate levels of nitrogen supply but gave no effects at the extreme concentrations of nitrogen used. They inferred that mycorrhizal infection could improve uptake by *Vaccinium macrocarpon* of ammonium nitrogen at low but not extremely low concentrations.

It appears, however, that improved uptake of ammonium alone by mycorrhizal plants is not enough for them to perform well, because ammonium is the only source of mineral N in heathland soil and is in short supply (Stribley and Read 1974). Stribley and Read (1974) labeled soil at room temperature for 197 days to encourage microbial turnover of ^{15}N. The soil was washed with acid in order to remove excess ammonium. Other basic elements were added to the labeled soil. Then the soil was sterilized by gamma-irradiation. Analyses revealed that in the sterilized soil the ^{15}N was higher in the inorganic fraction than in the organic fraction. Sterile seedlings of *Vaccinium macrocarpon* were aseptically transferred to the irradiated soil. To obtain mycorrhizal seedlings, the soil was inoculated with a suspension of *Hymenoscyphus ericae*. The final results showed that mycorrhizal plants attained higher dry weight and higher total N than non-mycorrhizal plants. But the labeled nitrogen concentration (atom %) in

mycorrhizal plants was lower than in non-mycorrhizal plants. This strongly suggested that ammonium was not the only N source available to the mycorrhizal plants and that organic nitrogen was involved in the nitrogen nutrition of mycorrhizal plants.

Tests of the capacity of mycorrhizal plants and non-mycorrhizal plants to utilize simple and complex nitrogen sources have been done by Stribley and Read (1980), and Bajwa and Read (1985) in the following two experiments. In the first, plants were grown in sand culture in an apparatus sterilized by gamma irradiation. The sand was saturated with 12 ml of a filter-sterilized nutrient solution of pH 5.8 in which one of the amino compounds, glucine, glutamine, glutamic acid, aspartic acid or alanine or no nitrogen was used as the only nitrogen source at a nitrogen concentration of 20.5 mg l^{-1}. Mycorrhizal plants of *Vaccinium macrocarpon* were obtained by inoculation of the sand culture with 5 mg of fresh macerated mycelium of *Hymenoscyphus ericae*. The results showed that mycorrhizal plants utilized all the amino acids as readily as they utilized ammonium, while non-mycorrhizal plants had restricted ability to utilize these organic N compounds. Comparisons between mycorrhizal and non-mycorrhizal plants of *V. macrocarpon* revealed a highly significant difference between the capacities to utilize organic nitrogen. In the second experiment, mycorrhizal and non-mycorrhizal seedlings of *V. macrocarpon* were transferred to sterile Rorison's nitrogen-free liquid medium containing one of glutathione, different chain lengths of alanine peptides, or ammonium as the nitrogen source at a nitrogen concentration of 20 mg l^{-1} for each nitrogen source instead of the amino acids used above. Analysis of the responses to the individual treatments showed that the mycorrhizal plants were heavier than the non-mycorrhizal plants. The results demonstrated that ericoid mycorrhizal plants are capable of utilizing peptides.

Recently it has been verified that ericoid mycorrhizal plants of *Vaccinium macrocarpon* can even use pure protein (Bajwa *et al.* 1985). In this study, mycorrhizal (*Hymenoscyphus ericae*) and non-mycorrhizal seedlings of *V. macrocarpon* were grown on a protein agar containing solutions of bovine serum albumin (BSA), casein-gelatin and gliadin each at 1% protein concentration in Rorinon's solution from which mineral nitrogen was excluded. The pH of each protein solution was adjusted to 4, 5 or 6. The seedlings were harvested after 45 days, oven-dried and weighed. The results showed that mycorrhizal colonization gave rise to significantly higher yields on all three proteins at pH 4.

All the research cited here shows that mycorrhizal colonization can stimulate the growth of the host plants by improving absorption of mineral nitrogen and utilizing organic nitrogen unavailable to non-mycorrhizal plants. *Hymenoscyphus ericae* itself is capable of utilizing amino acids (Stribley and Read 1980), peptides (Bajwa and Read 1985), pure protein (Bajwa *et al.* 1985; Leake and Read 1989a), and even chitin as sole source of nitrogen (Leake and Read 1990b). This fungus produces an active free acid proteinase enzyme which has pH optimum at 2.2 (Leake and Read 1989a).

Phosphorus nutrition and cation absorption

Hymenoscyphus ericae also intervenes in the uptake of phosphate. In 1971, Pearson's work showed that there was a higher phosphatase activity in mycorrhizal roots of *Calluna* than in the roots of non-mycorrhizal *Calluna*, and that the endophyte could use the organic phosphorus source, sodium phytate. Later studies confirmed that *Hymenoscyphus ericae* could improve uptake of phosphorus by *Calluna vulgaris* and *Vaccinium macrocarpon* even though there was a less significant increase of phosphorus than of nitrogen levels in the mycorrhizal plants

(Read and Stribley 1973). Pearson and Read (1973b) employed a split dish technique in which a big glass dish was used. Seedlings of *Vaccinium oxycoccus* were aseptically cultured in sterile soil on one side of the dish, while a small glass dish was put on the other side of the dish which contained distilled water agar (1%) on which *Hymenoscyphus ericae* grew. When hyphae from the colony grew out over the rim of the small glass dish, they could reach the seedling and colonize it. Then ^{32}P was applied to the small dish. After 72 hours incubation at 20^0C, extraction of the phosphorus was made from the seedlings. They found a great amount of ^{32}P in the seedlings which indicates that the labeled material was translocated by the endophyte to the seedlings.

Later, pure culture proved that *Hymenoscyphus ericae* could use organic phosphorus and possesses acid-phosphatase activity (Pearson and Read 1975). In this culture, Norkrans basic medium in a flask was adjusted to pH 5.8. For analysis of the utilization of organic phosphorus, all inorganic phosphorus in the basal medium was replaced by an equal concentration of organic phosphorus as the sodium salt of myo-inositol hexaphosphate (IHP) free of inorganic P. After autoclaving, the liquid culture was inoculated with macerated mycelium discs and incubated at 20^0C. After 18 days incubation, the mycelium was harvested. Growth was measured and expressed as the dry weight per flask. The results clearly showed that a high dry weight yield was obtained with the organic P as the sole phosphorus source. More evidence that the ericoid mycorrhizal endophyte could utilize organic P was provided by Mitchell and Read (1981). A great amount of polyphosphate both in pure cultures of the endophyte and mycorrhizal root systems of ericaceous seedlings has been observed and confirmed (Straker and Mitchell 1985). This kind of poly P is believed to function as storage. Extracellular phosphatase was demonstrated in culture and its high activity was

observed in the endophyte of *Erica hispidula* L. (Straker and Mitchell 1985, 1987). The phosphatases of the endophyte of *E. hispidula* could hydrolyze a wide range of phosphate esters (Straker and Mitchell 1985, 1987).

In addition to the advantages concerned with enhanced capture of major nutrients such as N and P, evidence has been provided that ericoid mycorrhizal colonization by *Hymenoscyphus ericae* can affect cation absorption by host plants. Leake and Read (1989b) showed that colonization could increase the efficiency of Ca uptake at low levels of Ca concentration.

Tolerance to soil toxins

Although many studies of the function of ericoid mycorrhizal colonization focus on nutritional aspects, recent studies have demonstrated that colonization may play a wider role, including enhancement of tolerance to soil toxins. Bradley *et al.* (1982) reported on the tolerance of both the endophyte *Hymenoscyphus ericae* and its hosts. In their experiment, a sand culture was employed which contained Rorison's nutrient solution. Copper sulphate at 0, 10, 25, 50 and 75 mg l^{-1} and zinc sulphate at 0, 25, 50, 100 and 150 mg l^{-1} were added to the solution. Seeds of *Calluna vulgaris* and *Vaccinium macrocarpon* were surface-sterilized and germinated aseptically. Sterile seedlings were transferred to Petri dishes of sterile soil, which were inoculated with *Hymenoscyphus ericae* and all plants were transferred to plastic pots containing acid washed sand and the above treated nutrient solutions. After 12 weeks, they harvested all the plants, analyzed the data and found the plants with ericoid mycorrhizae could grow well in soils containing high levels of heavy metals. Non-mycorrhizal plants failed to grow. It was inferred that the colonization reduced accumulation of metals in the shoot and avoided metal toxicity by increasing the sequestration of heavy metals through an

increase in the area of wall material available for the sites of complexing.

It appears that the success of the ericaceous plant, *Calluna vulgaris*, in the highly stressed heathland is due to its ability to form mycorrhizae with the ericoid mycorrhizal fungus, *Hymenoscyphus ericae*. It is this fungus that provides the plants with tolerance to stresses such as nutrient deficiency and soil toxicity and makes its host plants more competitive than other plants growing in the heathlands. Based on all the studies about *Calluna vulgaris* and its mycorrhizal fungus, *Hymenoscyphus ericae*, a general conclusion has been drawn that "the success of plants with ericoid mycorrhizas in some of the most stressed environments on earth is not attributable to their superior competitive ability but to their ability, largely provided by the fungal endophyte, to tolerate the stresses" (Read 1991). As mentioned earlier, there have been 13 species of fungi which have been found to form ericoid mycorrhizae with ericaceous plants. However, little is known of their roles in the growth of their host plants such as utilization of organic nutrients. In addition, there are about 103 genera and 3350 species of Ericaceae, widespread in the world (Mabberley 1987). Ericaceous plants such as *Kalmia angustifolia* L. and *Gaultheria shallon* Pursh are of ecological importance (Mallik 1991; Weetman *et al.* 1989a,b) in the temperate forests in North America, but there are few studies about their mycorrhizae. It is obvious that there is a need for more research about these ericaceous plants and their associated mycorrhizal fungi to determine how general is the postulated role of ericoid mycorrhizal fungi.

Chapter 2

Ericoid mycorrhizal fungi of *Gaultheria shallon*⋆

Introduction

Salal (*Gaultheria shallon* Pursh) is one of the most abundant forest undergrowth species on the west coast area of North America. Large areas of productive forest land are invaded by these vigorously growing plants, especially following clearcutting and slashburning (Bunnell 1990; Weetman *et al.* 1989a), and in the presence of salal, the growth of conifer plantations is dramatically reduced (Weetman *et al.* 1989a). Salal like some other ericaceous plants, such as *Calluna vulgaris* (L.) Hull (Handley 1963) and *Kalmia angustifolia* L. (Mallik 1991), is of ecological importance (Smith 1991), and therefore, it is of interest to understand the basis of the success of salal in such sites.

Ericoid mycorrhiza formation contributes to the success of ericaceous plants in stressed soils (Read and Bajwa 1985; Read 1984). Salal forms ericoid mycorrhizae (Largent *et al.* 1980); however, it is not known which fungi are involved. The purpose of the present study was to identify the mycorrhizal fungi of salal from a cutblock on northeast Vancouver Island by isolation of the fungi from roots and aseptic synthesis of mycorrhizae of salal with the isolates.

Materials and methods

Salal roots were collected September 20-24, 1990, from 3-year-old western hemlock (*Tsuga heterophylla* (Raf.) Sarge.) reforestation sites at Port McNeill on

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⋆: Mycologia 84: 470-471.

Vancouver Island, Canada. Sixty root samples from different locations were sorted to remove rhizomes and coarse debris, soaked in tap water in a flask for 10 min to loosen adhering fine debris, shaken vigorously in the flask, and washed in running tap water for 1 h. Roots were surface sterilized in a laminar flow hood by immersing them in 30% hydrogen peroxide in an autoclaved beaker for 30 sec. Sterilized roots were then washed repeatedly in sterile distilled water. Approximately 0.5 cm lengths were excised from the disinfected roots, and 10 pieces per Petri plate were placed onto 1/3 strength potato dextrose agar (PDA). Plates were incubated at 25^{0}C in the dark. Pure colonies were transferred onto modified Melin Norkrans agar (MMN) and incubated at 25^{0}C for subsequent inoculation and identification.

Seeds of salal purchased from Reid Collins Nurseries in Aldergrove, British Columbia were soaked in tap water in a refrigerator until the seeds sank to the bottom of the flask (about 1 wk). The seeds were then rinsed repeatedly in tap water, drained and transferred in the flow hood into an autoclaved beaker half-filled with 30% hydrogen peroxide and sterilized for 30 sec. After sterilization, the seeds were rinsed four times in sterilized, distilled water. The Petri plates were kept in a growth chamber at 25^{0}C and a light regime of 18 h light at 310 μ mol m^{-2} sec^{-1} (photosynthetic photon flux density) illumination and 6 h dark. After 2 wk the seeds started to germinate.

All fungal isolates recovered from plates were tested to form mycorrhizae with salal in pure culture. The synthesis was conducted in an 11 x 2.5 cm screw-capped vial. Two cm of the vial was filled with 1.5% water agar and autoclaved for 30 min. When the agar solidified, 10 ml of sterilized soil (autoclaved for 60 min twice with an interval of 24 h) was placed in the vial. Once the soil had moistened by absorbing water from the underlying agar, one germinant was aseptically planted in each vial and kept in the growth chamber at 25^{0}C with a

light regime of 18 h light at 310 μmol m^{-2} sec^{-1} illumination, 6 h dark. When the first post-cotyledonary leaves emerged, seedlings were aseptically inoculated with one rectangular block each cut from edges of colonies of the isolates from salal. The closed, inoculated vials were returned to the growth chamber.

After 1 month, seedlings that had on the average eight leaves and a shoot that was 6 cm tall were harvested. The root system was washed in running tap water, dried with paper towel, and immersed in FDA Blue No. 1 staining solution (Chapman 1992). The stained roots were mounted on a slide and examined under a compound microscope.

Results and discussion

Eight-three isolates of fungi were obtained from 60 root samples of field-grown salal and 24 of them produced typical ericoid mycorrhizae. These mycorrhizae were characterized by a weft of hyaline hyphae on the surfaces of the hair roots and crowded hyphal complexes inside the outer layer of cortical cells. Morphological differences between mycorrhizae formed by the different isolates were not evident under the light microscope.

All 24 fungal isolates which formed ericoid mycorrhizae with salal grew slowly during the culture on MMN and PDA media. Among them, 18 isolates formed grey-olive colonies on PDA, dirty-white on malt extract agar, and dark olive on MMN. They formed conidia and conidiophores on all media tested and were identified as *Oidiodendron griseum* Robak as described by Barron (1962). Cultures have been deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH). Three other isolates exuded yellow-brown pigment into malt extract agar medium. The colony of another was white. Since they did not sporulate, none of these other isolates could be identified.

Oidiodendron griseum, as a saprophyte, has been obtained from forest soil,

paper samples, toilet tissue, wood pulp (Barron 1962), tree leaves, humus, and rhizosphere soil (Dalpe 1986; Douglas *et al.* 1989; Stoyke and Currah, 1991). As an ericoid mycorrhizal endophyte, it was isolated from *Vaccinium* species (Couture *et al.* 1983). This is the first report of *O. griseum* as an ericoid mycorrhizal fungus of *Gaultheria.*

Chapter 3

The root-fungus associations of salal on CH and HA clearcuts

Introduction

There are about 103 genera and 3350 species of Ericaceae, a family that is widespread in arctic, temperate, and tropical climates (Mabberley 1987). In some areas, ericaceous species form pure heathlands (Specht 1978). Important forest lands have been overtaken by ericaceous plants after logging and burning in Europe (Gimingham 1972; Ellenberg 1988) and in North America (Meades 1986; Weetman *et al.* 1989a; Mallik 1991).

Root-associated fungi such as rhizosphere fungi (Kucey and Leggett 1989), mycorrhizal fungi (Harley and Smith 1983) and pseudomycorrhizal fungi (Haselwandter and Read 1982; Wilcox and Wang 1987) play very important roles in the growth of the plants they associate with. According to extensive work carried out by Read and coworkers (Read 1983, 1987a, 1991, Read and Bajwa 1985) on *Calluna vulgaris* and its mycorrhizal fungus, the ericoid mycorrhizal fungus plays a vital role in the dominance of this ericaceous plant. It is concluced that the most important contribution of ericoid mycorrhizal fungi to the dominance of their hosts is the direct nutritional benefits of access to organic forms of nutrients, particularly organic nitrogen in soil environments where the available nitrogen pool is very low (Read 1991). Thus ericoid mycorrhizal fungi not only provide their hosts with tolerance to stresses but also improve their competitive ability. Unfortunately virtually everything we know about ericoid mycorrhizae is based on only one ericoid mycorrhizal fungus, *Hymenoscyphus ericae,* in relation

to a few ericaceous plants such as *Calluna vulgaris* and *Vaccinium macrocarpon* (Read 1991). Clearly other species of ericoid mycorrhizal fungi in association with other ericaceous plants in different ecosystems around the world should be studied to determine how general is the postulated role of ericoid mycorrhizal fungi.

Relatively little is currently known about the root-associated fungi of salal, the mycorrhizal fungi in particular. Ericoid, arbutoid and ectomycorrhizae were reported on field salal roots from northern California (Largent *et al.* 1980). Roots of salal grown with Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco.) and western hemlock in pots containing field soil from the Oregon coast range developed ericoid and ectomycorrhizae but no arbutoid mycorrhizae (Smith 1993). This difference might be due to soil factors which are important in determining the nature of mycorrhizal associations (Dighton and Coleman 1992). Knowing the intensity of colonization of roots by mycorrhizal fungi is very important when assessing the influence of mycorrhizae on the growth of their hosts. Usually, good performance of mycorrhiza-forming plants is associated with high intensity of mycorrhizal colonization (Perry *et al.* 1987). Intensity of colonization of ericoid mycorrhizae varies in different ecosystems (Reed 1987) and is inversely proportional to levels of available nitrogen (Moore-Parkhurst and Englander 1982; Stribley and Read 1976). However, little is known of the intensity of mycorrhizal colonization of salal. We reported on a system we adapted to synthesize ericoid mycorrhizae with salal and on the identity of one of these fungi, *Oidiodendron griseum* (Xiao and Berch 1992).

The objectives of this study were to compare salal on CH and HA sites in relation to: 1) types of root-fungus associations and intensity of colonization, and 2) the fungi that form these associations.

Materials and methods

Study area and sampling

The study area is located between Port McNeill and Port Hardy on northern Vancouver Island, British Columbia, Canada (50°60'N, 127°35'W), and within CWHvh1 - Southern Very Wet Hypermaritime Coastal Western Hemlock Variant (Green and Klinka 1994). The area is undulating with elevations less than 300 m and annually receives an average precipitation of 1700 mm with daily temperatures varying from 3.0°C in January to 13.7°C in July. The soils have been reported to be Ferro-Humic Podzol with a thick layer (20-60 cm, but mostly >45 cm) of organic matter (Messier 1991) with an average pH ranging from 3.4-4.8 (Prescott *et al.* 1993c) in CH sites and Ferro-Humic Podzol with a relatively thinner (10-40 cm) organic matter layer (Messier 1991) with a average pH of 3.2-4.0 in HA sites (Prescott *et al.* 1993c). Nitrogen availability is lower in CH forest floors than in HA forest floors (Prescott *et al.* 1993c). The forests of the study site were clearcut in 1986 and broadcast burned in 1987. For HA and CH, two root samples of about 500 g (with rhizomes and soil) were collected with a shovel in September 1991 from each of 15 (16 x 16 m) plots. The samples were kept in plastic bags in a cooler when collected and transported to the University of British Columbia and stored in a refrigerator in the lab for use.

Percent colonization

Roots were subsampled by collecting roots from different individual rhizomes, washed with running tap water, cut into 1 cm lengths, and transferred to a grided tray (15 x 38 cm). A total of 50 pieces from each subsample was obtained by choosing 5 pieces from 10 randomly chosen grids. The root pieces were then mounted on a slide in FDA Blue No. 1 (Chapman 1992), and examined under a

compound light microscope at various magnifications. Percent colonization was assessed in two ways: root colonization and cell colonization. For percentage root colonization, 50 1 cm root pieces per sample were counted as colonized or non-colonized. One hundred contiguous cortical cells within each colonized root were counted as colonized or uncolonized for the percent cell colonization. The data were analyzed by a t-test.

The same root pieces used to determine colonization were examined for the morphology and structure of the colonization. Occasionally, additional cross and longitudinal hand sections were made for determination of detailed structure by freezing a piece of root on a freezing microtome stage and cutting it with a double-edged razor blade. Colonized roots of salal synthesized *in vitro* (see below) were also examined in the way described above.

Roots collected from the field, washed with tap water to remove soil particles, and two entire synthesized root systems were prepared for scanning electron microscopy by the methods of Tanaka and Mitsushima (1984) and Tanaka and Nagura (1981). The roots were first fixed with 0.5% paraformaldehyde with 0.5% gluteraldehyde in 0.1 M cacodylate with 0.475 M sucrose pH 7.4, then postfixed in 1% osmium tetroxide in cacodylate buffer. After soaking in dimethylsulphoxide (DMSO) solution, the roots were frozen on blocks in liquid nitrogen, and fractured with a razor blade. The exposed cell matrices were digested in buffered osmium tetroxide. Before critical point drying, the specimens went through dehydration and finally were mounted on stubs using rapid setting araldite and coated lightly with gold-palladium at 2KV 10 mA for 2 minutes. About 100 field roots and 20 synthesized roots were examined using Cambridge 250 scanning electron microscope (SEM).

Isolation of root-associated fungi

The sixty root samples were subsampled. The subsampled roots were washed for 30 min in running tap water, and surface sterilized in 30% hydrogen peroxide for 1 min. For each subsample, approximately 0.5 cm lengths were excised from the disinfected roots, and 20 pieces (10 per Petri plate) were plated out for fungal isolation using the methods previously described (Xiao and Berch 1992). All fungal isolates from salal field roots, except for *Penicillium*, *Aspergillus* and *Trichoderma* species were tested in axenic culture. Isolates that formed root-fungus associations with salal in axenic culture were, whenever possible, identified and some were sent out to specialists for identification.

