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Organic nitrogen use by salal ericoid mycorrhizal fungi from northern Vancouver Island and impacts on growth in vitro of *Gaultheria shallon*

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Abstract Salal (*Gaultheria shallon*) recovers quickly from rhizomes after clear-cut timber harvesting and dominates clearcuts of *Tsuga heterophylla* and *Thuja plicata* forests. Thus it contributes to considerable problems in regeneration of these sites in coastal British Columbia, Canada. Based on what is known about other ericaceous plants, we speculated that mycorrhizal fungi of salal play a vital role in the growth and dominance of salal by providing access to organic nitrogen. In this study, the ability of four species of fungi isolated from salal to use different forms of organic nitrogen was tested in pure culture and 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 maius*, *Acremonium strictum*, and two nonsporulating fungi. Inoculated plants always grew better than noninoculated plants regardless of nitrogen source. Glutamine was used as readily as ammonium nitrogen by all four fungi and the mycorrhizal plants of salal colonized by those fungi. There was considerable variation between fungus species or the plants inoculated with those fungi in using glutathione and BSA. Salal inoculated with *O. maius* grew better on glutathione than BSA, while *A. strictum* and unknown 1 produced significantly greater yields of salal on BSA. Colonization rates of salal by all four fungi was higher on glutathione or BSA than on ammonium or glutamine.

Key words *Gaultheria shallon* · Organic nitrogen use · Ericoid mycorrhiza · Salal

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Introduction

Salal (*Gaultheria shallon* Pursh) is considered a management problem in regeneration of certain forest types in coastal British Columbia, Canada, because it dominates cutblocks after timber harvesting and is involved in the poor growth of commercially planted western hemlock [*Tsuga heterophylla* (Raf.) Sarge.] (Weetman et al. 1989). This domination of cutblocks by salal is similar to the domination of heathlands by *Calluna vulgaris* (L.) Hull in Europe (Handley 1963) and the related growth check in Sitka spruce (Weatherall 1953).

The heathlands in Europe have been studied in great detail in relation to ericaceous plants and the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kernan, which provides its hosts with access to organic forms of nitrogen (Read 1983, 1987, 1991; Read and Bajwa 1985; Stribley and Read 1980). Bajwa and Read (1985) demonstrated after 30 days incubation in liquid culture that *H. ericae* grew as well on glutathione and various alanine peptides as on ammonium. Both *H. ericae* itself and *Vaccinium macrocarpon* Ait. inoculated with this fungus used proteins, especially bovine serum albumen (BSA), as sole nitrogen source (Bajwa et al. 1985) while nonmycorrhizal *V. macrocarpon* was totally unable to use protein.

At least four different mycorrhizal fungi are associated with salal in clearcuts on northern Vancouver Island, B.C. (Xiao and Berch 1996). Two groups of isolates from salal have remained sterile in culture (unknown 1 and unknown 2) but recent DNA sequence information indicates that these isolates are members of the Leotiales distinct from *H. ericae* (Monreal 1997). The other two known ericoid mycorrhizal fungi of salal are *Oidiodendron griseum* (now known to be *O. maius* Robak, Hambleton et al. 1998) and the unusual mycorrhizal fungus *Acremonium strictum* W. Gams (Xiao 1994).

Read (1992) pointed out that *H. ericae* was the only fungus isolated from the roots of ericaceous plants that

has been demonstrated to be a true mutualist, in that positive growth responses result from back inoculation of host plants. Because of the similarities between salal-dominated cutblocks in British Columbia and *Calluna* heathlands in Europe, we were interested in discovering whether the known ericoid mycorrhizal fungi of salal would similarly provide their host with access to organic forms of nitrogen.

Materials and methods

Fungus isolates

Four fungi isolated from field salal roots, *O. maius*, *A. strictum*, unknowns 1 and 2, and isolates of *H. ericae* from Europe were used in this study. *O. maius*, *A. strictum*, unknowns 1 and 2 and *H. ericae* formed typical ericoid mycorrhizae on salal in axenic culture (Xiao and Berch 1996). Four isolates of each species were used except for *H. ericae* (two isolates). These isolates were S4, S18, S45 and S80 (deposited at the University of Alberta Microfungus Collection) of *O. maius*; S214, S217, S220 and S232 (S232 was deposited at Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) of *A. strictum*; S9, S219, S234, S245 of unknown 1; and S203, S227, S246 and S255 of unknown 2. Isolates 100 and 101 of *H. ericae* were kindly provided by Dr. D.J. Read, University of Sheffield. All isolates were originally maintained on modified Melin Norkrans agar (MMN) and then transferred to nutrient agar without nitrogen before being used as inoculum to minimize the carry over of nitrogen to the experiment.