Synthesis of root-fungus associations

Fungal isolates from salal field roots were maintained on modified Melin Norkrans agar (MMN) and used as inoculum for the synthesis experiments in this study. The basal medium used in the synthesis chambers was MMN with the deletion of mineral nitrogen, malt extract, and glucose. These nutrient materials were deleted because, in a preliminary experiment with complete MMN, mycorrhizal colonization was very low, and the fungi overgrew the plates quickly. The pH used in the synthesis was 4 which is within the pH range (3.2-4.8) of the soils on the CH and HA sites. Eight g / l of agar was added to the basal medium, which was then autoclaved and 25 ml poured into Petri dishes. When the agar was solidified, half of the agar disc in each Petri dish was removed aseptically and these synthesis chambers were ready for further use.

Salal seeds were surface sterilized with 30% hydrogen peroxide and germinated on water agar (Xiao and Berch 1992). One germinant per Petri dish was planted at the center of the cut edge of the agar and incubated. When a real leaf emerged, 5

replicates per isolate were inoculated with about 2 mm^3 of colonized medium cut from the edge of a fungal colony. The plates were then sealed with parafilm and placed vertically in a growth chamber at 25^0C, 18 h light at 310 $\mu mol\ m^{-2}\ sec^{-1}$ illumination, and 6 h dark. Fourteen days after inoculation, we began checking the plants for colonization by placing the unopened chamber on the stage of a light microscope and examining the whole root system intact at 50-100x. This was possible because the nutrient agar used was transparent. Forty-five d after inoculation, the roots of the plants were harvested by gently pulling the root systems out of the agar, stained with FDA Blue No. 1, and examined at 400-1000x to confirm colonization. All isolates were re-isolated from the synthesized roots of the plants. Representatives of the isolates reisolated from synthesized salal roots were retested to form root-fungus association with salal in the same axenic culture system.

Results

The morphology of salal fine roots and mycorrhizae

Both field and lab-grown fine roots of salal range from 0.6 mm to 1 mm diam, are very simple in anatomy, consist of 5 to 6 layers of cells radially arranged in cross section (Fig. 3.1), and lack root hairs. Using the Petri plate culture system, it was also possible to observe undisturbed roots throughout their development (Fig. 3.2, 3.3). Typical ericoid mycorrhizal colonization was observed from both field and *in vitro* roots. The very tip of the apical region of *in vitro* non-mycorrhizal and mycorrhizal roots was covered by rounded root cap cells which sloughed off as the root elongated (Fig. 3.2). The apical cells were small and very dense. The apical region *in vitro* was surrounded by a continuous layer of mucigel. The stele was enclosed by two layers of cells and only the outer layer was colonized (Fig. 3.4) by mycorrhizal fungi. In some cases, a mantle-like structure of hyphae (Fig. 3.5) but no Hartig net was observed in field roots. The hypha became narrow when

penetrating the wall of the host cell (Fig. 3.6). Once inside the cell, it proliferated around the nucleus (Fig. 3.7), developing a hyphal complex and occupying all the cell space (Fig. 3.8). As seen under SEM, in colonized cortical cells hyphae were separated from the host cytoplasm by a continuous host plasmalemma (Fig. 3.9). No difference between the roots of the CH and HA sites was detected in terms of morphology of the salal mycorrhizae.

In axenic culture in the roots colonized by *Acremonium strictum* (see below), difference in colonization morphology has been noticed. As seen in the typical ericoid mycorrhizae, this fungus formed a weft of hyphae on the surface of the roots, penetrated some of the outermost cortical cells but did not produce typical hyphal complexes inside the cortical cells. Within the colonized cells, the hyphae of the fungus were loosely arranged. Because of the differences it is referred to here as "pseudomycorrhiza".

Percent colonization

The field roots of salal were highly colonized by the ericoid mycorrhizal fungi in terms of root colonization and cell colonization. The colonization rate was as high as 87% for root colonization and 91% for cell colonization. No statistically significant difference of root colonization between the two forest types was found (Table 3.1). Though the difference of cell colonization between the two forest types was not significant at $\alpha = 0.01$, the p value (0.013) is just 0.003 greater than the α.

The root-associated fungi

From the 560 root pieces per forest type, a total of two hundred and seventy-eight fungal isolates were retained. Among them, one hundred and seventy-five colonized salal roots in axenic culture. These root-associated fungal

Table 3.1 Comparison of colonization intensity of salal field roots.

Colonization	Site	Sample Size	Mean (%)	SD	T-test (*p*)
Cell	HA	30	89.2	2.76	0.013
	CH	30	90.8	1.86	
Root	HA	30	86.1	4.37	0.321
	CH	30	87.1	2.65	

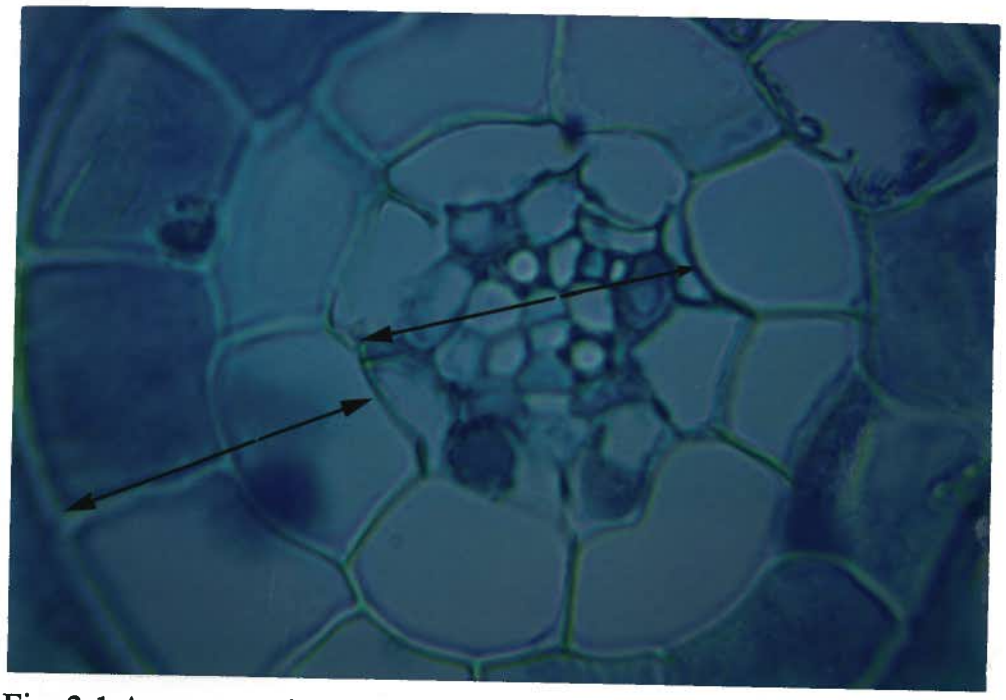

Fig. 3.1 A cross section of a fine root of salal showing the two layers of cortical cells and the simple stele, x 400.

Fig.3.2 A surface view of a root of salal, showing the sloughed root cap cells, x 250.

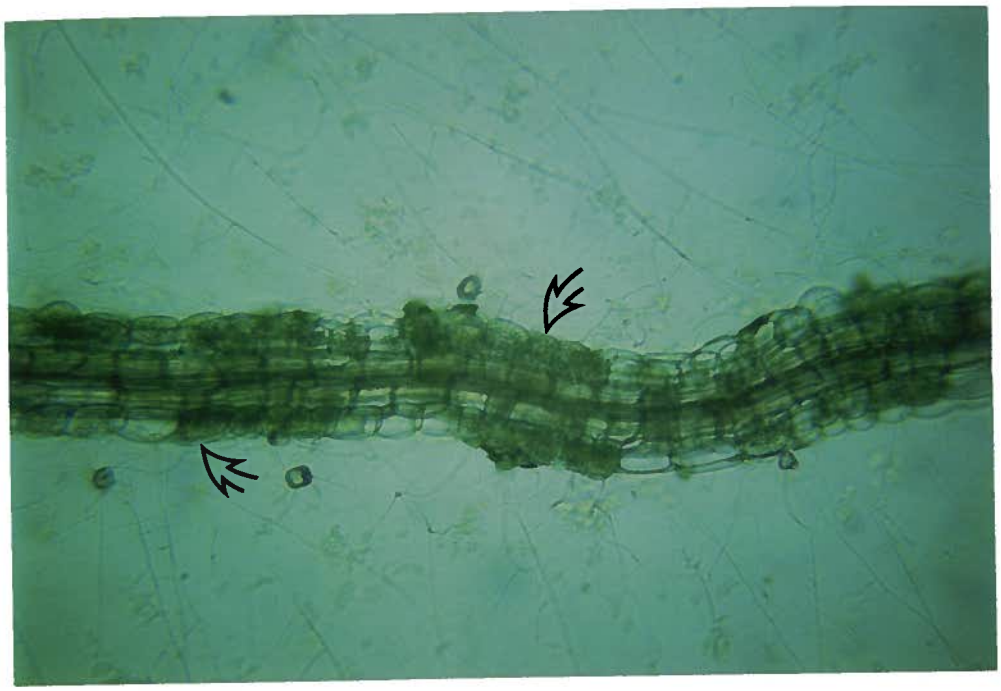

Fig. 3.3 A surface view of an *in vitro* salal root colonized by its mycorrhizal fungi, x 250.

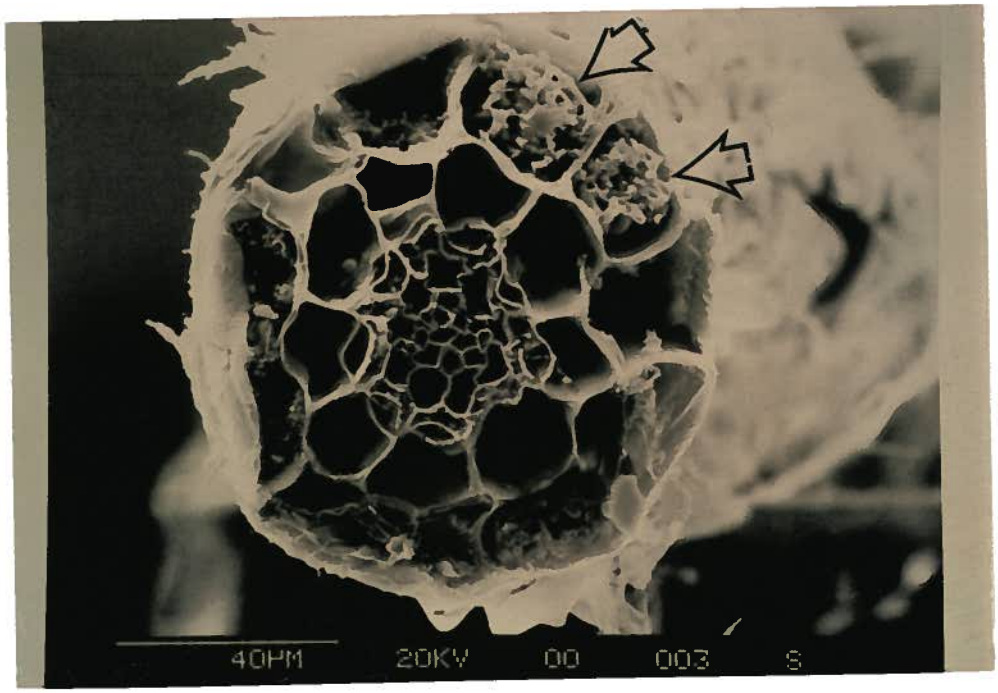

Fig.3.4 A field salal root, showing the outer layer of the cortical cells colonized by ericoid mycorrhizal fungi

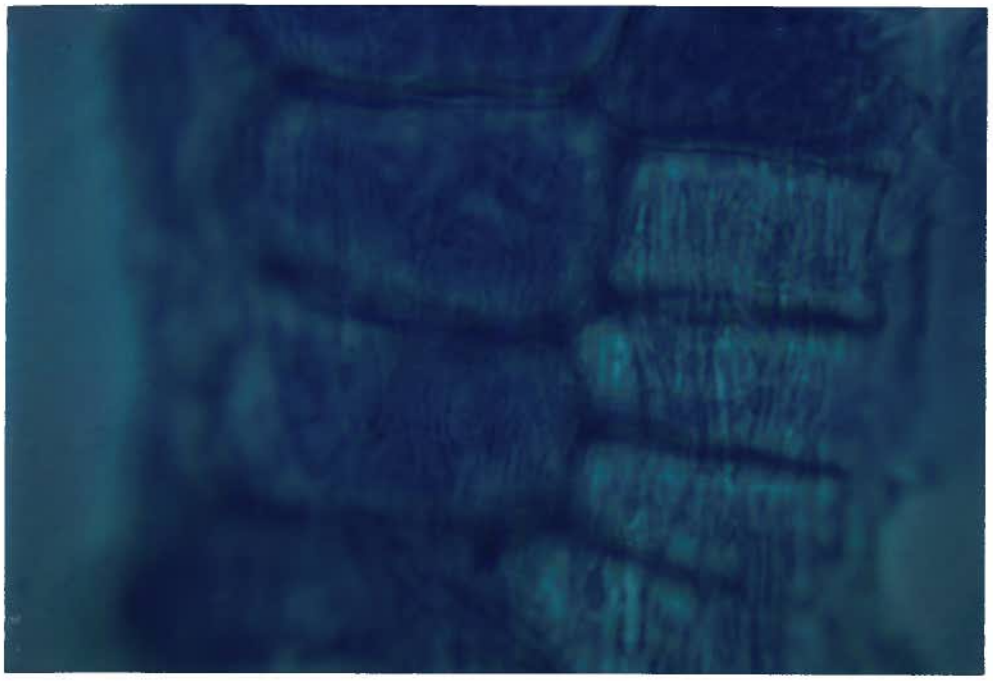

Fig. 3.5 A mantle-like structure of hyphae on the surface of field roots, x 1000.

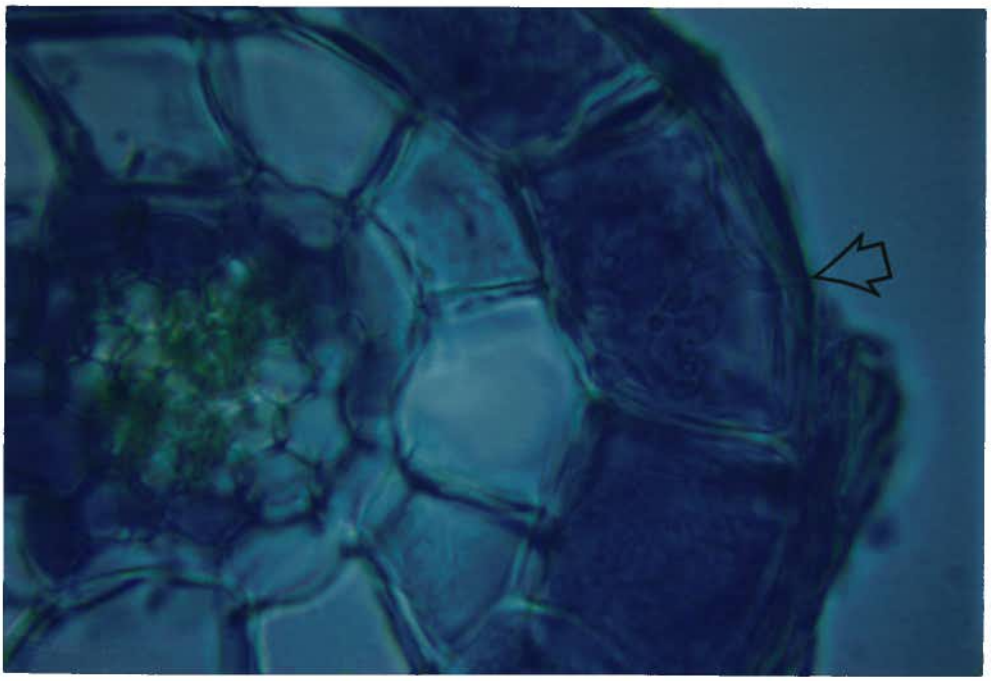

Fig. 3.6 A cross section of a field salal root, showing a hypha becoming narrow when penetrating the wall of the host cell, x 400.

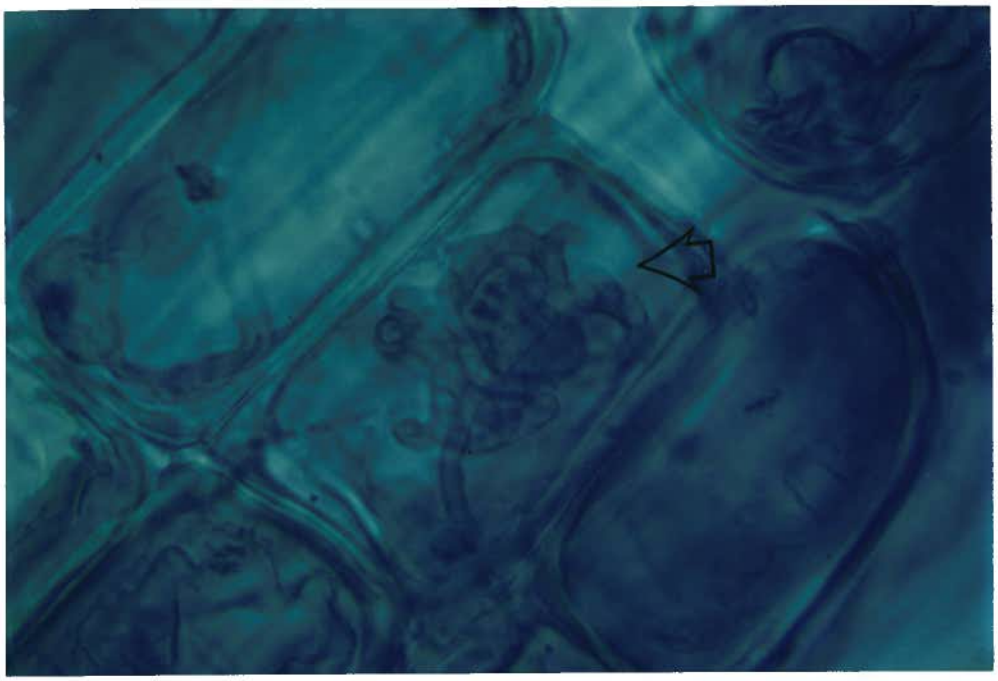

Fig. 3.7 A colonized cortical cell of a salal field root, showing hyphae proliferating around the nucleus, x 1000.

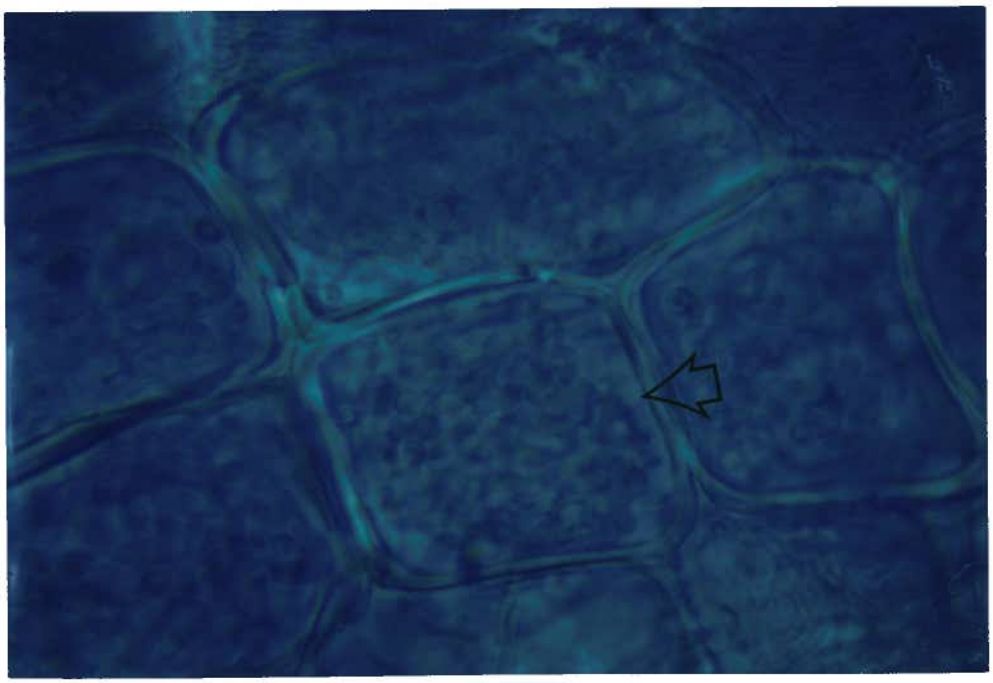

Fig. 3.8 Cortical cells of a salal field root, showing hyphal complexes occupying all the cell space, x 1000.

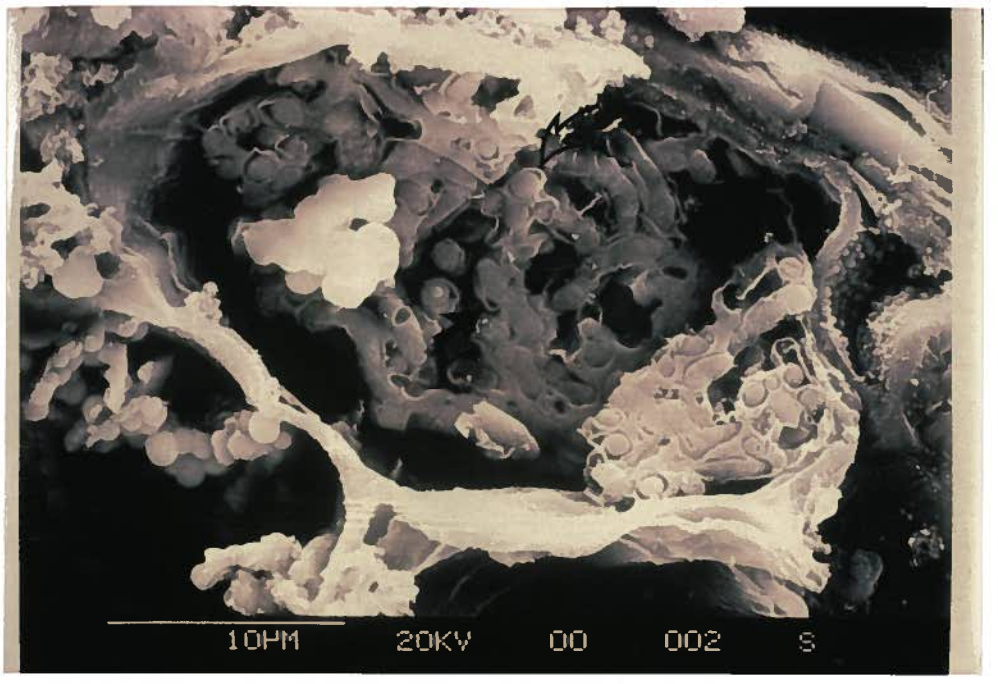

Fig. 3.9 A colonized cortical cell, showing hyphae separated from the host cytoplasm by a continuous host plasmalemma.

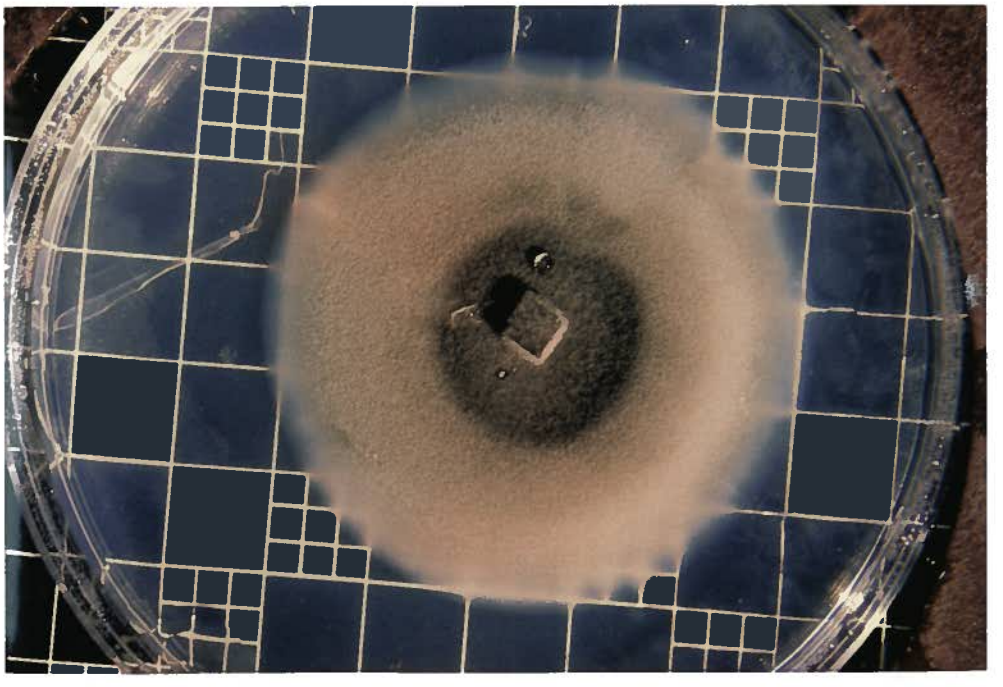

Fig. 3.10 A colony of *Oidiodendron griseum* on MMN.

isolates were grouped by the morphology of their colonies and asexual fruiting bodies whenever possible and probably belong to four different species, two of which sporulated in culture and two of which did not. The most common was identified as *Oidiodendron griseum* (Xiao and Berch 1992). The second sporulating species was identified as *Acremonium strictum* W. Gams by Dr. Walter Gams, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The two unknown species were not identified due to the lack of any fruiting bodies. These fungi were isolated from field salal roots with a similar frequency on HA and CH cutblocks (Table 3.2).

In axenic culture, *Oidiodendron griseum* and the 2 unknowns formed a light weft of hyphae on the root surface and extensive and dense hyphal complexes in the outer layer of salal root cortical cells. *Acremonium strictum,* though also forming light weft of hyphae on the root surface, colonized the outer layer of salal root cortical cells less densely and formed no dense hyphal complexes.

Oidiodendron griseum Robak (Figs. 3.10-3.12)

Colonies on MMN reached 2 cm diam in 14 d and 5 cm diam in 30 d at 25^{0}C. They are white at first, later olive-greenish, distinctly zonate (Fig. 3.10) and sporulate with long stalked conidiophores usually arising from hyphae at the agar surface (Fig. 3.11, 3.12). Colonies on PDA reached 2 cm diam in 14 d and 4 cm diam in 30 d at 25^{0}C. They are whitish-grey, cottony with a mealy appearance due to abundant production of conidia, domed, wrinkled. On MEA, colonies reached a diameter of 2 cm in 14 d and 3.5 cm in 30 d at 25^{0}C. Colonies are flat and hyaline. No conidiophores or conidia are produced. Conidiophores on MMN are 161-390 µm long and 2-4.5 µm broad, brown, smooth, cylindrical, unbranched in the lower part (Fig. 3.11). Conidia are grey-green, smooth to finely roughened and subglobose or ovoid, commonly 2.5 by 3 µm (Fig. 3.12). Isolates examined were

Table 3.2 Frequency of root-associated fungi isolated from 560 salal rootpieces each from **CH** and **HA.**

Fungal species	CH	HA
Oidiodendron griseum	28	31
Acremonium strictum	21	20
Unknown 1	18	18
Unknown 2	19	22
Total	87	91

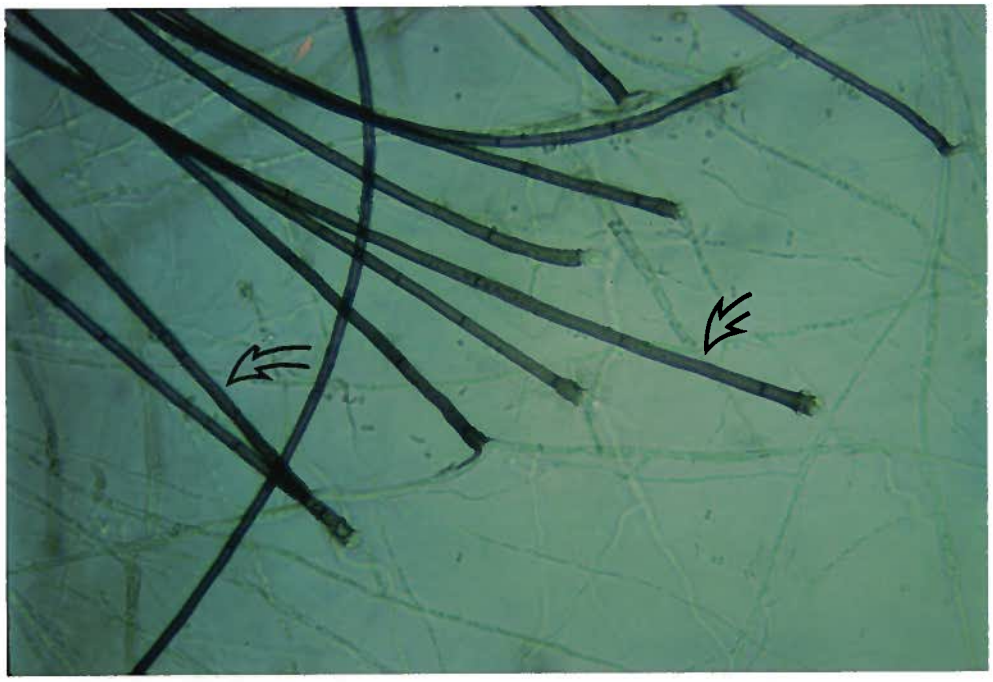

Fig. 3.11 Conidiophores of *Oidiodendron griseum* arising from hyphae at the agar surface, with a smooth, cylindrical unbranched lower part, x1000.

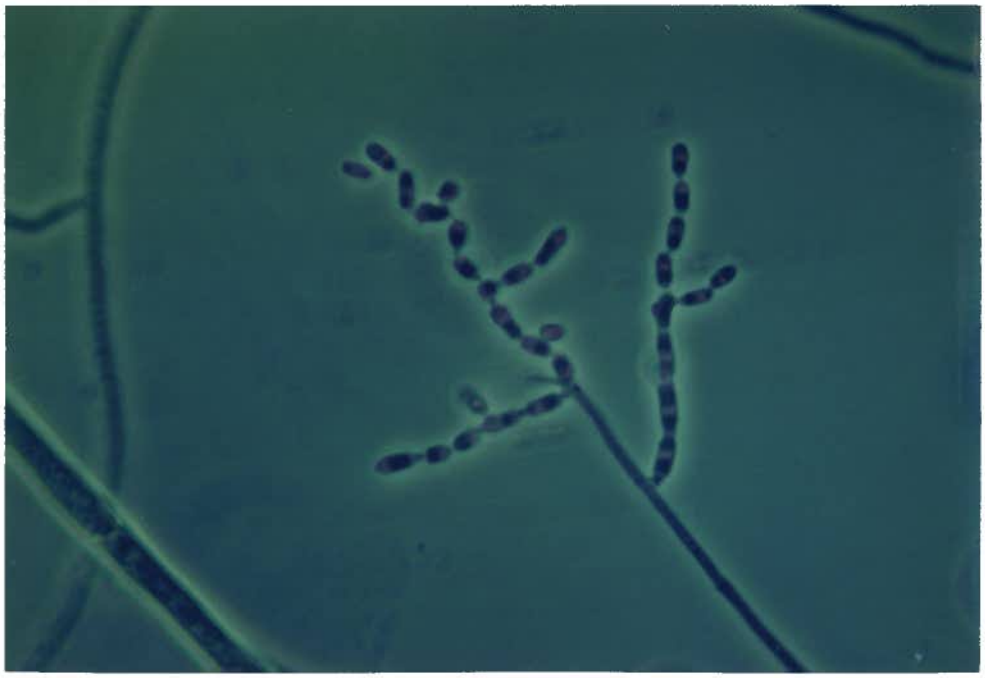

Fig. 3.12 A conidiophore and conidia of *Oidiodendron griseum*, x 1000.

S4, S18, S45 and S80.

Acremonium strictum W. Gams (Figs. 3.13).

Colonies reached 2 cm diam in 14 d and 4 cm in 30 d at 25^{0}C on MMN. Colonies are white-creamy with a pinkish tint, flat, usually moist (Fig. 3.13). The isolates show a slight radial wrinkling (Fig. 3.13). *Acremonium strictum* possesses the conidial heads characteristic of *Phialemonium* (Gams and McGinnis 1983). Phialide pegs are simple and arise from submerged or slightly fasciculated aerial hyphae. Conidia are mostly cylindrical, 3 x 1 μm. On PDA, colonies reached a diameter of 3 cm in 14 d and 5 cm in 30 d at 25^{0}C. On MEA, colonies reached a diameter of 2.8 cm in 14 d and 4.5 cm in 30 d at 25^{0}C. Colonies are very thin, flat, and hyaline with a pinkish tint. Isolates examined were S214, S217, S220, and S232.

Unknown 1 (Figs. 3.14)

Colonies reached a diameter of 2 cm on MMN in 14 d and 4 cm in 30 d at25^{0}C. They are olive-brown, distinctly zonate with a lustrous surface (Fig. 3.14). On PDA, colonies reached 2 cm in 14 d and 5 cm in 30 d at 25^{0}C. They are raised in the center, wrinkled and distinctly zonate. Colonies are grey, black, and white from the center to the edge with a powdery look. No spores and no pigment are produced on MMN and PDA. On MEA, colonies reached a diameter of 2 cm in 14 d and 4 cm in 30 d at 25^{0}C. Violaceous brown pigment was released into the media so that the whole agar disc was stained brown-purple. The whole colony is olive-greenish, flat, and smooth. Isolates examined were S9, S219, S234, and S245.

Unknown 2 (Fig. 3.15)

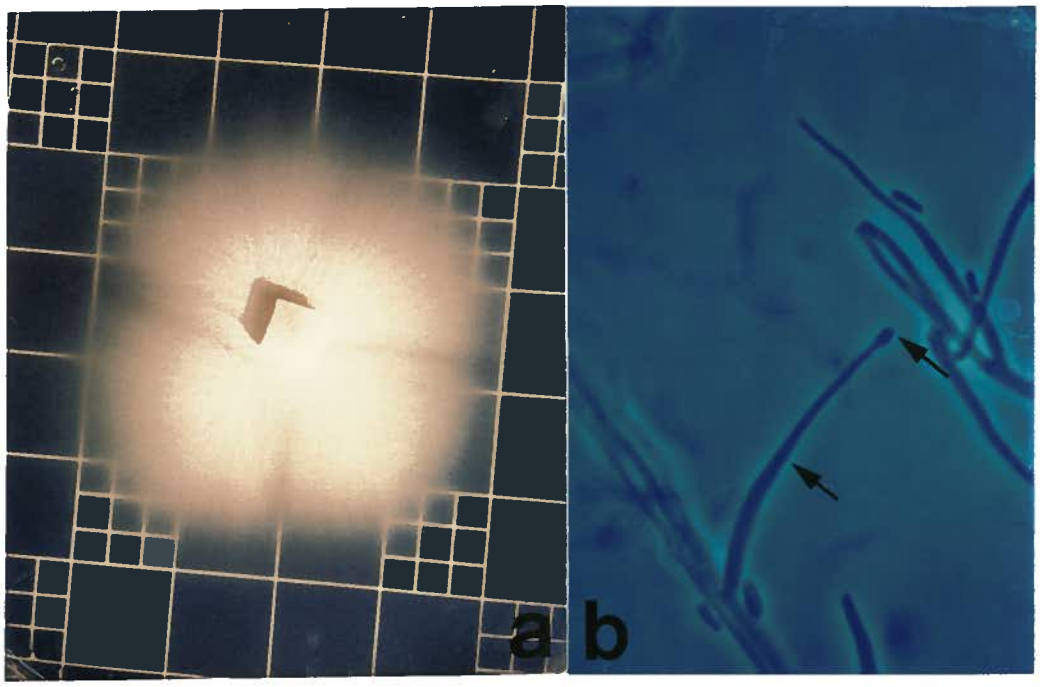

Fig. 3.13 (a) A colony of *Acremonium strictum* MMN, white-creamy with a pinkish tint, flat, usually moist. (b) A conidiophore and a conidia.

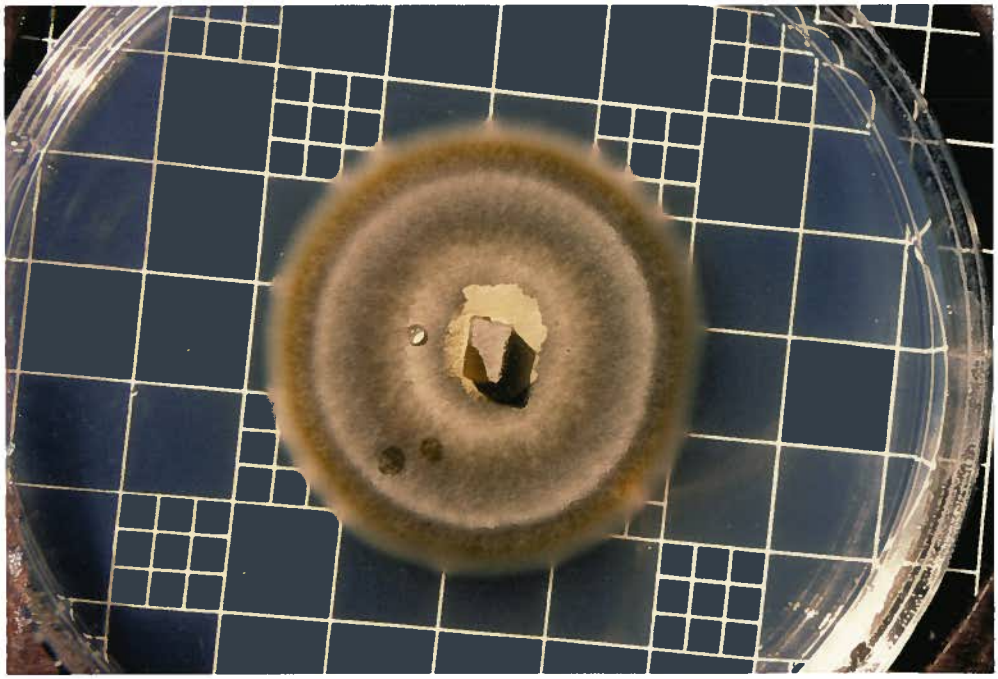

Fig. 3.14 A colony of Unknown 1 on MMN, olive-brown, distinctly zonate with a lustrous surface.

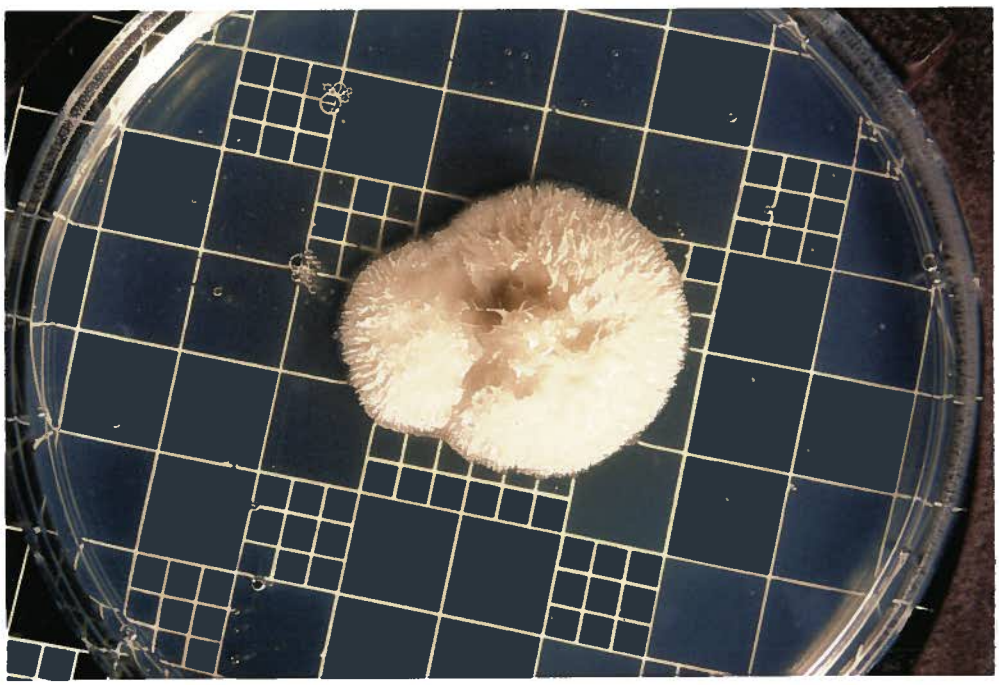

Fig. 3.15 A colony of Unknown 2 on PDA, creamy, moist, domed and funiculose with ropes radiating out.

On MMN, colonies reached a diameter of 2 cm in 14 d and 3 cm in 30 d at 25^{0}C. Two types of zonate colonies were produced. One was creamy with a dark center and droplets of cream-colored liquid scattered over the surface. The other was in general grey and has four zones, alternating floccose and light grey, dark grey with a peach fuzz, olive green and off white from the center to the edge. A marked radial wrinkling is exhibited. Colonies reached 1.5 cm diam in 14 d and 3 cm diam in 30 d on PDA at 25^{0}C. They are creamy, moist, domed and funiculose with ropes radiating out (Fig. 3.15) . On MEA, colonies reached a diameter of 2 cm in 14 d and 3.5 cm in 30 d at 25^{0}C. They are funiculose and olivaceous-black. No pigment and spores were produced in any media. Isolates examined were S203, S227, S246, and S255.

Colonies of these isolates resembled pure culture of isolates 100 and 101 from Dr. D. J. Read, of *Hymenoscyphus ericae* in overall appearance and growth rate.

Discussion

Salal formed two kinds of root-fungus associations, ericoid mycorrhizae and pseudomycorrhizae in axenic culture. The morphology and structure of salal roots, whether colonized or non-colonized, are in general similar to the roots of other ericaceous plants, such as *Calluna vulgaris*, *Rhododendron ponticum* L. and *Vaccinium myrtillus* L. The roots are very fine, as thin as 0.6 mm in diameter and typical of the "hair roots" in ericaceous plants (Beijerinck 1940). Like other ericaceous plants mentioned above, salal roots do not have any root hairs. Read (1983) characterized the fine roots of ericaceous plants as having one to three layers of cells, the number varying according to species, surrounding a narrow central stele. Salal roots differ somewhat from the fine roots of *Calluna vulgaris* (Bonfante-Fasolo and Gianinazzi-Pearson 1982) and *Rhododendron ponticum* (Duddridge and Read 1982) which have a single layer of cortical cells surrounding

a simple stele, because in salal there are two layers of cells outside the endodermis. In the colonized roots, only the outer layer of cells is colonized by root-associated fungi and the colonization does not extend into the stele or the meristematic region of the root. Bonfante-Fasolo *et al.* (1981) found that there were one or two layers of cells surrounding the stele in the fine roots of *Vaccinium myrtillus* and that some of the cells were colonized by a fungus, however, it is not clear if any cells of the second layer were colonized.

No attempt was made here to investigate why the root-associated fungi only colonize the first of the two layers of the cortical cells. What the difference between the two outermost layers cells is, whether the host sets up a defence line between the two layers, whether the first of the two layers of the cell is epidermis and the second layer of cells is cortex are not known.

Cells appeared collapsed behind the apical region of non-mycorrhizal roots of *Calluna vulgaris* (Bonfante-Fasolo and Gianinazzi-Pearson 1982) and *Vaccinium myrtillus* (Bonfante-Fasolo *et al.* 1981). The collapsed appearance was also observed in this study but might be due to processing for electron microscopy because no sign of collapse was found on live and intact mycorrhizal and non-mycorrhizal roots of salal behind the apical region.