Utilization of organic nitrogen by the fungi

This experiment was conducted in a liquid culture system. The basal medium was MMN without mineral nitrogen and the pH of the medium after autoclaving was 4. This pH was used because it is within the range of the soil pH (3–4) of the salal sites. Five different nitrogen treatments, BSA, glutathione, glutamine, $(NH_4)_2HPO_4$ and nitrogen free, were applied. The nitrogen solutions 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 of each nitrogen treatment were aseptically transferred to 50-ml beakers. The beakers were separately inoculated with about 30 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. For unknowns 1 and 2, *O. maius*, and *A. strictum*, we used one replicate per isolate, except for S9, S4, S232, and S246 for which we used 2 replicates. For *H. ericae*, we used 3 replicates of isolate 100 and 2 replicates of 101. Cultures were incubated at 25 °C in the dark, harvested 45 days after inoculation by filtration of the culture solution through oven-dried pre-weighed filter paper, oven-dried at 80 °C for 24 h, and weighed.

Utilization of organic nitrogen by salal

The experiment was carried out in 100 × 15 mm Petri dishes with half of the agar disc removed (Xiao and Berch 1992). The MMN growth medium contained 8 g l^{-1} of Difco Bacto-agar and was modified by the omission of mineral nitrogen, malt extract and glucose. 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. We found that the final pH at harvest had dropped to 3.5–3.9.

One experimental plant per Petri dish was obtained by planting a germinated surface-sterilized seed at the center of the cut edge of the agar disc. This plant was inoculated when it had its first true leaf with about 30 mm^3 inoculum cut from the edge of a colony of the fungal isolates grown on nitrogen-free media. Noninoculated controls received about 30 mm^3 of agar without inoculum. Five replicate plates per treatment combination (same number of replicates per isolate as above) were sealed with Parafilm and placed vertically in a growth chamber at 25 °C and a light regime of 18 h light at 310 μmol m^{-2} sec^{-1} illumination and 6 h dark. Two weeks after inoculation, we started to check all the plants for colonization by mounting the inverted plate on the platform of a compound light microscope. At harvest the entire root system was gently pulled out of the agar, mounted intact on a glass slide with a drop of water, and covered by a glass coverslip for determination of colonization.

Three root sections each with 100 contiguous cortical cells of each colonized root system were counted for the percentage of cells colonized, scoring + for colonized cells and – for noncolonized. After examination for colonization, the entire plants were oven-dried for 24 h at 80 °C.

Statistical analysis

Data were analysed as a completely randomized factorial design using ANOVA. Differences between the treatment means were judged statistically by Tukey's (HSD) Test. Residuals for percent colonization data were normally distributed (SAS proc univariate, $W = 0.98$ $p = 0.88$), so the data were not transformed. A level of significance of $\alpha = 0.05$ was used throughout for inferring statistical significance, except for the five sets of linear contrasts (noninoculated versus inoculated for each fungus) where the level of significance was set to $\alpha = 0.05/5 = 0.01$ to control type I error. The Pearson Correlation Coefficient r was used to examine the relationship between salal biomass and fungus biomass or mycorrhizal colonization by fungus species.

Results

Yield of fungi

Both fungus and nitrogen source had significant effects on fungus biomass and there was a significant interaction between the two factors (Table 1, $R^2 = 0.85$). Overall, fungus growth was best on ammonium and glutamine, moderate on glutathione and BSA, and poor when no nitrogen was added (Table 1). However, each fungus showed somewhat different preferences for the various nitrogen sources. *A. strictum*, for instance, grew as well on BSA as on the two simple nitrogen sources, while *O. maius*, *H. ericae*, unknown 1 and unknown 2 all grew as poorly on BSA as they did when no nitrogen was added.

Yield of plants

Similar to the situation for fungus response, both fungus and nitrogen source had significant effects on salal biomass and there was a significant interaction between the two factors (Table 2, $R^2 = 0.90$). In general, salal growth was best on ammonium and glutamine, moderate on glutathione and BSA, and poor when no nitrogen was added (Table 2). Without inoculation, salal

Table 1 Mean biomass (mg) of ericoid mycorrhizal fungi on different nitrogen sources. Values with the same letter are not significantly different ($p \leq 0.001$)

	Overall	Unknown 1	*Oidiodendron maius*	*Acremonium strictum*	Unknown 2	*Hymenoscyphus ericae*
Ammonium	10.2 a	10.3 a	14.4 a	9.2 ab	8.0 ab	9.1 b
Glutamine	11.1 a	12.1 a	9.7 b	10.2 a	11.7 a	11.9 a
Glutathione	4.5 b	2.3 b	6.9 bc	4.8 cb	5.6 bc	3.0 c
Bovine serum albumen	5.0 b	4.2 b	3.3 cd	13.3 a	2.3 c	2.1 c
No nitrogen	2.7 c	1.9 b	2.3 d	4.1 c	2.6 c	2.5 c

Results from ANOVA performed on fungus biomass on different nitrogen sources

	df	Mean square	F	*p*
Fungus	4	0.00002902	8.18	0.0001
Nutrient	4	0.00034509	97.23	0.0001
Fungus*Nutrient	16	0.00003459	9.74	0.0001
Error	100	0.00000355		

Table 2 Mean salal biomass (mg) inoculated with different ericoid mycorrhizal fungi and grown on different nitrogen sources. Values with the same letter are not significantly different ($p \leq 0.001$)

	Overall	Not inoculated	Unknown 1	*Oidiodendron maius*	*Acremonium strictum*	Unknown 2	*Hymenoscyphus ericae*
Ammonium	73.6 a	55.8 a	74.9 a	76.6 a	91.4 a	84.2 a	58.7 b
Glutamine	78.6 a	28.7 b	84.5 a	82.7 a	77.2 a	92.5 a	105.82 a
Glutathione	26.6 b	9.4 c	11.0 c	63.5 a	27.4 b	26.1 b	22.3 c
Bovine serum albumen	32.7 b	8.9 c	33.2 b	30.3 b	91.3 a	16.9 b	15.4 c
No nitrogen	15.2 c	10.9 c	11.7 c	11.6 b	26.6 b	14.4 b	15.8 c

Results from ANOVA performed on salal biomass inoculated with different fungi and grown on different nitrogen sources (for contrasts $p < \alpha = 0.01$)

	df	Mean square	F	*p*
Fungus	5	0.00441742	29.72	0.0001
Nutrient	4	0.02494314	167.79	0.0001
Fungus*Nutrient	20	0.00167461	11.26	0.0001
Error	120	0.00014866		
Contrasts:				
Inoculated versus noninoculated on ammonium	1	0.00191309	12.87	0.0005
Inoculated versus noninoculated on glutamine	1	0.01492609	100.41	0.0001
Inoculated versus noninoculated on glutathione	1	0.00177091	11.91	0.0008
Inoculated versus noninoculated on bovine serum albumen	1	0.00338723	22.79	0.0001
Inoculated versus noninoculated on nitrogen-free	1	0.00010736	0.72	0.3971

grew best on ammonium, moderately on glutamine, and poorly on glutathione and BSA and when no nitrogen was added.

Salal grew significantly better when inoculated than when not inoculated except on nitrogen-free medium (Table 2). Overall, the response of inoculated salal to nitrogen source was similar to the response of the fungi to the same nitrogen source (Pearson Correlation Coefficient $p \leq 0.0001$ for salal and fungus biomass on all nitrogen sources: $r = 0.91$ for unknown 1, $r = 0.79$ for *O. maius*, $r = 0.69$ for *A. strictum*, $r = 0.72$ for unknown 2, and $r = 0.89$ for *H. ericae*). As seen with the fungi themselves, salal inoculated with different fungi showed somewhat different preferences for nitrogen source. Salal inoculated with *A. strictum*, for instance, grew as well on BSA as it did on ammonium and glutamine. With *H. ericae*, *O. maius*, and unknown 2, salal grew as poorly on BSA as it did when no nitrogen was added, while unknown 1 permitted significantly better growth on BSA than on nitrogen-free medium.

Mycorrhizal colonization

Both fungus and nitrogen source had significant effects on percent colonization of salal roots and there was a significant interaction between the two factors (Table 3, $R^2 = 0.98$). In general, colonization rates were significantly lower on ammonium and glutamine than on glutathione and BSA or when no nitrogen was added (Table 3). There was a strong negative correlation between mycorrhizal colonization and salal biomass (Pearson

Table 3 Mean percent root cell colonization by different ericoid mycorrhizal fungi grown on different nitrogen sources. Values with the same letter are not significantly different ($p \leq 0.001$)

	Overall	Unknown 1	*Oidiodendron maius*	*Acremonium strictum*	Unknown 2	*Hymenoscyphus ericae*
Ammonium	17.3 b	18.8 b	20.0 c	11.2 c	18.6 c	17.8 b
Glutamine	17.8 b	20.6 b	19.2 c	11.8 c	17.0 c	20.4 b
Glutathione	85.2 a	95.8 a	80.4 b	83.8 a	82.2 b	84.0 a
Bovine serum albumen	84.0 a	96.2 a	94.4 a	63.4 b	84.6 ab	81.4 a
No nitrogen	88.8 a	95.2 a	93.2 a	86.2 a	90.4 a	79.2 a

Results from ANOVA performed on percent root cell colonization by different ericoid mycorrhizal fungi grown on different nitrogen sources

	df	Mean square	F	*p*
Fungus	4	693.53	44.82	0.0001
Nutrient	4	35258.03	2278.83	0.0001
Fungus*Nutrient	16	170.35	11.01	0.0001
Error	100	15.47		

Correlation Coefficient $p \leq 0.0001$ for salal biomass and mycorrhizal colonization on all nitrogen sources: $r = -0.90$ for unknown 1, $r = -0.80$ for *O. maius*, $r = -0.70$ for *A. strictum*, $r = -0.93$ for unknown 2, and $r = -0.83$ for *H. ericae*).