In order to classify their mycorrhizal types, Largent *et al.* (1980) defined ecto- and arbutoid mycorrhizae: mycorrhizae with a mantle and a Hartig net are called ectomycorrhizae; and, mycorrhizae with a mantle, a Hartig net and intracellular hyphal penetration are arbutoid mycorrhizae. They found ericoid, arbutoid, and ectomycorrhizae on field salal roots from northern California. Roots of salal grown in pots containing field soil developed ericoid mycorrhizae, trace amount of ectomycorrhizae, and but no arbutoid (Smith 1993). Neither ecto- nor arbutoid mycorrhizae were observed from either field or synthesized salal roots in this study. However, a mantle-like fungal structure was occasionally found on the

surface of field salal roots with or without intracellular colonization. Having a mantle can give the superficial impression of an ectomycorrhiza, but without a Hartig net a colonized root cannot be considered a true ectomycorrhiza. Similarly, without a Hartig net, a mycorrhizal root with a mantle and intracellular colonization cannot be considered a true arbutoid mycorrhiza either.

Salal roots from both CH and HA sites were highly colonized by ericoid mycorrhizal fungi and differences between the two sites in terms of root colonization or cell colonization intensity could not be detected. Studies have shown that low availability of mineral nitrogen and high organic nitrogen content can result in high intensity of colonization of ericoid mycorrhizae (Reed 1987) and that application of inorganic nitrogen can suppress ericoid mycorrhiza formation (Brook 1952; Morrison 1957; Stribley and Read 1976; Moore-Parkhurst and Englander 1982). Most of the work on colonization intensity of ericoid mycorrhizal fungi has centered on mycorrhizal plants in the lab. Stribley and Read (1976) found that 70% of the root cortical cells of *Vaccinium macrocarpon* were colonized by *Hymenoscyphus ericae*. In an axenic culture, nine percent of the root cortical cells of *V. angustifolium* were colonized by *Oidiodendron rhodogenum*, 12% by *O. cerealis* and 21% by *O. griseum* (Dalpe 1986). No information is available on mycorrhiza colonization intensity of ericaceous plants in relation to nitrogen availability and organic nitrogen concentration in the field, so no comparison can be made to this study.

A number of species of root-associated fungi of salal were isolated from both CH and HA sites with similar frequency. *Acremonium strictum* is first reported here as a pseudomycorrhizal fungus. The genus *Acremonium* contains over 100 species, many of which are soilborne fungi (Domsch *et al.* 1980). The species placed in *Acremonium* section *Albo-lanosa* Morgan-Jones & Gams live in grasses and sedges (Morgan-Jones and Gams 1982). Many species of grasses infected by

Acremonium have been reported to be toxic to livestock and insects (Siegel *et al.* 1987; Clay 1989) and nematodes (West *et al.* 1987). In addition, infection by *Acremonium* has also been reported to be beneficial to the host by increasing drought tolerance (Belesky *et al.* 1987) and allelopathy (Quigley *et al.* 1990). *Acremonium strictum* is a ubiquitous fungus which occurs in soil, plant wheatfield debris, rhizosphere, plant surfaces, excrements, hay, stained wood, atmosphere, iron ore tailings, fuel and fuel filters (Domsch *et al.* 1980; Wong *et al.* 1978). It has an extremely wide range of activities: plant pathogen (Kang and Singh 1977; Hesseltine and Bothast 1977; Chase 1978, Chase and Munnecke 1980; Seemueller 1976; Natural *et al.* 1982); parasite on eggs of the sugar beet cyst nematode (Nigh 1979), mycoparasite on fungi such as rusts, powdery mildews, agarics, *Scleroderma*, polypores (Domsch *et al.* 1980), and inhibitor of fungi (McGee *et al.* 1991). However, comparison of colony description of our isolates of *Acremonium strictum* identified by Dr. W. Gams to that of the same species by Domsch *et al.* (1980) and by McGee *et al.* (1991) revealed that our description is consistent with the description by Gams but significantly different from the description by McGee *et al.* in that the colonies of our isolates were pink, moist and smooth, while the colonies of McGee's isolates were velutinous to cottony. Since *A. strictum* has the least number of differential characters (Domsch *et al.* 1980) and probably is difficult to identify, we feel that McGee *et al.* (1991) might not be dealing with *A. strictum* but something totally different. Unfortunately, this cannot be verified because no vouchers were deposited in herbaria.

In axenic culture with salal, *Oidiodendron griseum* and the 2 unknowns formed typical ericoid mycorrhizae, a light weft of hyphae on the root surface and extensive and dense hyphal complexes in root cortical cells (Read 1983). However, *Acremonium strictum,* though also forming a hyphal weft on the root

surface, colonized the outer layer of salal root cortical cells less densely and formed loose hyphal complexes inside the cortical cells. Therefore, the colonization of salal roots by *Acremonium strictum* is different from those by the other three fungi in this study. Based on the difference from the typical ericoid mycorrhizae formed by other fungi in this study, *Acremonium strictum* is considered to be a pseudomycorrhizal fungus of salal in this thesis.

Twenty years ago, a single fungal species was isolated from *Calluna vulgaris* as an ericoid mycorrhizal fungus (Pearson and Read 1973). No other ericoid mycorrhizal fungi have since been reported from *C. vulgaris*. Couture *et al.* (1983) isolated a single ericoid mycorrhizal fungus, *Oidiodendron griseum* Robak from *Vaccinium angustifolium* Ait. and Douglas *et al.* (1989) isolated a single species of ericoid mycorrhizal fungus, *O. maius*, from *Rhododendron.* This might give the impression that an ericaceous species in a certain ecosystem forms ericoid mycorrhizae with no more than one species of fungus and, if so, that this fungus plays a very important role in the adaptation and dominance of its host in the stressed soil. For the first time, this paper reports that several ericoid mycorrhizal fungi were isolated from a single ericaceous plant. This indicates that the real situation might be that many fungi form mycorrhizae with an ericaceous species in any ecosystem, a situation more similar to that observed for most other mycorrhizal types and plant species.

The diversity of root-associated fungi, high intensity of colonization and good performance of salal suggest that root-associated fungi are involved in the dominance of salal. *Oidiodendron griseum* has been isolated from a variety of substrates (Burgeff 1961; Barron 1962; Wong *et al.* 1978; Domsch *et al.* 1980; Couture *et al.* 1983; Dalpe, 1986; Douglas *et al.* 1989; Stoyke and Currah 1991),

demonstrated to be antagonistic to the saprophytic fungi isolated from its original habitat (Dickinson and Boardman 1970), and shown to be able to use chitin as a nitrogen source (Leake and Read 1990b). Another root-associated fungus of salal, *Acremonium strictum*, is mycoparasitic (Gandy 1979). This suggests that the root-associated fungi of salal could be able to utilize organic nitrogen and directly inhibit the growth of the ectomycorrhizal fungi of the planted trees growing around salal.

Chapter 4

The potential mycorrhizal fungi of salal

Introduction

Like other ericaceous plants, including *Calluna vulgaris* (L.) Hull. in western Europe and *Kalmia angustifolia* L. var. *angustifolia* in eastern Canada, salal produces numerous fine, hair roots. In the field, these roots are heavily colonized by mycorrhizal fungi, which are thought to be very important in the dominance of cutblocks by salal (see Chapter 3). A number of species of fungi have been shown to form mycorrhizae with salal: *Oidiodendron griseum* Robak and two nonsporulating species (see Chapter 3). *Oidiodendron griseum* has been isolated from soil, rhizosphere, plant tissue and atmosphere (Barron 1962; Wong *et al.* 1978; Domsch *et al.* 1980; Dalpe, 1986; Douglas *et al.* 1989; Stoyke and Currah 1991). As an ericoid mycorrhizal endophyte, *Oidiodendron griseum* was isolated from *Vaccinium* species (Couture *et al.* 1983). This fungus is widespread, versatile in substrate utilization, tolerant to broad ranges of pH and temperatures, and sporulates easily. Such characters render it easy to isolate and manipulate under laboratory conditions at room temperature on artificial media. As such procedures are often selective and favor the isolation of this type of fungus, I have good reason to believe that there might be other fungal species that are mycorrhizal with salal but that have not been isolated yet. These fungi, not reacting well under lab conditions, are still to be studied.

There are, to date, 14 fungi which have been proven to form ericoid mycorrhizae with ericaceous plants in axenic culture (Table 4.1), although *Scytalidium vaccinii* and *Hymenoscyphus ericae* are now considered to be anamorph and teleomorph states of a single species (Egger and Sigler 1993) which reduces the total number of species to 13. The purpose of this study was to identify the potential ericoid mycorrhizal fungi of salal among the known ericoid mycorrhizal fungi. Their ability to form ericoid mycorrhizae with salal was tested in axenic culture.

Methods and materials

Twelve species of ericoid mycorrhizal fungi used were obtained from the University of Alberta Microfungus Collection Herbarium (UAMH). *Oidiodendron periconioides* Morrall was not available when this study was carried out. Fungal isolates originally maintained on modified Melin Norkrans agar (MMN) were retrieved and used as inoculum for the synthesis experiments. For synthesis, the same culture system as described in Chapter 3 was used.

A non-mycorrhizal seedling per Petri dish was obtained by germinating the seeds on water agar after surface-sterilizing with 30% hydrogen peroxide (Xiao and Berch 1992) and planting at the center of the cut edge of the agar (Fig. 4.1). Five seedlings per fungal species were inoculated when a real leaf emerged with about 2 mm^3 inoculum cut from the edge of a colony. The plates were then sealed with parafilm and placed vertically in a growth chamber (Fig. 4.2) under the same growth conditions as described in Chapter 3. The seedlings were examined for colonization by mounting the plate on the stage of a light microscope and examining the whole root system intact from 14 d after inoculation up to harvesting. By 45 d after inoculation, the roots of the plants were gently pulled

Table 4.1 Currently known ericoid mycorrhizal fungi and their host used in synthesis.

Fungal species	Host Species used in Mycorrhiza Synthesis	Reference
Gymnascella dankaliensis (Castellani) Currah	*Vaccinium angustifolium* Ait.	Dalpe 1989
Hymenoscyphus ericae (Read) Korf and Kernan	*Calluna, Erica, Gaultheria Rhododendron and Vaccinium*	Douglas *et al.* 1989
Myxotrichum setosum (Eidam) Orr, Kuehn and Peunkett	*Vaccinium angustifolium* Ait.	Dalpe 1989
Oidiodendron chlamydosporicum Morrall	*Vaccinium angustifolium* Ait.	Dalpe 1991
Oidiodendron citrinum Barron	*Vaccinium angustifolium* Ait.	Dalpe 1991
Oidiodendron flavum Szilvinyi	*Vaccinium angustifolium* Ait.	Dalpe 1991
Oidiodendron griseum Robak	*Vaccinium* spp.	Couture *et al.* 1983
	Vaccinium angustifolium Ait.	Dalpe 1986
	Gaultheria shallon Pursh	Xiao and Berch 1992
Oidiodendron maius Barron	*Rhododendron* cv. 'Pink Pearl'	Douglas *et al.* 1989
Oidiodendron periconioides Morrall	*Vaccinium angustifolium* Ait.	Dalpe 1991
	Rhododendron brachycarpum G. Don.	Currah *et al.* 1993
Oidiodendron rhodogenum Robak	*Vaccinium angustifolium* Ait.	Dalpe 1986
Oidiodendron scytaloides W. Gams and Soderstrom	*Vaccinium angustifolium* Ait.	Dalpe 1991
Pseudogymnoascus roseus Raillo	*Vaccinium angustifolium* Ait.	Dalpe 1989
Scytalidium vaccinii Dalpe, Litten and Sigler	*Vaccinium angustifolium* Ait.	Dalpe *et al.* 1989
Stephanosporium cerealis (Thum.) Swart	*Vaccinium angustifolium* Ait.	Dalpe 1986

out of the agar (this is possible because only 8 g /L of agar was applied), stained with FDA Blue No. 1 and examined at 400-1000x to confirm colonization and morphology of the mycorrhizae. Occasionally, additional cross and longitudinal hand sections were made for determination of detailed structure by freezing a piece of root on a freezing microtome and cutting it with a double-edged razor blade. All isolates were reisolated from the synthesized mycorrhizal plants.

Percent mycorrhizal colonization

Mycorrhizal colonization was assessed in two ways: root colonization and cell colonization. For percentage root colonization, all roots of a seedling were counted as colonized or non-colonized. One hundred contiguous cortical cells of each of 3 colonized roots were counted for the cell percent colonization.

Results

Six species of the tested fungi formed typical ericoid mycorrhizae with salal in axenic culture. Five previously unreported are *Hymenoscyphus ericae*, *Oidiodendron flavum*, *O. maius*, *Pseudogymnoascus roseus* and *Scytalidium vaccinii* (Table 4.2). In all cases, only the outermost layer of cortical cells was colonized by mycorrhizal fungi. In some cases, a hyphal weft was observed on the roots. Inside the colonized cell, the hyphae proliferated, developed a hyphal complex and occupied almost all the space of the cell. No difference among the mycorrhizae formed by different fungi could be detected in terms of the morphology of the hyphal complex. The hyphal complex consists of relatively undifferentiated hyphae. The mycorrhiza-forming fungi did not alter the root morphology.

Colonization of the cortical cells took place 2 wk after inoculation and fanned out over the root system from the base of a seedling where inoculum was applied.

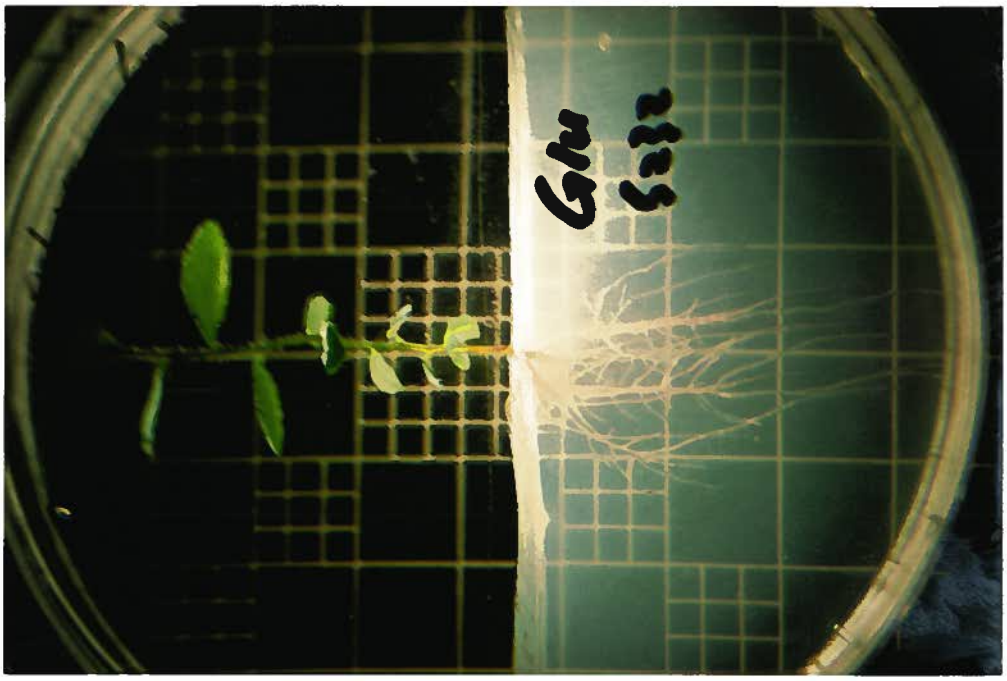

Fig. 4.1 The synthesis culture system.

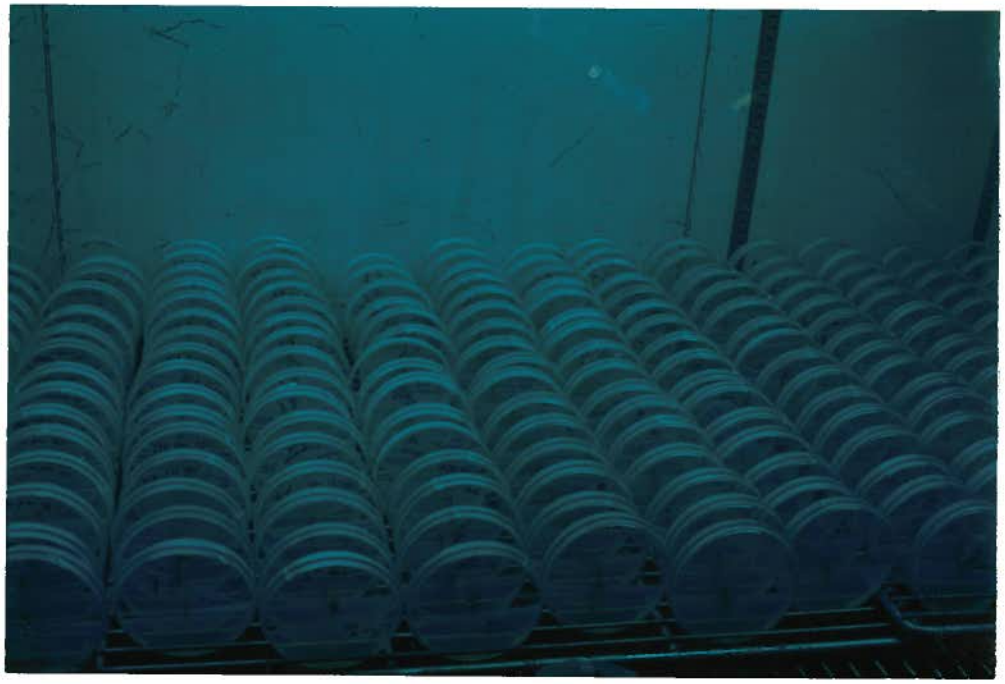

Fig. 4.2 Petri dishes vertically placed in a growth chamber.

Table 4.2 Fungal species used in the synthesis study together with their mycorrhiza potential with salal and their isolation data.

Fungal species	Isolate Number	Mycorrhiza Formation with Salal	Isolation Data
Gymnascella dankaliensis	3531(UAMH)	--	Soil (Arizona)
Hymenoscyphus ericae	6735(UAMH)	+	Roots of *Calluna vulgarius* (UK)
	100	+	
	101	+	
	(From D. J. Read UK)		
Myxotrichum setosum	3835(UAMH)	--	Soil (Mt. Allen Kananaskis, Alta.
Oidiodendron chlamydosporicum	6520(UAMH)	--	Soil under conifers (Candle Lake, Sask.)
Oidiodendron citrinum	1525(UAMH)	--	Soil of cedar bog (Aberfoyle, Ont.)
Oidiodendron flavum	1524(UAMH)	+	Soil of cedar bog (Aberfoyle, Ont.)
Oidiodendron griseum	S4	+	Roots of salal (Vancouver Island, B.C.)
	S18	+	
	S24	+	
	S80	+	
Oidiodendron maius	1540(UAMH)	+	Peat soil of cedar bog (Guelph, Ont.)
Oidiodendron rhodogenum	1405(UAMH)	--	Sludge in pulp strainers (Kistefoss Mills Norway)

Table 4.2 Continued.

Fungal species	Isolate Number	Mycorrhiza Formation with Salal	Isolation Data
Oidiodendron scytaloides	6521(UAMH)	--	Forest soil (Sweden)
Pseudogymnoascus roseus	1658(UAMH)	+	Rhizosphere of a conifer tree
Scytalidium vaccinii	5826(UAMH)	+	Roots of *Vaccinium angustifolium* (Washington, Co., Maine)
Stephanosporium cerealis	1522(UAMH)	--	Peat soil (Bradford Marsh, Ont.)

ascomycetes and hyphomycetes. Some of the fungi, such as *Hymenoscyphus ericae* (Read 1987) and *Pseudogymnoascus roseus* (Dalpe 1989), are proven to be able to use organic forms of nitrogen and degrade cellulose. Formation of ericoid mycorrhizae with a variety of fungi which have various capabilities including the capability of utilizing organic nitrogen might make a major contribution to the success of salal in highly nutrient-stressed sites.

Table 4.3 Percent mycorrhizal colonization of *Gaultheria shallon* by its potential mycorrhizal fungi.

Fungal species	Root colonization per root system	Cell colonization per hundred cells
Hymenoscyphus ericae	17%	43%
Oidiodendron flavum	23%	67%
Oidiodendron maius	27%	76%
Pseudogymnoascus roseus	11%	32%
Scytalidium vaccinii	20%	50%

Chapter 5

Utilization of organic forms of nitrogen by the root-associated fungi and plants of *Gaultheria shallon* inoculated with the fungi

Introduction

The CH situation is similar to one in the heathlands in Europe. The heathland in Europe is dominated by *Calluna vulgaris* (Handley 1963), while CH in northern part of Vancouver Island is dominated by salal (Messier and Kimmins 1991). The types of soil are similar: low pH, low nutrient availability and high organic matter content, and the major growth limiting nutrient is nitrogen (Weetman *et al.* 1989a). *Calluna vulgaris* and salal are both ericaceous plants and form ericoid mycorrhiza associations (Handley 1963; Xiao and Berch 1992). With the dominance of *Calluna* in the heathlands, spruce displayed growth "check" (Mcvean 1963), and in the presence of salal, the growth of plantations of western hemlock in CH is dramatically reduced (Weetman *et al.* 1989a).

The heathlands in Europe have been studied in great detail in relation to *Calluna vulgaris*. It has been well documented (Read 1983, 1987a, 1991, Read and Bajwa 1985) that the ericoid mycorrhizal fungus *Hymenoscyphus ericae* plays a vital role in the success and dominance of the heath, *Calluna vulgaris,* most importantly by providing its host with access to organic forms of nitrogen which is a critical element for the growth of plants in those soils. The utilization of organic nitrogen by ericoid mycorrhizal fungi and mycorrhizal plants has been convincingly demonstrated with *H. ericae* and *C. vulgaris* (Bajwa *et al.* 1985; Bajwa and Read 1985; Stribley and Read 1980). Because of the similarities between CH and *Calluna* heathlands, it is suggested that ericoid mycorrhizal fungi

might play a vital role in the establishment and dominance of salal. However, the ecology of the ericoid mycorrhizal fungi and their mycorrhizal associations with salal in CH ecosystems is poorly known. Knowledge of whether ericoid mycorrhizal fungi benefit salal growth and, if so, how, is required for a proper understanding of the check problem in CH clearcuts.

At least four different species of root-associated fungi are involved in the dominance of salal on CH clearcuts (Chapter 3) and others that we did not isolate and identify might also be involved. Based on the good performance of salal in the nitrogen-deficient soils, it was speculated that the root-associated fungi of salal might be capable of utilizing organic forms of nitrogen and provide salal with access to organic forms of this nutrient. The objective of this paper was to test if the root-associated fungi and and plants of salal in association with the fungi can use different forms of organic nitrogen.

Materials and methods

Fungal isolates

Four fungi isolated from field salal roots, *Oidiodendron griseum*, *Acremonium strictum*, Unknown 1 and 2, and *Hymenoscyphus ericae* from Europe were used in this study. *Oidiodendron griseum*, Unknown 1 and 2 formed typical ericoid mycorrhizae in axenic culture (see Chapter 3). *Hymenoscyphus ericae* is a well-known ericoid mycorrhizal fungus. For each species, except for *Hymenoscyphus ericae* (two isolates 100 and 101 were used), four isolates were used. These isolates were S4, S18, S45 and S80 (deposited at the University of Alberta Microfungus Collection) of *Oidiodendron griseum*, S214, S217, S220 and S232 (S232 was deposited at Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) of *Acremonium strictum*, S9, S219, S234, S245 of Unknown 1, S203, S227, S246 and S255 of Unknown 2. The isolates 100 and 101 of *Hymenoscyphus*

ericae were kindly provided by Dr. D.J. Read at the University of Sheffield. *Hymenoscyphus ericae* is a well-studied fungus in terms of using organic forms of nitrogen and was used here for comparison. All isolates including *Hymenoscyphus ericae* were originally maintained on modified Melin Norkrans agar (MMN) and then transferred to nutrient agar with the deletion of any nitrogen sources before they were used as inoculum.

Utilization of organic N by the fungi

This experiment was conducted and repeated once in a liquid culture system. The basal medium was MMN with deletion of mineral nitrogen. The solutions were sterilized by autoclaving. The pH level of the solutions after autoclaving was 4. This pH value was selected because it is within the range of the soil pH (3 - 4) of the CH sites. Five different nitrogen sources, bovine serum albumin (BSA), glutathione, glutamine, $(NH_4)_2HPO_4$ and nitrogen free were applied. The solutions of BSA (a protein), glutathione (a peptide), glutamine (an amino acid) and $(NH_4)_2HPO_4$ were filter-sterilized and added to the autoclaved basal medium separately to give a nitrogen concentration of 0.016 g l^{-1}. Five replicate 30 ml aliquots at each nitrogen treatment were aseptically transferred to 50 ml beakers. The beakers were separately inoculated with 2 mm^3 of inoculum taken from the edge of a colony of one of the isolates of each test fungus and covered with aluminum foil. Cultures were incubated at 25^0C in the dark, harvested 45 days after inoculation by filtration of the culture solution through oven-dried preweighed filter paper, oven-dried at 80^0C for 24 hr, and weighed with a fine electronic balance. The filtered culture solutions were measured with a pH meter to obtain final pH value after harvesting to see if the pH of the medium was changed by the activity of the growing fungi.

Utilization of organic nitrogen by plants

The experiment was carried out in 100 x 15 mm Petri dishes with half of the agar disc cut out (see Chapter 3). The growth medium contained MMN with mineral nitrogen, malt extract and glucose deleted, 8 g l^{-1} of Difco Bacto-agar. Before pouring, solutions of BSA, glutathione, glutamine and $(NH_4)_2HPO_4$ were filter-sterilized and then added to the autoclaved medium separately to give a nitrogen concentration of 0.016 g N l^{-1}. The pH of the medium was 4 after autoclaving.

An experimental plant per Petri dish was obtained by planting a germinated seed at the center of the edge of the agar disc (see Chapter 3). This plant was inoculated when it had its first true leaf with 2 mm^3 inoculum cut from the edge of a colony of the fungal isolates maintained on nitrogen-free media. Noninoculated controls received 2 mm^3 of agar without inoculum. Ten plants for each treatment were prepared. The plates were sealed with parafilm and placed in a growth chamber at 25^0C and a light regime of 18 h light at 310 µmol m^{-2} sec^{-1} illumination and 6 h dark. Two wk after inoculation, we started to check all the plants for colonization by mounting the plate on the platform of a light compound microscope (see Chapter 3). The colonization intensity of their root systems was expressed in cell colonization. The entire plants were harvested by gently pulling them out of the agar. The entire root system was mounted intact on a glass slide with a drop of water and covered by a glass cover slip for clear examination of colonization. Three root sections each with 100 contiguous cortical cells of each colonized root were counted for the cell percent colonization. After examination for colonization intensity, the entire plants were oven-dried for 24 h at 80^0C. The pH of the growth medium after harvesting was checked by inserting a pH probe into the half agar disc at several locations to see if the activity of the plants and fungi influenced the pH of the growth medium.

Statistical analysis

The utilization of organic nitrogen by the fungi was a completely randomized 5 x 5 factorial experimental design with 5 replicates for each treatment (Table 5.1). Utilization of organic nitrogen by plants was a completely randomized 5 x 6 factorial experimental design with 10 replicates for each treatment (Table 5.2). All differences between the treatment means for each experiment were judged statistically by Tukey's Studentized Range (HSD) Test at Alpha = 0.05. All analyses were done with SAS (SAS 6.03 Edition, SAS Institute Inc. 1988).

Results

Yield of fungi

The total dry weights of the fungi in the liquid culture study after 45 d are presented in Table 5.3 in which each number is the mean yield of all test isolates of the same species. Dry weight produced by the fungus Unknown 1 on glutamine as a sole nitrogen source was more than on ammonium, and 6 times more than on nitrogen free but its dry weight yields on glutathione, BSA and nitrogen-free media were not significantly different. Ammonium, glutamine and glutathione gave greater yields in dry weight of *Oidiodendron griseum* relative to the control nitrogen-free treatment. *Acremonium strictum* grew as well on glutamine as on ammonium and much better on BSA but its growth was limited on glutathione. There were significantly higher dry weight yields of Unknown 2 on ammonium, glutamine and glutathione. The growth of Unknown 2 on BSA was not significantly different from the growth on nitrogen free. *Hymenoscyphus ericae* grew very well on both simple nitrogen sources though better on glutamine but no significant growth occurred on the two more complicated organic nitrogen sources, glutathione and BSA. In the absence of any nitrogen source, all fungi tested produced yields which were not statistically different from each other (Table 5.3).

Table 5.1 The layout of the fungal isolates used in the experiment of the growth of the root-associated fungi of salal on different sources of nitrogen.

Fungi	Nitrogen Sources				
	Ammonium	**Glutamine**	**Glutathione**	**BSA**	**N-free**
Unknown 1	S9	S9	S9	S9	S9
	S9	S9	S9	S9	S9
	S219	S219	S219	S219	S219
	S234	S234	S234	S234	S234
	S245	S245	S245	S245	S245
O. griseum	S4	S4	S4	S4	S4
	S4	S4	S4	S4	S4
	S18	S18	S18	S18	S18
	S45	S45	S45	S45	S45
	S80	S80	S80	S80	S80
A. strictum	S232	S232	S232	S232	S232
	S232	S232	S232	S232	S232
	S214	S214	S214	S214	S214
	S217	S217	S217	S217	S217
	S220	S220	S220	S220	S220
Unknown 2	S246	S246	S246	S246	S246
	S246	S246	S246	S246	S246
	S203	S203	S203	S203	S203
	S227	S227	S227	S227	S227
	S255	S255	S255	S255	S255
H. ericae	100	100	100	100	100
	100	100	100	100	100
	100	100	100	100	100
	101	101	101	101	101
	101	101	101	101	101

Table 5.2 The layout of the fungal isolates used in the experiment of the growth of salal plants in association with its root-associated fungi on different nitrogen sources.

Plants inoculated with	Nitrogen Sources											
	Ammonium		**Glutamine**		**Glutathione**		**BSA**		**N-free**	
Unknown 1	S9	S9	S9	S9	S9	S9	S9	S9	S9	S9
	S9	S9	S9	S9	S9	S9	S9	S9	S9	S9
	S219	S219	S219	S219	S219	S219	S219	S219	S219	S219
	S234	S234	S234	S234	S234	S234	S234	S234	S234	S234
	S245	S245	S245	S245	S245	S245	S245	S245	S245	S245
O. griseum	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
	S18	S18	S18	S18	S18	S18	S18	S18	S18	S18
	S45	S45	S45	S45	S45	S45	S45	S45	S45	S45
	S80	S80	S80	S80	S80	S80	S80	S80	S80	S80
A. strictum	S232	S232	S232	S232	S232	S232	S232	S232	S232	S232
	S232	S232	S232	S232	S232	S232	S232	S232	S232	S232
	S214	S214	S214	S214	S214	S214	S214	S214	S214	S214
	S217	S217	S217	S217	S217	S217	S217	S217	S217	S217
	S220	S220	S220	S220	S220	S220	S220	S220	S220	S220
Unknown 2	S246	S246	S246	S246	S246	S246	S246	S246	S246	S246
	S246	S246	S246	S246	S246	S246	S246	S246	S246	S246
	S203	S203	S203	S203	S203	S203	S203	S203	S203	S203
	S227	S227	S227	S227	S227	S227	S227	S227	S227	S227
	S255	S255	S255	S255	S255	S255	S255	S255	S255	S255
H. ericae	100	100	100	100	100	100	100	100	100	100
	100	100	100	100	100	100	100	100	100	100
	100	100	100	100	100	100	100	100	100	100
	101	101	101	101	101	101	101	101	101	101
	101	101	101	101	101	101	101	101	101	101
Fungus-free	Fungus-free		Fungus-free		Fungus-free		Fungus-free		Fungus-free	

Table 5.3 The growth of root-associated fungi of salal on different sources of nitrogen.

Fungi	Nitrogen Sources				
	Ammonium	**Glutamine**	**Glutathione**	**BSA**	**N-free**
Unknown 1	10.3cde	12.1abc	2.3jk	4.2ijk	1.9k
	*(1.7)	(2.8)	(0.1)	(0.5)	(0.2)
O. griseum	14.4a	9.7cde	6.9fgh	3.3ijk	2.3jk
	(4.3)	(1.4)	(1.2)	(0.3)	(0.2)
A. strictum	9.2def	10.2cde	4.8hij	13.3ab	4.1ijk
	(4.4)	(2.2)	(0.6)	(1.3)	(0.3)
Unknown 2	8.0efg	11.7bcd	5.6ghi	2.3jk	2.6jk
	(1.1)	(3.9)	(1.8)	(0.2)	(0.5)
H. ericae	9.1def	11.9abc	3.0ijk	2.1jk	2.5jk
	(1.7)	(1.9)	(0.2)	(0.0)	(0.2)

Means (in mg) with the same letter are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=5. *: Standard Deviation.

Growth of all fungi on ammonium and glutamine was overall much greater than on any other nitrogen source. All fungi utilized glutamine as readily as ammonium, even better than ammonium in the cases of Unknown 2 and *H. ericae*. Glutathione was good for *O. griseum* and Unknown 2 as well, but an unsatisfactory N source for Unknown 1, *A. strictum* and *H. ericae*. Though best for *A. strictum*, BSA was an unsuitable nitrogen source for all other species of the tested fungi.

Yield of plants

The total dry weights of the plants of salal are shown in Table 5.4. In general , the pattern is similar to the one in Table 5.3. In the absence of nitrogen, plant dry weight was lowest and colonized and non-colonized plants were not statistically different from each other. Non-colonized plants grew better on ammonium and glutamine than on any other nitrogen source. There is a trend that the fungi can improve the growth of salal plants even on ammonium though dry weights were not statistically different from those of non-colonized plants on this nitrogen source except the plants inoculated with *A. strictum*. Non-colonized plants grown on glutathione, BSA and no nitrogen were very small, dying or dead when harvested (Fig. 5.2). So were colonized plants growing on N-free (Fig. 5.1) compared to the colonized plants growing on any one of the nitrogen sources, for example on the simple form of organic nitrogen glutamine (Fig. 5.4) and the most complicated form of nitrogen used in this study BSA (Fig. 5.3). Although growth of non-colonized plants on glutamine appeared to be better than on the more complex forms, there was no significant difference.

The colonized plants grew as well on glutamine as on ammonium except for those inoculated by *Hymenoscyphus ericae* which grew much better on glutamine than on ammonium. All plants, whether colonized or non-colonized, did well on

Table 5.4 The growth of the fungus-associated plants of salal on different sources of nitrogen.

Plants inoculated with	Nitrogen Sources				
	Ammonium	**Glutamine**	**Glutathione**	**BSA**	**N-free**
Unknown 1	74.8bc	84.5abc	10.9f	33.2def	11.7f
	*(16.0)	(17.3)	(1.1)	(5.3)	(2.4)
O. griseum	76.6abc	82.7abc	63.5bc	30.3def	11.6f
	(8.9)	(22.3)	(10.1)	(5.0)	(2.3)
A. strictum	91.4ab	77.2abc	27.4ef	91.3ab	26.6ef
	(17.4)	(14.6)	(7.6)	(10.9)	(5.5)
Unknown 2	84.2abc	92.5ab	26.1f	16.9f	14.3f
	(7.4)	(28.7)	(9.5)	(2.6)	(4.5)
H. ericae	58.7cd	105.8a	22.3f	15.4f	15.8f
	(27.8)	(18.0)	(7.6)	(4.2)	(5.5)
Fungus-free	55.7cde	28.7ef	9.4f	8.9f	10.9fg
	(9.1)	(5.0)	(2.6)	(2.0)	(2.4)

Means (in mg) with the same letter are not significantly different at $\alpha = 0.05$ (by Tukey's Studentized Range Test). n=10. *: Standard Deviation.

Fig. 4.1 The synthesis culture system.

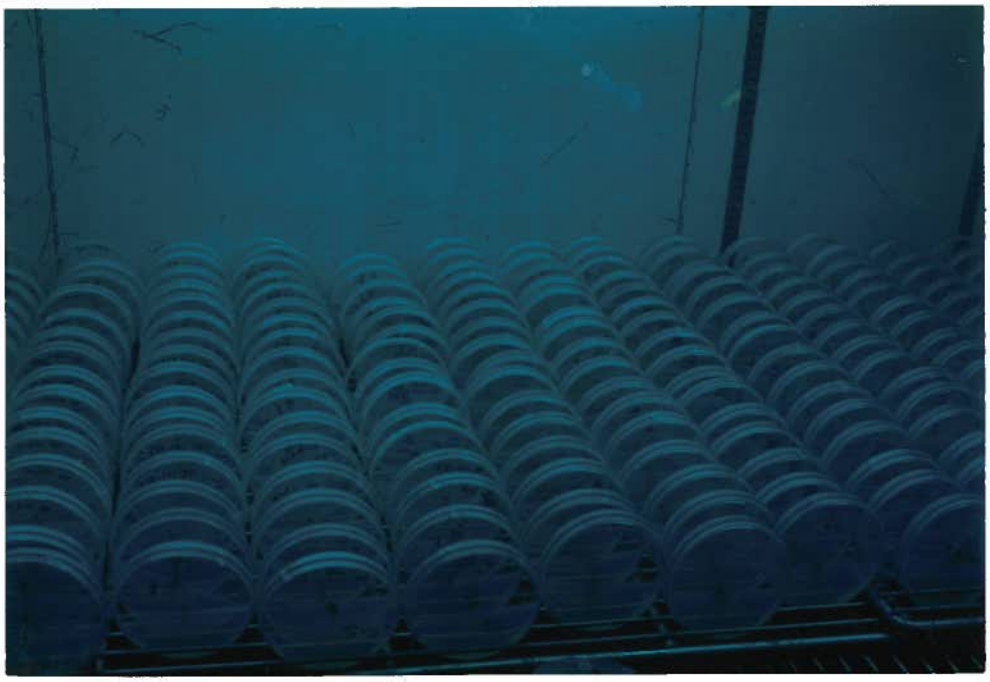

Fig. 4.2 Petri dishes vertically placed in a growth chamber.

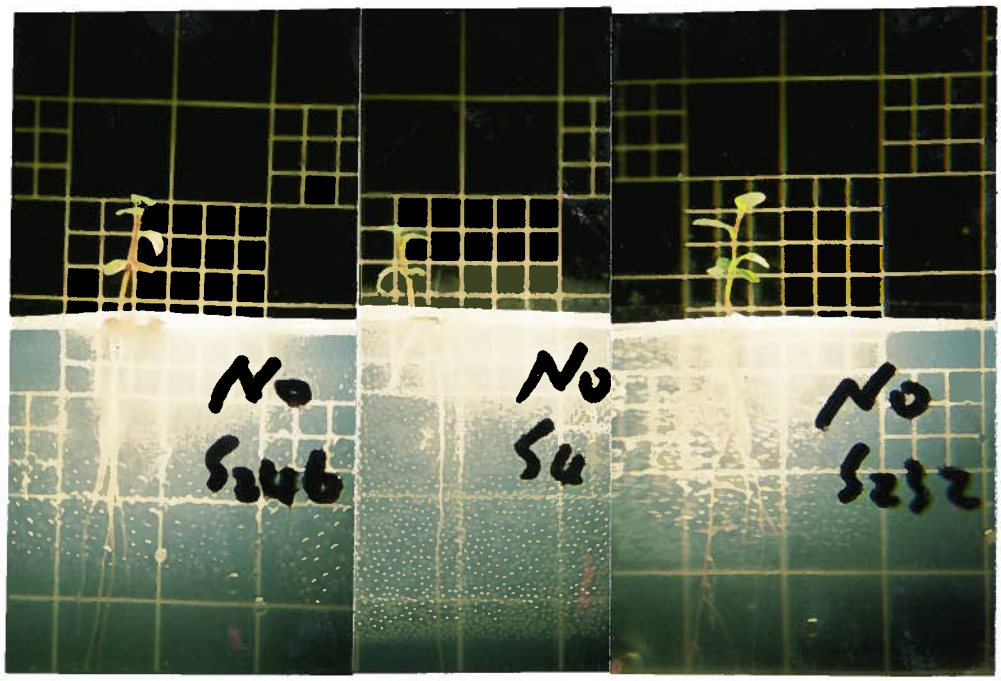

Fig. 5.1 The growth of the mycorrhizal plants on N-free media, showing poor growth.

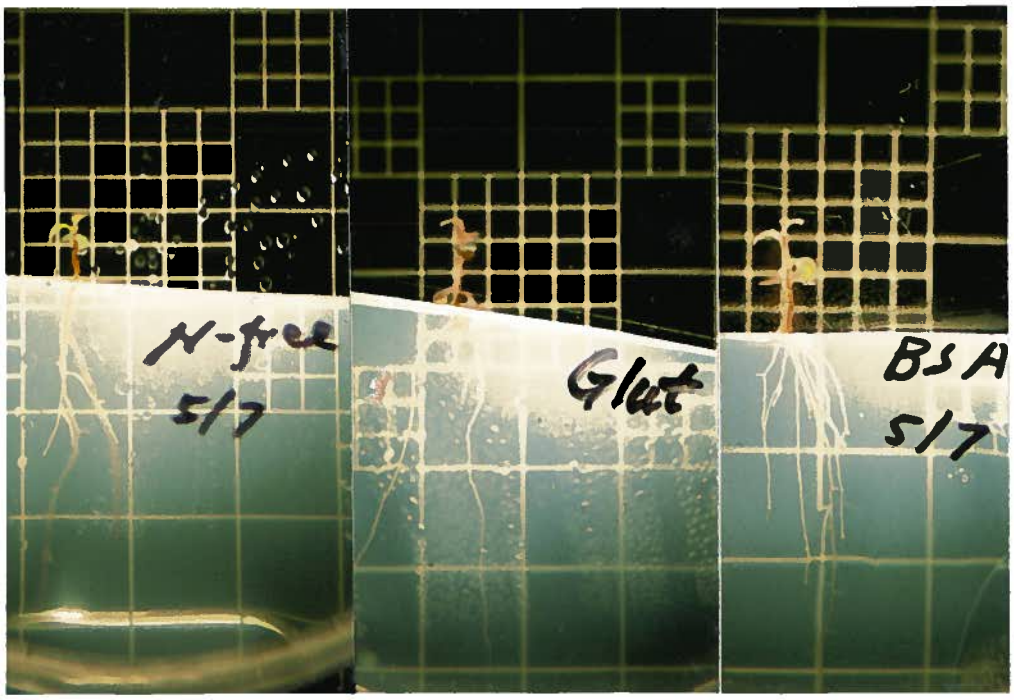

Fig. 5.2 The growth of non-mycorrhizal plants, showing poor growth.

Fig. 5.3 A seedling of salal inoculated with *A. strictum* on BSA.