Discussion

Considering four ericoid mycorrhizal fungi not previously tested for their ability to provide their host with access to organic N, we have expanded the known mutualistic mycorrhizal fungi of ericaceous plants beyond *H. ericae*. Regardless of which mycorrhizal fungus colonized the roots and which nitrogen source was provided, mycorrhizal plants grew better than nonmycorrhizal plants.

Although ammonium and glutamine were the preferred nitrogen sources for virtually all the salal ericoid mycorrhizal fungi tested in this study and the plants colonized by them, three of the four fungi and salal inoculated with these fungi were able to use various organic nitrogen sources. Noninoculated plants were unable to use the most complex nitrogen sources provided (glutathione and BSA) and grew less well on glutamine than on ammonium.

There were, however, differences among the fungi in use of the peptide and the protein. For instance, salal colonized by *O. maius* grew better on glutathione than on BSA. *A. strictum* and salal colonized by this fungus grew as well on BSA as it did on ammonium and glutamine. Given that salal roots are colonized by all four of these fungi on the same site, the potential is created for salal, through its mycorrhizal symbionts, to access nitrogen in the variety of forms that would actually exist in the forest floor. In the salal-dominated western redcedar and western hemlock forest systems from which we isolated the four fungi studied here, there is very little available nitrogen in the forest floor although total nitrogen content is high (Prescott et al. 1993). In the highly controlled system we used here, percent colonization of the root system was not positively correlated with growth response of the plants. However, good growth of salal in the presence of ample available nitrogen (ammonium and glutamine) is clearly irrelevant to an ecosystem where there is little or no available nitrogen. It is clear that noninoculated salal cannot use complex nitrogen forms as nitrogen sources and this would be a major limitation to the success of salal in the absence of its mycorrhizal fungi. In the field, low mineral nitrogen availability and high organic matter content probably lead to a shift in plant status that permits high levels of colonization by mycorrhizal fungi. Salal roots are, in fact, highly colonized in the field (Xiao and Berch 1996).

We recommend that complex organic nitrogen sources instead of mineral nitrogen be used when investigating whether fungi can form ericoid mycorrhizae in monoxenic culture. Stribley and Read (1976) found a decline in root colonization in *Vaccinium* on media containing high levels of ammonium sulfate. In our study, salal grown on ammonium or glutamine had lower mycorrhizal colonization rates than on more-complicated nitrogen sources. Sole use of complex organic nitrogen in vitro would shift the situation in favour of mycorrhiza formation.

As pointed out by Bajwa et al. (1985), ericoid mycorrhizal fungi are also good saprophytes and able to use protein as sole source of nitrogen and carbon. We do not know if mycorrhiza formation is even necessary for plants to respond positively to inoculation in the monoxenic culture system we used. Perhaps the presence of any fungus capable of breaking down organic nitrogen and releasing some simple nitrogen into the medium is sufficient to cause a positive plant growth response. We recommend that this highly artificial monoxenic system be used not for in-depth exploration of the physiology of the host-fungus relationship, but rather to confirm ability to form mycorrhizae and for simple exploration of the impacts of inoculation.

Further limitations of this culture-based system are demonstrated by the poor performance on complex nitrogen sources of the European isolates of *H. ericae* used for comparison purposes. As cited earlier, there is extensive evidence that this fungus readily uses complex nitrogen sources and its poor performance in our system may have resulted from the use of old cultures.

Acremonium strictum is an unusual ericoid mycorrhizal fungus in that it is suspected of also being a fungal endophyte of grasses that could deter herbivory (McGee et al. 1991). Given the simplicity of its conidiophores and phialides, it would not be surprising to discover that the name *A. strictum* is applied to phylogenetically diverse fungi with superficial morphological similarity. Because the roots of salal appear to be a very different habitat from the leaf bases of grasses, it would be interesting to know whether the same species of fungus really does inhabit both. In addition, Monreal (1997) found that, as a member of the Hypocreales, *A. strictum* is distinct from the two other groups of known ericoid mycorrhizal fungi: (1) *H. ericae*, its anamorph *Scytalidium vaccinii* Dalpé, Litten and Sigler and a large number of nonsporulating isolates (e.g. unknowns 1 and 2) in the Leotiales and (2) *Oidiodendron* species and teleomorphs such as *Pseudogymnoascus roseus* Raillo in the Onygenales. The ability of this little-known ericoid mycorrhizal fungus to enhance the growth of salal on complex nitrogen sources suggests that other fungi with a similar ability are yet to be discovered.

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