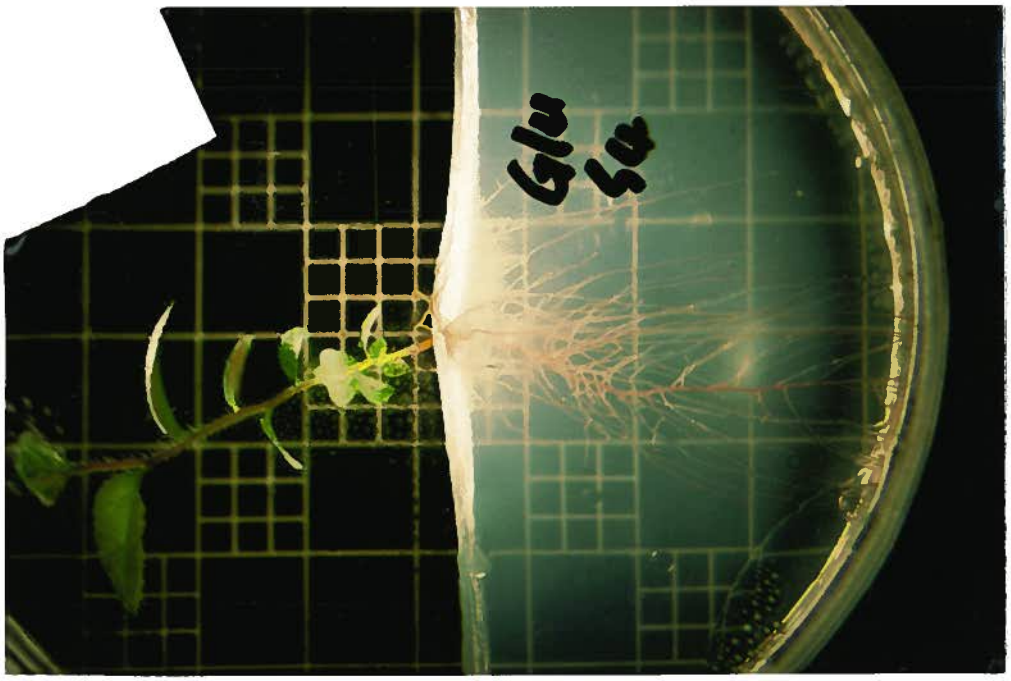

Fig. 5.4 A seedling of salal inoculated with *Oidiodendron griseum* on on glutamine.

ammonium as the sole nitrogen source. The plants inoculated with Unknown 1 produced significant dry weight yield on ammonium and glutamine but inoculation with this fungus did not significantly improve the growth of its colonized plants on glutathione and BSA though the dry weight on BSA was three times higher than that of the plants on nitrogen free. Yields of the plants on ammonium, glutamine, and glutathione inoculated with *Oidiodendron griseum* were not statistically different from each other but were much greater than that of the plants growing without nitrogen. However the plants inoculated with this fungus grew as poorly on BSA as on nitrogen free. No statistically significant difference in the growth of the plants inoculated with *Acremonium strictum* could be detected between the most complicated form of nitrogen, protein, and the two simple nitrogen sources, ammonium and glutamine. The dry weight yields of these plants were much higher than that of the plants on either glutathione or nitrogen free. In addition, colonization by *A. strictum* improved the growth of salal plants on ammonium compared to the non-colonized plants on this mineral nitrogen. Plants inoculated with Unknown 2 and *H. ericae* performed very well on ammonium and glutamine but very poorly on the two complex forms of nitrogen, glutathione and BSA.

Colonization rate

The colonization rates of the plants of salal inoculated with the fungi are shown in Table 5.5. In general, colonization rates were lower on simple nitrogen sources, e.g. ammonium and glutamine, than on the more complicated forms of nitrogen, protein and peptide, or even no nitrogen at all. High colonization rates are not associated with high total dry weight of colonized plants. This pattern can be seen particularly in the case of *A. strictum* in which the plants inoculated with this fungus produced significantly higher dry weight yield on ammonium, glutamine and BSA than on glutathione and N-free but the pattern of cell percent

colonization rate of the plants was the opposite. Unknown 1 and *Oidiodendron griseum* were the most aggressive in terms of colonization rate.

Discussion

The root-associated fungi isolated from salal field roots and the plants of salal in association with these fungi can utilize different forms of organic nitrogen sources. The simple form of organic nitrogen, the amino acid glutamine, was used as readily as ammonium by all the fungi tested in the study and the colonized plants. There was a large difference among the root-associated fungi of salal and their corresponding colonized plants in terms of utilization of the peptide and the protein. Only *Acremonium strictum* used BSA; *Oidiodendron griseum* and Unknown 2 utilized glutathione as sole nitrogen. It has been reported that ericoid mycorrhizal fungus *Hymenoscyphus ericae* utilizes protein and peptide as a sole nitrogen source through the production of an extracellular proteinase and that its production and activity are regulated by pH and appropriate substrates (Leake and Read 1989a, 1990a). Ericoid endophytes isolated from different hosts growing in soils of different pH perform differently in response to pH and the optimal pH for the activity of the proteinase of one endophyte can totally inhibit the activity of the proteinase of the other (Leake and Read 1990a). However, it is not clear that the failure of *Hymenoscyphus ericae* or the colonized plants inoculated with it to use BSA in this study is due to unsatisfactory pH or substrates.

The ericoid endophyte *Hymenoscyphus ericae* preferred certain amino acids as sole nitrogen source and the fungus grew relatively poorly on glycine, an amino acid utilized by most fungi (Pearson and Read 1975). The mycorrhizal plants of *Vaccinium macrocarpon* inoculated by the ericaceous endophyte *Hymenoscyphus ericae* produced much less yield on aspartic acid than on other amino acids (Stribley and Read 1980). This might suggest that failure of a fungus or a

Table 5.5 The cell percent colonization of salal plants inoculated with five different root-associated fungi.

Plants inoculated with	Nitrogen Sources				
	Ammonium	**Glutamine**	**Glutathione**	**BSA**	**N-free**
Unknown 1	18gh *(3)	20g (3)	95a (2)	96a (2)	95a (2)
O. griseum	20gh (6)	19gh (2)	80de (7)	94ab (3)	93abc (5)
A. strictum	11h (2)	11gh (2)	83de (4)	63f (6)	86bcde (2)
Unknown 2	18gh (2)	17gh (2)	82de (5)	84cde (4)	90bcde (4)
H. ericae	17gh (2)	20gh (3)	84cde (5)	81de (6)	79e (7)

Figures with the same letter are not significantly different at $\alpha = 0.05$ (by Tukey's Studentized Range Test). n=10. *: Standard Deviation.

mycorrhizal plant to use a specific peptide does not necessarily mean that the fungus or the plant can not use this peptide. It might be that the organisms are capable of producing the right enzyme to break down the peptide but the products are not suitable for the organism. The peptide glutathione containing three amino acids, glutamine, cysteine, and glycine, was a very poor nitrogen source for the majority of the test fungi and plants colonized by them except for *Oidiodendron griseum*, Unknown 2, and the plants inoculated by *O. griseum* in this study. The failure of the fungi to use this peptide in terms of yield production might be due to unsuitable amino acids it contains. Even though the fungi or the plants associated with them might be able to break down the peptide they can not produce significant yield because the amino acids it contains are not satisfactory to them.

Without mycorrhiza formation, the plants of salal have no access to any of the organic nitrogen sources, from simple amino acid to more complicated forms of nitrogen such as peptide and protein. Therefore, the mycorrhizal fungi of salal are essential to the survival and growth of the salal plants by providing their hosts with access to the organic nitrogen sources.

One of the 12 known ericoid mycorrhizal fungi, *Hymenoscyphus ericae,* has been well studied in terms of organic nitrogen utilization in relation to the heath, *Calluna vulgaris*. This fungus was first isolated from *C. vulgaris* (Pearson and Read 1973a), demonstrated to have the ability to use amino acids (Stribley and Read, 1980), peptides, (Bajwa and Read, 1985) and proteins (Bajwa *et al.* 1985), whether in pure culture or associated with its host plants. It now appears that some root-associated fungi of salal have the same capability. This capability is considered to be very important in the survival and dominance of *Calluna vulgaris* in highly nitrogen-stressed heathland soils. In the soil, amino acids (Abuarghub

and Read 1988) and peptides (Bremner 1965; Biederbecy and Paul 1973) arise from the degradation of proteins released from plant, animal and microbial material in the course of decomposition. The role of root-associated fungi of salal is to provide the hosts with access to these types of organic nitrogen (Read and Bajwa 1985). An extracellular proteinase was isolated from *Hymenoscyphus ericae* (Leake and Read 1989a), which suggests that this fungus is involved in direct nitrogen cycling (Dighton 1991) by deriving available nitrogen from organic resources such as organic matter. Often a thick layer of organic matter overlying the soils is considered to be a sign of slow decomposition, indicating low nutrient availability. This might not hold true for salal plants because their root-associated fungi can take nutrients from organic matter which is usually not available to trees. This might also explain why in the ecosystems with surface litter accumulation (Read 1991) ericaceous plants predominate and the proliferation of ericoid mycorrhizae is favored.

That the well-studied ericoid mycorrhizal fungus, *Hymenoscyphus ericae*, did not have access to the two complicated forms of nitrogen sources, peptide (glutathione) and protein (bovine serum albumin), whether in pure culture or associated with salal plants is in conflict with the observation that the fungus has the ability to utilize peptides and protein (Bajwa and Read 1985; Bajwa, *et al.* 1985). In the study of utilization of protein by the fungus (Bajwa, *et al.* 1985), BSA was used and the medium was adjusted with acid to pH 3, 4, 5 or 6. The highest yields of the mycorrhizal endophyte and the mycorrhizal plants were produced at pH 4 (Bajwa *et al.* 1985, Read and Bajwa 1985) which is the same pH we used. No buffer was used and the pH levels of the growth medium were not checked when harvesting. In this case, the pH of the medium could be dramatically changed, perhaps lowered, during the experiment because of the activities of the fungus growing in the medium. Therefore, the highest yields of

the endophyte and the mycorrhizal plants were not necessarily produced at pH 4. Leake and Read (1989a), using buffered medium, found that the proteinase of *Hymenoscyphus ericae* has a sharp pH optimum at 2.2 with little activity above pH 4.0. To explain the difference between the pH optima of proteinase activity reported by Read and Bajwa (1985) for the ericoid mycorrhizal fungus and those observed in their study, Leake and Read (1989a) proposed that the pH responses obtained by Bajwa and Read actually represented the combined effect of pH on enzyme production and activity because Bajwa and Read used intact mycelium rather than isolated free enzymes in their enzyme assays.

In our study, we did not use buffered medium. The final pH of the medium varied between 3.5 to 3.9 when checked at harvesting. The total dry weights of *Hymenoscyphus ericae* and the dry weights of salal plants colonized by this fungus are higher than the controls but the difference is not statistically significant. The failure to utilize peptide and protein by *Hymenoscyphus ericae* and the mycorrhizal plants of salal colonized by this fungus in our study might be due to the unfavorable pH of the medium. There is a possibility that physiological changes may occur in the fungus during culture maintenance and different levels of compatibility with different hosts are involved. As mentioned before, this fungus was originally isolated from the heath *Calluna vulgaris*. Though it does not appear to show any specificity, it might have low compatability with salal. Therefore salal plants mycorrhizal with this fungus might not be able to utilize those complicated forms of nitrogen used in this study.

We recommend that organic nitrogen sources should be used instead of mineral nitrogen when synthesizing ericoid mycorrhizae in axenic culture. This is because mycorrhizal plants of salal on peptide and protein in this study, though majority of them did not grow well on these two forms of nitrogen, had high cell mycorrhizal colonization rate. Stribley and Read (1976) found a decline in cell

colonization in *Vaccinium* roots in inorganic nutrients containing high levels of ammonium sulfate. In our study, mycorrhizal plants grown on medium containing simple nitrogen, ammonium or glutamine, had lower mycorrhizal colonization rates than ones grown on more complicated nitrogen sources, peptide or protein, or nitrogen free. This indicates that application of mineral nitrogen can lower mycorrhizal association between ericaceous plants and fungi but not necessarily be harmful to either partner since both can grow well on mineral nitrogen as the sole nitrogen source. High colonization rates are associated with complicated organic nitrogen forms or low level of mineral nitrogen and low colonization rate was associated with mineral nitrogen source. Perhaps, low mineral nitrogen availability and high organic matter content signal to ericaceous plants that mycorrhizal association is needed. High mycorrhizal colonization rate did not result in high yields, indicating that plants highly colonized by its mycorrhizal fungi do not necessarily perform well.

It has been frequently reported that application of nitrogenous fertilizers reduces biomass of ericaceous plants such as species of *Calluna, Erica, Kalmia, Vaccinium* (Albrektson *et al.* 1977; Tamm 1991; Persson 1981; Heil and Diemont 1983; Kumi 1984; Aerts and Berendse 1988; Aerts *et al.* 1990). Reductions in salal cover by fertilization have been reported by Stanek *et al.* (1979), Heilman (1961) and Prescott *et al.* (1993a). Salal cover was eliminated by application of high level of N (Prescott *et al.* 1993a). Prescott *et al.* (1993a) found that in order to reduce salal cover, application of nitrogen fertilizers must be repeated. They speculated that increased concentration of available nitrogen through fertilization in forest floors may render salal less competitive or may interfere with ericoid mycorrhizae, resulting in salal reduction. In the present study, evidence shows that colonization rate can be lowered by application of mineral nitrogen. Stalder and Schutz (1957) found that the endophyte of *Calluna vulgaris* was eradicated in

cultivation by the application of fertilizers. Perhaps, salal and its root-associated fungi are dissociated at a critical level of mineral nitrogen in the growth medium and at the same time this level of nitrogen might not provide salal with good growth. Thus salal might suffer nutrient stress and its biomass may be reduced.

The root-associated fungi of salal can live as saprophytes without association with salal, but when conditions are favorable, e.g. low availability of mineral nitrogen, these fungi may become associated with salal again . Therefore, to keep root-associated fungi dissociated from salal, a certain level of mineral nitrogen must be maintained in the soil. This would explain why repeated application of nitrogen fertilizers is needed in order to reduce salal cover.

High level of available nitrogen in the soil, for example 1540 kg N/ha (Prescott *et al.* 1993a), might be toxic and lethal to salal. Field observations are not available on the effects on the plant root-fungal associations. But this can be tested in a controlled culture system by introducing different levels of mineral nitrogen into the media where the fungi grow and checking their biomass to see how those fungi respond to the application of different levels of mineral nitrogen.

Chapter 6

In vitro interactions among the root-associated fungi of *Gaultheria shallon* and the mycorrhizal fungi of *Tsuga heterophylla*

Introduction

Interactions among species are one of the most important factors influencing the organization of communities (Roughgarden and Diamound 1986). The ways in which fungi interact in soil are based on the mycelial nature of most fungi and their similar physical, chemical and biological requirements. These interactions play a very important role in determining species spatial distributions, mycelial organization and function (Rayner and Webber 1984; Wicklow 1986). Three possible interactions among fungi have be identified (Cooke and Rayner 1984; Rayner and Boddy 1988): (1) neutral intermingling of hyphae, (2) deadlock, in which neither individual mycelium enters territory occupied by the other, and (3) invasion of domain, with one mycelium partially or completely replacing the other. The knowledge of interactions among fungi has been an essential tool to understand the structure and functioning of fungal communities (Rayner and Boddy 1988; Christensen 1989). Interactions among fungi have been applied to combat fungal diseases of plants (Cook and Baker 1983; Dubos 1987; Lockwood 1986; Martin *et al.* 1985). For example, a saprophytic fungus, *Hypholoma* sp., resulted in complete or partial exclusion of *Armillaria luteobubalina* Watling & Kile, the most pathogenic *Armillaria* in Australia, from stumps in a field *Armillaria* biological control experiment (Pearce and Malajczuk 1990). Mycorrhizal fungi offer protection to their host plants against root diseases by inhibiting the growth of the disease fungi. Though in some cases antibiosis by

some mycorrhizal fungi is due to acidification of the growth media (Rasanayagam and Jeffries 1992), many mycorrhizal fungi have been reported to produce antibiotics, including antifungal compounds, in pure culture (Marx 1973) or in mycorrhizal association (Krywolap *et al.* 1964; Marx 1969; Garrido *et al.* 1982) that inhibit the growth of competing fungi. The ericoid mycorrhizal fungus *Oidiodendron griseum* has been reported to be antagonistic to the fungi which were isolated from its original peat habitat (Dickinson and Boardman 1970). *Acremonium strictum,* which is a root-associated (pseudomycorrhizal) fungus of salal (see Chapter 3) strongly inhibited the growth of *Mycogone perniciosa* Magn (Gandy 1979). The ectomycorrhizal fungus, *Pisolithus tinctorius* (Mich. ex Pers.) Coker and Couch, is strongly antagonistic to some other fungi (Kope and Fortin 1990). Greenhouse tests revealed that a species of *Lactarius*, which forms ectomycorrhizae with *Tilia americana* L., was strongly inhibitory to some fungi which cause damping-off of pine seedlings (Park 1970). In both cases, an antifungal compound was produced. The successful colonization of roots by mycorrhizal fungi, and the protection of plants from root-invading pathogens by mycorrhizal fungi have been attributed to such antagonistic effect (Zak 1964; Marx 1969; Duchesne *et al.* 1989).

Mutualistic root-fungus associations are very common in terrestrial ecosystems. Root-associated fungi, particularly mycorrhizal fungi, are important for the survival and growth of plants by providing their hosts with enhanced nutrient uptake, in some cases access to organic nutrients, protection from diseases and detoxification of heavy metals (Kucey and Leggett 1989; Haselwandter and Read 1982; Wilcox and Wang 1987; Read 1991). Good performance of a root-associated fungus (or fungi) may improve the competitiveness of its host. Therefore root-associated fungi might influence composition and succession of plant communities (Janos 1980; Trappe 1981; Allen 1984) by regulating

competition between plants since competition between plants has been considered as the main mechanism determining the course of succession (Connell and Slatyer 1977). Any factors influencing the performance of a root-associated fungus, including interactions among root-associated fungi, may affect indirectly the survival and growth of its associated plants by changing the competitiveness of the plants.

Gautheria shallon (Ericaceae) grows very well on the CH clearcuts. Western hemlock forms ectomycorrhizae and performs very poorly on the same sites. I speculated that the root-associated fungi of salal might negatively influence the growth of the ectomycorrhizal fungi of western hemlock and therefore indirectly inhibit the growth of western hemlock. The objectives of this study were to examine and characterize the *in vitro* interactions among the root-associated fungi of salal and the ectomycorrhizal fungi of western hemlock to provide insight on how these interactions may influence the growth of the two plants in the field.

Materials and Methods

Fungal isolates

Two groups of fungi, ectomycorrhizal fungi of western hemlock and root-associated fungi of salal, used in this study are listed in Table 6.8. All root-associated fungi of salal were isolated from field salal roots and confirmed to form root-fungus associations with salal in axenic culture (see Chapter 3). The isolates of root-associated fungi of salal used in this study are S4 of *Oidiodendron griseum*, S232 of *Acremonium strictum*, S9 of Unknown 1 and S246 of Unknown 2. Three ectomycorrhizal fungi used in this study are mycorrhizal with western hemlock (Kropp and Trappe 1982) and kindly provided by Jane Smith, U.S. Department of Agriculture, Corvallis, Oregon. The isolates used are Culture # Pt 210 of *Pisolithus tinctorius*, Herbarium #Trappe 11313 of *Suillus lakei* (Murr.)

Smith & Thiers, and Herbarium #Trappe 11569 of *Rhizopogon semireticulatus* Smith. These ectomycorrhizal fungi were chosen because they grow with a reasonable rate on artificial media. All isolates were maintained in Petri dishes on modified Melin Norkrans agar (MMN).

Dual cultures

Interactions were examined using dual cultures on two growth media, normal MMN and buffered MMN containing 20 mM 2-[N-morpholino]ethanesulphonic acid (MES). The pH of both media was adjusted to 6 using 1 M-NaOH before autoclaving. Each root-associated fungus of salal was grown opposite each of the ectomycorrhizal fungi of western hemlock and each fungus was grown opposite itself on both normal and buffered MMN. The inoculum was cut from the edge of a colony of the fungal isolates and placed at 3 cm away from each other. All treatments were replicated three times. Cultures were incubated at about 25^0C in the dark and observed periodically for up to 90 days.

Observations and measurements

Two measurements of colony expansion in Petri dish cultures were made periodically after inoculation of the Petri dishes until the hyphae of the paired fungi met or elongation of the hyphae towards the opposing colony stopped. These measurements were mycelial growth toward the other colony and mycelial growth away from the other colony, and were used to determine colony expansion. For each species, colony expansion was statistically compared to its self-pairing (the control) to see if the growth of the fungus, when paired with other fungi, was inhibited. Appearance of space between the paired colonies, mycelial intermingling and overlapping, and colony restriction were recorded.

Table 6.1 Measurements of mycelial expansion of *Pisolithus tinctorius* in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
16.0 (*1.0)a	14.0 (1.0)a	15.3 (2.1)a	17.7 (0.58)a	*Pisolithus tinctorius*
16.3 (0.58)a	10.7 (0.58)b	17.0 (1.0)a	18.7 (1.2)a	*Acremonium strictum*
15.3 (1.5)a	14.3 (1.2)a	13.7 (0.58)a	15.0 (1.0)b	*Oidiodendron griseum*
15.0 (1.0)a	15.0 (1.0)a	15.7 (0.58)a	15.3 (0.58)ab	Unknown 1
15.0 (1.0)a	13.7 (0.58)a	14.7 (2.1)a	16.3 (1.2)a	Unknown 2

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.
*: Standard Deviation.

Table 6.2 Measurements of mycelial expansion of *Rhizopogon semireticulatus* in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
12.7 (*0.58)a	9.3 (0.58)a	14.7 (0.58)a	14.7 (0.58)a	*Rhizopogon semireticulatus*
7.3 (0.58)c	5.7 (0.58)c	9.0 (1.0)b	9.0 (1.0)b	*Acremonium strictum*
7.3 (0.58)c	7.3 (0.58)b	7.3 (0.58)b	8.0 (1.0)b	*Oidiodendron griseum*
10.7 (0.58)b	9.7 (0.58)a	13.3 (0.58)a	14.0 (1.0)a	Unknown 1
11.7 (0.58)a	10.7 (0.58)a	8.7 (0.58)b	13.7 (0.58)a	Unknown 2

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.
*: Standard Deviation.

Table 6.3 Measurements of mycelial expansion of *Suillus lakei* in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
20.3 (*0.58)a	20.3 (0.58)a	21.0 (1.0)a	21.3 (1.2)a	*Suillus lakei*
16.3 (2.3)b	8.3 (0.58)c	11.3 (0.58)b	13.7 (0.58)b	*Acremonium strictum*
13.0 (1.7)b	13.7 (1.5)b	10.3 (0.58)b	11.3 (0.58)b	*Oidiodendron griseum*
15.0 (1.0)b	15.3 (0.58)b	11.3 (0.58)b	11.7 (0.58)b	Unknown 1
20.7 (0.58)a	15.7 (1.2)b	10.3 (0.58)b	14.0 (1.0)b	Unknown 2

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.
*: Standard Deviation.

Table 6.4 Measurements of mycelial expansion of *Acremonium strictum* in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
21.3 (*0.58)a	11.3 (1.2)b	10.3 (0.58)b	16.3 (0.58)a	*Acremonium strictum*
22.3 (0.58)a	18.7 (0.58)a	10.3 (0.58)b	16.7 (0.58)a	*Pisolithus tinctorius*
22.3 (2.5)a	22.0 (2.7)a	13.7 (1.2)a	16.0 (2.0)a	*Rhizopogon semireticulatus*
21.7 (0.58)a	18.3 (0.58)a	14.3 (1.0)a	17.0 (1.0)a	*Suillus lakei*

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.
*: Standard Deviation.

Table 6.5 Measurements of mycelial expansion of *Oidiodendron griseum* in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth toward interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
13.7 (*0.58)a	12.3 (0.58)a	11.0 (1.0)b	15.3 (0.58)a	*Oidiodendron griseum*
12.7 (0.58)a	12.3 (0.58)a	10.3 (0.58)b	16.7 (0.58)a	*Pisolithus tinctorius*
12.7 (0.58)a	12.7 (0.58)a	13.7 (1.2)a	13.7 (1.2)ab	*Rhizopogon semireticulatus*
13.7 (0.58)a	13.3 (0.58)a	13.7 (0.58)a	14.7 (0.58)ab	*Suillus lakei*

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.

*: Standard Deviation.

Table 6.6 Measurements of mycelial expansion of Unknown 1 in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth towards interacting colony (mm)	Mycelial growth towards interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
11.7 (*0.58)b	11.7 (0.58)b	11.7 (0.58)b	12.6 (0.58)b	Unknown 1
11.3 (0.58)b	11.3 (0.58)b	10.3 (0.58)b	11.7 (0.58)b	*Pisolithus tinctorius*
14.3 (0.58)a	14.3 (0.58)a	13.3 (0.58)a	15.3 (0.58)a	*Rhizopogon semireticulatus*
10.7 (0.58)b	10.3 (0.58)b	11.3 (0.58)b	11.7 (0.58)b	*Suillus lakei*

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.
*: Standard Deviation.

Table 6.7 Measurements of mycelial expansion of Unknown 2 in dual culture.

on MES		on MMN		
Mycelial growth away from interacting colony (mm)	Mycelial growth towards interacting colony (mm)	Mycelial growth towards interacting colony (mm)	Mycelial growth away from interacting colony (mm)	Opposing fungus
12.3 (*1.5)a	11.7 (1.2)a	11.3 (2.5)a	12.3 (0.58)a	Unknown 2
10.0 (1.7)a	11.0 (1.7)a	11.0 (1.0)a	11.0 (1.0)a	*Pisolithus tinctorius*
13.7 (0.58)a	14.0 (1.0)a	13.7 (0.58)a	13.7 (0.58)a	*Rhizopogon semireticulatus*
11.7 (0.58)a	11.3 (0.58)a	11.7 (0.58)a	11.7 (0.58)a	*Suillus lakei*

Means with the same letter in column are not significantly different at α=0.05 (by Tukey's Studentized Range Test). n=3.

*: Standard Deviation.

Results

The measurements (the mean of three replicates) of the expansion of both fungal colonies in dual culture were presented in Tables 6.1- 6.7. The growth of *Pisolithus tinctorius* (Table 6.1), when paired with each of the root-associated fungi of salal, was not significantly different from that of its self-pairing except that the mycelial growth towards the colony of *Acremonium strictum* on MES and away from the colony of *O. griseum* was significantly reduced. The growth of an entire colony of *Rhizopogon semireticulatus* (Table 6.2) was decreased in the presence of *Acremonium strictum* and *Oidiodendron griseum* on both media, and its mycelial growth towards the colony of Unknown 2 on MMN and away from Unknown 1 on MES was restricted. Growth reduction of an entire colony of *Suillus lakei* was dramatic when paired with *A. strictum*, *O. griseum* and Unknown 1 on both MES and MMN, and Unknown 2 on MMN (Table 6.3). On MES, its mycelial growth towards the colony of Unknown 2 was limited. The growth of the root-associated fungi of salal is quite different from that of the ectomycorrhizal fungi in dual culture. Mycelial growth of *Acremonium strictum* towards itself was tremendously reduced on both MES and MMN. In contrast, its mycelial growth towards the colony of *Rhizopogon semireticulatus* and *Suillus lakei* on both media and *Pisolithus tinctorius* on MES was favored (Table 6.4). So were the mycelial growth of *O. griseum* towards the colony of *Rh. semireticulatus* and *S. lakei* on MMN (Table 6.5) and the mycelial growth of Unknown 1 towards *Rh. semireticulatus* on both media (Table 6.6). However, the presence of ectomycorrhizal fungi showed no significant influence on the growth of Unknown 2 on both types of media (Table 6.7).

Interactions between paired fungi are summarized in Table 6.8. All the interactions among the paired fungi in this study fall into three categories, intermingled, inhibited and deadlocked. Two of the categories, intermingling and

Table 6.8 Observations on the colony interactions of the fungi in dual culture 88 days after inoculation.

One of the two interacting fungi in a pair	Medium	Interaction	One of the two interacting fungi in a pair
Acremonium strictum	MES	Deadlock	*Acremonium strictum*
	MMN	Deadlock	
Acremonium strictum	MES	*Pisolithus tinctorius* was inhibited.	*Pisolithus tinctorius*
	MMN	Deadlock	
Acremonium strictum	MES	*Rh. semireticulatus* was inhibited.	*Rhizopogon semireticulatus*
	MMN	*Rh. semireticulatus* was inhibited.	
Acremonium strictum	MES	*Suillus lakei* was inhibited.	*Suillus lakei*
	MMN	*Suillus lakei* was inhibited.	
Oidiodendron griseum	MES	Deadlock	*Oidiodendron griseum*
	MMN	Deadlock	
Oidiodendron griseum	MES	Intermingle	*Pisolithus tinctorius*
	MMN	Intermingle	
Oidiodendron griseum	MES	*Rh. semireticulatus* was inhibited.	*Rhizopogon semireticulatus*
	MMN	*Rh. semireticulatus* was inhibited.	
Oidiodendron griseum	MES	*Suillus lakei* was inhibited.	*Suillus lakei*
	MMN	*Suillus lakei* was inhibited.	
Unknown 1	MES	Deadlock	Unknown 1
	MMN	Deadlock	
Unknown 1	MES	Deadlock	*Pisolithus tinctorius*
	MMN	Deadlock	
Unknown 1	MES	Deadlock	*Rhizopogon semireticulatus*
	MMN	Deadlock	
Unknown 1	MES	*Suillus lakei* was inhibited.	*Suillus lakei*
	MMN	*Suillus lakei* was inhibited.	

Table 6.8 Continued.

One of the two interacting fungi in a pair	Medium	Interaction	One of the two interacting fungi in a pair
Unknown 2	MES	Deadlock	Unknown 2
	MMN	Deadlock	
Unknown 2	MES	Deadlock	*Pisolithus tinctorius*
	MMN	Deadlock	
Unknown 2	MES	Deadlock	*Rhizopogon semireticulatus*
	MMN	*Rh. semireticulatus* was inhibited.	
Unknown 2	MES	*Suillus lakei* was inhibited.	*Suillus lakei*
	MMN	*Suillus lakei was inhibited.*	
Pisolithus tinctorius	MES	Intermingle	*Pisolithus tinctorius*
	MMN	Deadlock	
Rhizopogon semireticulatus	MES	Deadlock	*Rhizopogon semireticulatus*
	MMN	Deadlock	
Suillus lakei	MES	Intermingle	*Suillus lakei*
	MMN	Intermingle	

deadlock, were described by Cooke and Rayner (1984) and Rayner and Boddy (1988), and were applied here. Inhibition was used to represent the third category in this study instead of "Replacement" a category title in the spectrum of interactions used by those workers. This is because the definition of replacement described by those workers as "one mycelium grows into the other and begins to consume and replace it" does not fit the case in this study where one mycelium does not grow into the other, does not replace it, and instead, restricts the growth of the other. These three categories are:

(1) Neutral intermingling-one or both colonies grow into the other with no apparent adverse effect on the mycelium of either (Fig. 6.1).

(2) Inhibition- the growth of one mycelium is reduced by the presence of the other. The reduction of the inhibited colony might occur on the mycelial growth towards the interacting colony (Fig. 6.2) or the size of the whole inhibited colony might be reduced compared to its control, self-pairing (Fig. 6.3).

(3) Deadlock-the mycelial growth towards interacting colony is restricted and neither mycelium can enter territory occupied by the other (Fig. 6.4).

Generally speaking, growth of the majority of the fungi tested in this study was in some way affected by the presence of every other fungus in paired cultures on both MMN and MES. Some patterns of the interactions among the fungi can however be seen. For example, the measurements of the pair of *Acremonium strictum* against itself on MES (Table 6.4) showed that the mycelial growth away from interacting colony is 21.3 mm on MES and 16.3 mm on MMN, and the mycelial growth of both colonies towards each other is reduced (11.3 mm on MES and 10.3 mm on MMN). This is the pattern of deadlock reflected in Table 6.8 for the same pairing.

Growth responses of the majority of the fungi on the two media are similar except that the growth of *Acremonium strictum* was favored by MES because its

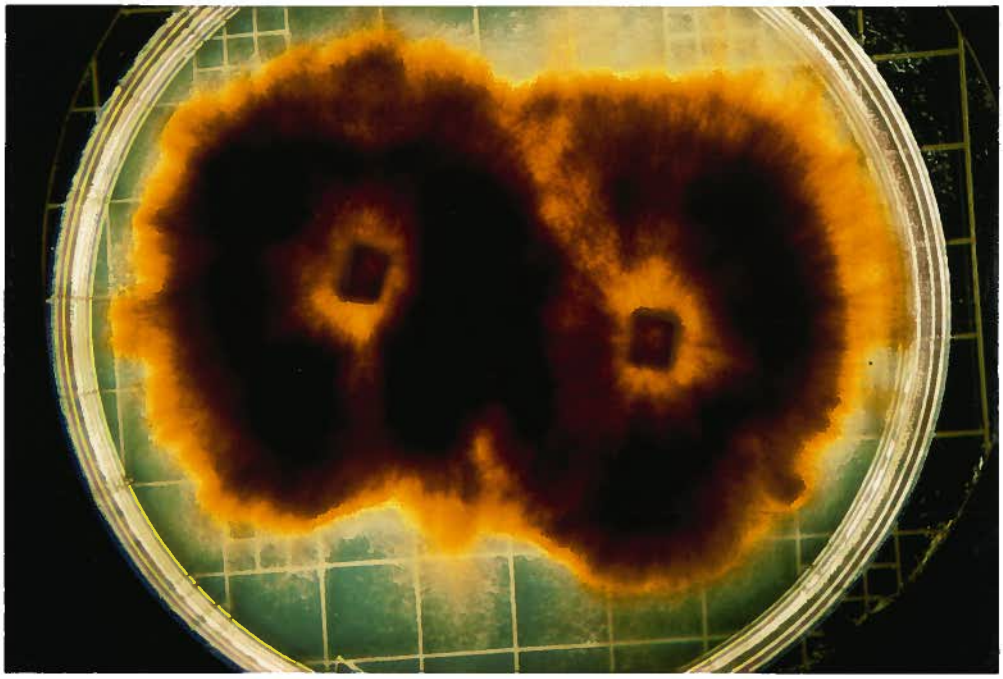

Fig. 6.1 Two colonies of *Suillus lakei*, intermingling with each other.

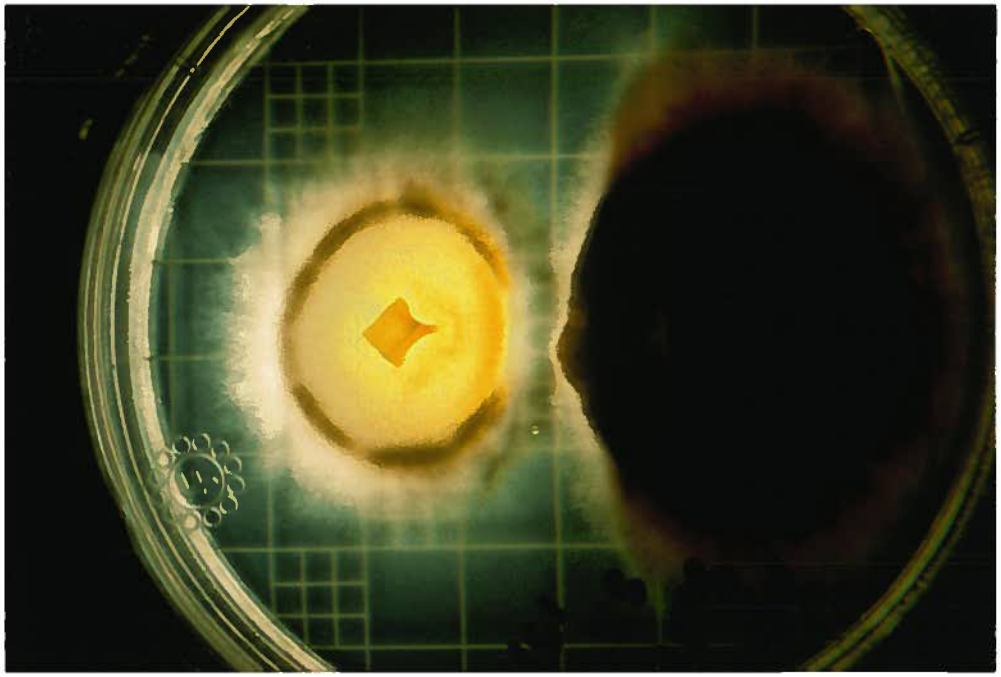

Fig. 6.2 Inhibition of colony expansion of *Suillus lake* (black) by the fungus Unknown 2 on MMN.

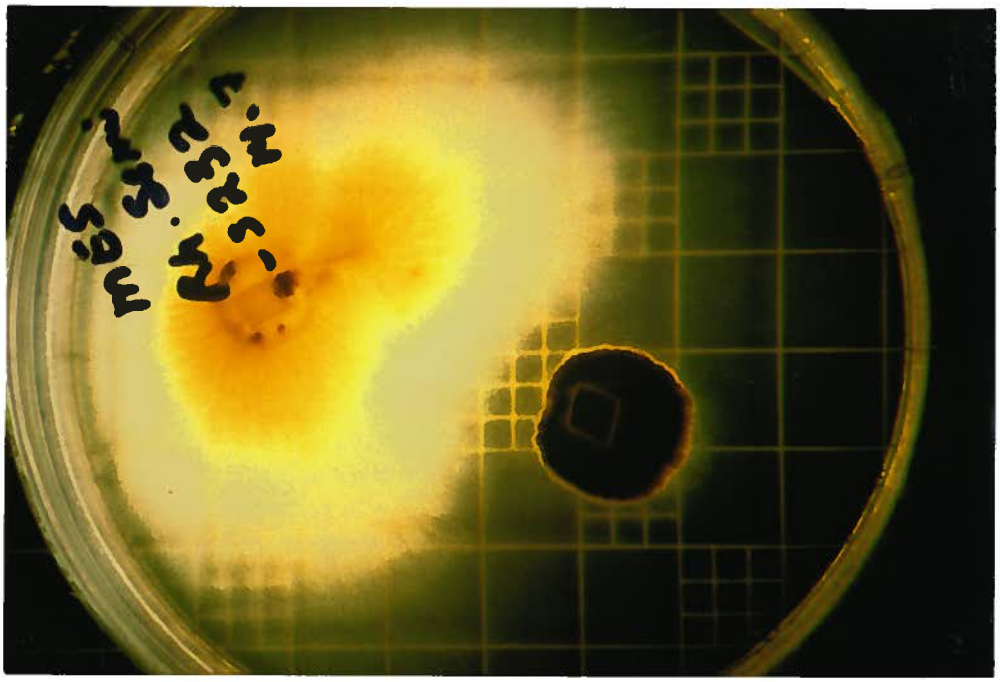

Fig. 6.3 Inhibition of colony growth of *Rhizopogon semireticulatu* (black) by *Acremonium strictum* on MES.

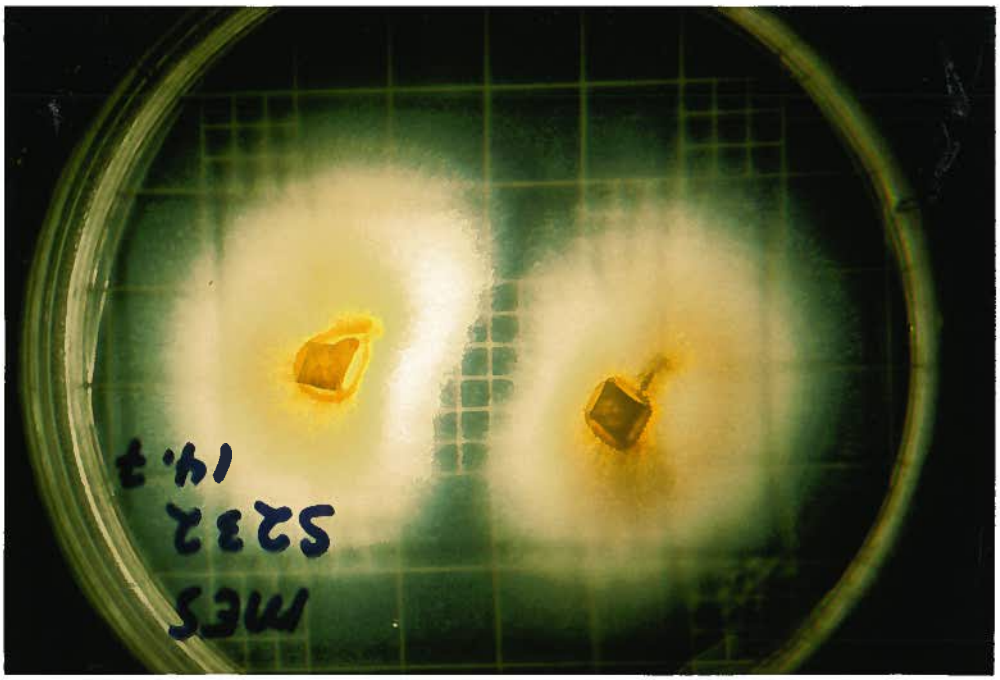

Fig. 6.4 Two colonies of *Acremonium strictum* on MMN, showing deadlock interaction between the two colonies.

colony diameter was always greater (not statistically) on MES than on MMN (Table 6.4). Responses of some of the pairings on the two media differed in terms of interaction outcome. *Pisolithus tinctorius* was inhibited on MES in the pairing with *Acremonium strictum* while the interaction between the two was deadlock on MMN. Different interaction outcomes occurred in the self- pairing of *P. tinctorius*, intermingle on MES and deadlock on MMN (Table 6.8). *Suillus lakei* (Table 6.3) was inhibited by *A. strictum* on both media but the inhibition was more severe on MES (not statistically) .

Among all the pairings of the fungi on the two media (Table 6.8), deadlock, seen in 50% of all the matches, was the most commonly recorded outcome in inter- and intraspecific interactions. Inhibition, seen in 37%, was the second and intermingle, 13%, the least. Intraspecific matches revealed two types of outcomes, deadlock and intermingle. Seventy percent (11 out of 14) of the intraspecific pairings resulted in deadlocked colonies. All three interaction outcomes, intermingle, deadlock and inhibition, occurred in the 24 interspecific pairings and among them, inhibition was the most common outcome (58%, 14 out of 24 pairings). Deadlock was the result in 33% of all interspecific matches. In the interspecific matches, all three ectomycorrhizal fungi were inhibited by root-associated fungi of salal, but none of the four root-associated fungi was inhibited by any of the ectomycorrhizal fungi and in fact in some cases the growth of root-associated fungi of salal was enhanced (Table 6.8). *Acremonium strictum* was the most aggressive of the root-associated fungi. It inhibited all three ectomycorrhizal fungi on MES and all but *Pisolithus tinctorius* on MMN. *Oidiodendron griseum* was the second in terms of aggressiveness to the ectomycorrhizal fungi. It inhibited *S. lakei* and *Rh. semireticulatus*, and intermingled with *P. tinctorius* on both media. *S. lakei* and *Rh. semireticulatus* were kept in check by 3 of the 4 root-associated fungi of salal on both media (Table 6.8).

Discussion

The majority of the interactions between two colonies of the same fungus resulted in some degree of self-inhibition or deadlock in spite of the fact that both mycelia in intraspecific interactions were started as hyphal tips from the same colony. Self-inhibition or intraspecific competition has been reported commonly among fungi. Shearer and Zare-Maivan (1988) reported self-inhibition in 88% of their intraspecific trials. Dickinson and Boardman (1970) found that the majority of their fungi isolated from peat were self-inhibitory, including *O. griseum*. Among plants and animals, intraspecific competition not only exists but also is more pronounced than is interspecific competition (Connell 1983).

Recent studies have revealed that soil pH is a decisive factor in determining the functional effectiveness of fungi (Danielson and Visser 1989). In this study, only the growth of *Acremonium strictum* was favored by MES. This might be due to the pH difference between the two media since MES was buffered at pH 6 and the pH of the unbuffered MMN might be lowered due to the activity of the fungi growing on it. Rasanayagam and Jeffries recently (1992) found that in some cases, acidification of growth media is the cause of inhibition. In this study, responses of some of the pairings of the fungi on the two media slightly differed in terms of interaction outcome. In the pairing of *Acremonium strictum* against *P. tinctorius*, *Pisolithus tinctorius* was inhibited on MES while the interaction between the two is deadlock on MMN. Kope *et al.* (1991) have reported the production of *p*-hydroxybenzoylformic acid and (*R*)-(-)-*p*-hydroxymandelic acid by *Pisolithus tinctorius*. These compounds have been shown to produce inhibitory effects in buffered media at pH 4.3 (Kope and Fortin 1990). The explanation of the different responses of *P. tinctorius* on the two media in this study is that the compounds released by *P. tinctorius* might not be effective on the MES buffered media because the pH was too high (pH 6) but function well on MMN whose pH

might be lowered due to fungal activity.

Different interaction outcomes occurred in the self-pairing of *P. tinctorius*, intermingle on MES and deadlock on MMN. *Suillus lakei* was inhibited by *A. strictum* on both media but the inhibition was severe on MES. This seems to indicate that acidification of the media influenced the outcome of interactions among some of the fungi. The effects of pH in this study points out that the study of fungal interactions is complicated and that there are many other physical and chemical factors that might contribute, including incubation temperature.

All the ectomycorrhizal fungi were inhibited to some degree by one or more of the root-associated fungi of salal and none of the root-associated fungi of salal was inhibited by any of the ectomycorrhizal fungi. Inhibition of one fungus by the other might result from competition for nutrients or production of antagonistic compounds (Rasanayagam and Jeffries 1992). In this study, inhibition of one fungus by the other between the two interacting fungi does not seem to be due to competition for nutrients because the growth of the inhibited fungus was not reduced when it was self-paired. This might indicate that the inhibition is due to production of antagonistic compounds. Reports have shown that both *Acremonium strictum* and *Oidiodendron griseum* are inhibitory to other fungi (Dickinson and Boardman 1970; Gandy 1979). In both cases, an antagonistic compound was considered to be involved. Gandy (1979) isolated an antifungal antibiotic from *A. strictum*. Based on the results of this study, it is clear that the fungi isolated from field salal roots have the capability to inhibit the growth of one or more of the ectomycorrhizal fungi of western hemlock.

In attempting to explain the poor performance of mycorrhizal associations of non-ericaceous plants on the heathlands in Europe, Handley (1963) first put forward that *Calluna vulgaris* could produce a factor toxic to the mycorrhizal fungi of trees, and isolated the factor. Robinson (1972) confirmed that a fungitoxic

factor is produced by the living root system of the heather though its identity is still not known. It was concluded that the fungitoxin secreted by the roots of *C. vulgaris* plays a crucial role in maintaining the dominance of the heather even though it is not known whether the fungitoxin is secreted by the mycorrhizal roots or by the fungal symbiont (Handley 1963). This study has shown that fungal symbionts can produce fungitoxin. This capacity of root-associated fungi to benefit salal through production of fungitoxin active against the mycorrhizal fungi of western hemlock might be of great ecological importance. These root-associated fungi of salal, by producing fungitoxin and providing salal with access to organic nutrients or other benefits, regulate the competition between the two species of plants and result in the dominance of salal on the CH sites. The root-associated fungi can improve the competitiveness of their host plants by directly interfering with the growth of mycorrhizal fungi associated with other plants.

The roots of western hemlock and salal intermingle in the litter layer of the soils of CH (Messier 1991). This creates a situation where the root-associated fungi of these two plant species can interact in the field in ways revealed in this study. However, it must be stressed that the results of this study are only suggestive of how these fungi might interact in the field. This is because field conditions such as physical, chemical and biological factors are quite different from those of the lab and unpredictable. This field environment for fungal growth can influence the outcome of the interactions between the fungi and it might be considered that this study is of limited value. Nevertheless, this study can provide a basis from which further detailed examinations may be made of field interaction patterns of the fungi associated with their hosts.

Chapter 7

General conclusion and discussion

Introduction

The two types of forests, CH and HA, between Port McNeill and Port Hardy in northern Vancouver Island are quite different in terms of dominant tree and understorey species. The HA is dominated by western hemlock and amabilis fir with insignificant understorey growth of salal and the CH by western red cedar and western hemlock with understorey densely covered by salal. After harvesting, salal rapidly recovers and dominates the CH clearcuts by sprouting from its old rhizomes. Plantations of western hemlock on HA clearcuts and western red cedar on CH clearcuts grow reasonably well but the plantations of western hemlock exhibit serious growth problem on CH clearcuts. This difference in productivity unveiled a hidden difference between the CH and HA. That is that nutrient availability is lower on CH than on HA (Keenan 1993; Prescott *et al.* 1993c Weetman *et al.* 1989a,b). As mentioned in Chapter 1, low nutrient availability is the cause of the growth problem of western hemlock seedlings on the CH. Salal intensifies the nutrient-stressed situation by outcompeting planted trees for nutrients and immobilizing scarcely available nutrients in its living tissue (Messier 1991; Weetman *et al.* 1989a,b). Therefore, the growth problem on the CH is actually attributed to two factors: low nutrient availability and salal competition.

The cause of the growth problem on CH

There are three hypotheses intended to explain the difference in nutrient availability between the two sites: disturbance hypothesis (Lewis 1982; de Montigny 1992), salal competition hypothesis (Messier 1991), and litter quality

hypothesis (Keenan 1993).

The disturbance hypothesis (de Montigny 1992) recognizes two major differences between the CH and HA sites. One is that the HA forests are on drier ridgetops and the CH forests are on lower, wetter situations. This difference alone can result in difference in nutrient availability because it has profound impact on soil physical and chemical properties regulating decomposition rate of organic matter deposited on both sites. The second is that HA has repeatedly experienced windthrow disturbance because of its higher topographic position, while CH has existed for several thousand years without this natural disturbance due to its lower position. Windthrow changes soil physical properties such as aeration conditions by mixing mineral soil and organic matter, which benefits decomposers (Armson 1977; Ugolini *et al.* 1990). Without disturbance, the CH soils are compacted with the presence of hardpans which causes annual periods of anaerobic soil conditions and large amount of nutrient poor woody humus (de Montigny 1992). This leads to lower decomposition activity, and consequently results in lower nutrient availability. Thus trees grow slowly and less densely, resulting in the understorey dominated by salal because the openness of the CH permits light to penetrate its canopy and stimulates salal growth. The presence of salal further lowers nutrient availability of the sites by producing tannins, lipids and phenolic acids which decrease decomposition.

The second hypothesis proposed that immobilizing available nutrients in large amount of living tissue produced annually by salal is the cause of low nutrient availability on CH sites (Messier 1991). Salal recovers rapidly following disturbance on CH sites by resprouting from its old rhizomes. In just a few years, it can reach maximum cover and completely dominate a site (Messier 1991). The plants produce tremendous amount of both belowground and aboveground biomass in eight years after harvesting of the sites (Messier and Kimmins 1991). This

amount of biomass ties up nutrients absorbed from the soil with a scarce source of nutrients. This hypothesis considers the decomposition rate to be low on both CH and HA because of the unfavorable weather, such as low temperature, and poor soil conditions, for example low pH. The organic material input by salal contains chemicals such as phenolic acids and is resistant to decomposition. So salal, by immobilizing nutrients in its biomass, lowers the site fertility and consequently reduces productivity on the CH.

The litter quality hypothesis does not consider that windthrow disturbance plays an important role in nutrient availability in this area. Keenan (1993) concluded "there was little consistent difference between the two forest types that either (i) indicated that differences in topographic position and soil drainage could explain the differences nutrient availability and productivity, or (ii) indicated that there had been major differences in anti-horizonation processes such as disturbance due to windthrow" (a conclusion drawn by de Montigny 1992). His study showed (i) that there is a significant accumulation of organic matter on and in both CH and HA, (ii) that the two types of forests occur in similar topographic situations, (iii) that there are no hardpans within the B horizon as described by de Montigny (1992) to suggest that there is greater waterlogging in the CH type, (iv) that a standard substrate, lodgepole pine needles, decomposed at almost the same rate in each type, indicating that the decomposition rate is similar in both sites (Keenan 1993). The litter quality hypothesis focuses on litter quality of the dominant species of canopy and understorey in which litter quality is defined as nitrogen availability to decomposers (Keenan 1993). Keenan (1993) pointed out that the lower N availability is a product of the lower rate at which N is mineralized in the forest floor of the CH type and that the lower mineralization rate is due to poor quality of foliar litter in the species dominating the CH forests because of higher rates of resorption of nutrients. He found that "the rate of nutrient resorption at the

time of leaf senescence by western red cedar was higher than that of hemlock on the CH type, and the rate of resorption by hemlock in the CH type was greater than that of either hemlock or amabilis fir in the HA type". So the overall litter quality is much lower on the CH than on HA. It is this lower litter quality that decreases the decomposition rate and consequently results in lower nutrient availability and lower productivity on the CH sites (Keenan 1993).

What the three hypotheses have in common is that lower decomposition rate is the cause of the lower nutrient availability particularly nitrogen on CH sites, but they differ in mechanisms by which the lower nutrient availability is brought about. The disturbance hypothesis may explain how disturbance can change the soil physical properties and how this could benefit the growth of western hemlock on the HA sites. But it has no explanation for the fact that hemlock is a dominant species in old growth of CH forests, and that salal and cedar grow well on CH clearcuts. If the CH has lower nutrient availability due to lack of disturbance, why do these three species do well in the old growth, and cedar and salal grow well on the clearcut of this type of forests? With the salal competition hypothesis, we can understand that in the old growth the influence from salal is in check because light is the major limiting factor for its growth. When the canopy is cleared, the limiting factor is lifted, and salal grows well, produces large amount of biomass. Therefore, plantations of western hemlock face severe competition from salal. However, if the CH sites are nutrient-deficient because salal biomass immobilizes scarcely available nutrient and the lower nutrient availability affects the growth of hemlock, why are salal itself and cedar immune to this kind of nutrient stress? For the litter quality hypothesis, the key evidence on which the hypothesis is based is nutrient resorption of the dominant species. Cedar and hemlock on the CH sites resorb much of the nutrients from their leaves before senescence, make their litterfall less valuable to decomposers and consequently lower nutrient availability.

Cedar and hemlock can survive and dominate the CH sites because they are tolerant of low nutrient availability created by themselves even though hemlock on CH is less tolerant of the growth stress than cedar. On the contrary, hemlock, the same species as in CH, and amabilis fir retrieve much less nutrient from their leaves. The turnover of the nutrients in their litterfall upgrades the site fertility, therefore the dominant species can grow fast and occupy the sites before any other species. As stated by the author (Keenan 1993), this is quite speculative. It does not explain why salal can grow well on the nutrient poor sites . The whole growth problem is far from completely understood.

The high N concentration of salal foliage led to the suggestion that its growth is not generally limited by N in this area (Keenan 1993). The contradiction that nitrogen-deficient CH soil regardless of low decomposition rate supports a good growth of salal with high concentration of nitrogen might indicate that the availability of nutrients, nitrogen in particular, to salal on the CH sites may not be dependent on decomposers to mobilize the nutrients in organic matter. It may also suggest that there are some other mechanisms involved in the nitrogen cycling of salal, for example, root-associated fungi, mycorrhizal fungi in particular.

Read (1991) pointed out that the quality of the soil nutrient resource is far more important than climate at the local level in influencing the type of plant community, and that mycorrhizal fungi are a key factor in determining the structure of plant communities. From the equator to the poles, soil changes with increasing organic matter content, vegetation varies from herbaceous plants, through deciduous, mixed and coniferous forests to ericaceous vegetation, and dominant mycorrhiza types shift from vesicular-arbuscular mycorrhizae through the ectomycorrhizae to the ericoid mycorrhizae (Read 1991). Mycorrhizal fungi are very important to their hosts because they play a crucial role in plant nutrition and particularly provide plants with access to growth-limiting nutrients (Read

1991). They not only enhance uptake of nutrients from the soil but may also provide access to organic forms of nutrients which are usually not available to plants, a direct nutrient cycling. Direct nutrient cycling was proposed by Went and Stark (1968) as fungi associated with plants obtain nutrients for their host directly from organic resources in the soil. The hypothesis of direct nutrient cycling was inspired by field observations: i) woody fruits readily colonized by fungi in the region of mycorrhizal roots, ii) mycelium and roots confined to the litter layer. Since then, it has been frequently reported that ericoid and ectomycorrhizal fungi, in pure culture or associated with their hosts, are capable of mineralizing organic resources such as organic carbon, organic phosphorus and organic nitrogen for nutrients and energy by producing enzymes (Todd 1979; Trojanowski *et al.* 1984; Dighton 1983; Dighton *et al.* 1987 Mitchell and Read; 1981; Straker and Mitchell 1985; Abuzinadah *et al.* 1986; Abuzinadah and Read 1986; Antibus *et al.* 1986; Bousquet *et al.* 1986; Calleja and D'Auzac 1982; Calleja *et al.* 1980; Coupe *et al.* 1982; Heal and Dighton 1986; Herrera *et al.* 1978; Ho and Zak 1979; Kroehler *et al.* 1988; Lacaze 1983; Lundeberg 1970; Mejstrik and Krause 1973; Mousin *et al.* 1988; Mousin and Salsac 1982; Pearson and Read 1975; Stribley and Read 1980; Theodorou 1971, 1968). It has been well documented (Read 1983, 1987a, 1991, Read and Bajwa 1985) that the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kernan plays a vital role in the success and dominance of the heath, *Calluna vulgaris,* most importantly by providing its hosts with access to organic forms of nitrogen which is a critical element for the growth of plants in those soils. Direct nutrient cycling would be of advantage to the plants growing in CH, where nutrient availability is low and organic matter is rich. Equipped with mycorrhizal fungi which can derive nutrients from organic matter and provide their hosts with access to such nutrient-rich materials, the plants might survive, grow vigorously and become dominant

with little or no requirement for available nutrient resources. This kind of species can be considered as a superior competitor by the resource-ratio hypothesis (Tilman 1988; Wedin and Tilman 1993) because it might require available resource the least. However, this mycorrhizal fungi-mediated direct nutrient cycling puts mycorrhizal fungi in a critical position in the growth of their hosts. If they are suppressed by any physical, chemical and biological factors, for example, by other mycorrhizal fungi associated with other plant species, their host plants would experience serious nutrient crisis, and become less competitive.

Therefore, my hypothesis is that root-associated fungi, particularly mycorrhizal fungi, might play a critical role in each plant species of interest. My explanation of the performance of each species of interest is described as follows.

Salal has been reported to form ericoid mycorrhizae (Largent *et al.* 1980, Smith 1993). The ericoid mycorrhizal fungus, *Hymenoscyphus ericae*, as mentioned above, has been demonstrated to be involved in direct nutrient cycling with the plants of *Calluna vulgaris* in the heathlands. To date, there have been 12 species of ericoid mycorrhizal fungi described (see Chapter 4). These fungi are ubiquitous in distribution, versatile in nutrition. Some of them have been found to be capable of degrading organic forms of nitrogen such as protein (Bajwa *et al.* 1985) and chitin (Leake and Read 1990). Some of them are also strongly antagonistic to other soil fungi (Dickinson and Boardman 1970). Salal would be a superior competitor associated with fungi of this kind. Salal is associated with four different species of fungi and forms ericoid mycorrhizae with three of them. These fungi were isolated from field collected salal roots with similar frequency on HA and CH cutblocks. Three of them were proven to form ericoid mycorrhizae in axenic culture and one of them is a root-associated fungus with salal, specifically pseudomycorrhizal with salal. These include *Oidiodendron griseum*, *Acremonium strictum*, which is here reported for the first time as an

pseudomycorrhizal fungus, and at least two nonsporulating unknown fungal species, which were described in culture. Five out of twelve known ericoid mycorrhizal fungi developed a hyphal complex in the outer layer of the cortical cells of salal roots and a hyphal weft on the surface of the roots, typical of ericoid mycorrhizae. These fungi are *Hymenoscyphus ericae* (Read) Korf and Kernan, *Oidiodendron flavum* Szilvinyi, *O. maius* Barron, *Pseudogymnoascus roseus* Raillo, and *Scytalidium vaccinii* Dalpe, Litten and Sigler. The fungi isolated from salal, *Oidiodendron griseum, Acremonium strictum,* and two nonsporulating unknown fungi and the plants of salal inoculated by those fungi used glutamine as readily as ammonium nitrogen. There was a considerable variation between fungal species or the plants inoculated with those fungi in terms of using glutathione and BSA. *Oidiodendron griseum* on glutathione and *Acremonium strictum* on BSA produced significant yields in pure culture or as associated with salal. The plants of salal inoculated by all four fungi had higher colonization rate on glutathione or BSA than on ammonium or glutamine. The colonization of salal roots by its fungi was suppressed by application of available nitrogen and simple organic nitrogen and favored by more complicated organic nitrogen.

In vitro interactions between species of 4 root-associated fungi of salal, *Acremonium strictum*, *Oidiodendron griseum* and 2 unknown, isolated from field salal roots and 3 ectomycorrhizal fungi proven to be mycorrhizal with western hemlock, *Pisolithus tinctorius*, *Rhizopogon semireticulatus* and *Suillus lakei*, were examined and characterized on buffered and unbuffered modified Melin Norkrans agar (MMN). Three interaction patterns were revealed, neutral intermingling, deadlock and inhibition. Inhibition, in which the growth of one mycelium was reduced by the other, was the predominant outcome of the pairing of ectomycorrhizal fungi opposing the root-associated fungi of salal. All three ectomycorrhizal fungi were inhibited by the root-associated fungi of salal, but

none of the four fungi was inhibited by any of the ectomycorrhizal fungi. *Acremonium strictum* was the most aggressive of the root-associated fungi of salal. It inhibited all three ectomycorrhizal fungi in 5 of 6 interspecific pairings on both media. *Oidiodendron griseum* was the second in terms of aggressiveness to the ectomycorrhizal fungi. *Suillus lakei* and *Rhizopogon semireticulatus* were kept in check by 3 of the 4 root-associated fungi of salal on both media. The results suggested that the root-associated fungi of salal are inhibitory to the ectomycorrhizal fungi.

This study has demonstrated that salal does form root-fungus associations with fungi which can provide it with access to organic nutrient resources and inhibit the growth of mycorrhizal fungi of western hemlock which are critical in the growth of the plants of the species so that salal can outcompete its opponents in the case of CH clearcuts where nitrogen is the growth-limiting factor. But in the situation such as the old growth of CH where light is a limiting growth factor, salal might be kept in control. Its adverse influence on other plants through root-associated fungi might be minor. The salal check in the old growth of CH forests might be explained, in terms of root-associated fungi involvment, that colonization by fungi will effectively increase relative growth rate only when photosynthesis is limited by nutrient supply from the soil. If growth was limited by light intensity, colonization by root-associated fungi would have negative impact on the growth of the host by the removal of the photosynthate by the fungus.

Western hemlock forms ectomycorrhizae with many ectomycorrhizal fungi (Kropp and Trappe 1982), at least 9 types of ectomycorrhizae have been characterized from the seedlings growing on both CH and HA clearcuts (personal unpublished data). Some ectomycorrhizal fungi are capable of using organic nitrogen (Abuzinadah and Read 1986). Messier (1991) found that both field and pot-grown seedlings of western hemlock were highly colonized by

ectomycorrhizal fungi and that there was no difference in total percentage mycorrhizal colonization among the seedlings, whether growing with or without salal. Western hemlock grows poorly on the CH clearcuts. The fact that well-colonized mycorrhizal seedlings of western hemlock grow poorly might imply that mycorrhiza is not a factor influencing the growth of western hemlock, or at most, a minor one in the situation of the CH clearcuts. However, this view might be too simplistic because having high percentage mycorrhizal colonization does not necessarily mean that a tree can perform well. High mycorrhizal colonization intensity can only indicate that mycorrhizal fungi are involved in the growth of their hosts but does not tell how they are involved, negatively or beneficially. High mycorrhizal colonization intensity can be beneficial to mycorrhizal plants only when the mycorrhizal fungi function normally. If the mycorrhizal fungi do not perform normally, high mycorrhizal colonization intensity would benefit the fungal partner of the mycorrhizal association. But by the removal of the photosynthate by the fungus, the fungus might become a parasite, therefore decrease the growth of the host. This respect of mycorrhizae has been well addressed by Harley and Smith (1983). This study has demonstrated that three tested ectomycorrhizal fungi of western hemlock were inhibited by the root-associated fungi of salal, suggesting that the growth of western hemlock is in check possibly due to the inhibition of its mycorrhizal fungi by the root-associated fungi of salal.

Western red cedar forms VA mycorrhizae (Parke *et al.* 1983). VA plant species are usually associated with soils of low phosphorus availability (Harley and Smith 1983; Read 1991). Unfortunately, there is little information about their ability to derive nutrients from organic matter. As Keenan (1993) mentioned, this species can tolerate low N availability, so, N is probably not a limiting growth factor. As the CH soils are also deficient in available phosphorus (Weetman *et al.* 1989a,b),

perhaps it might be the phosphorus availability which limits the growth of cedar. Cedar can survive and achieve dominance on the CH sites, possibly because it forms VA mycorrhizae with VA fungi which might be able to improve its phosphorus nutrition. This needs to be studied.

This research was carried out under laboratory conditions. The results of the present research are only suggestive when field management is considered. This is because there is a difference between the laboratory conditions and the field situations. Field situations are far more complex and unpredictable, while laboratory conditions are usually simple and defined. Therefore, there is a need to demonstrate degradation of organic substrates in the field by mycorrhizal fungi when they are associated with their hosts and to also look at their interactions.

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