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SHORT NOTE

Detection of extracellular protease activity in different species and genera of ectomycorrhizal fungi

Cajsa M. R. Nygren · Johan Edqvist · Malin Elfstrand · Gregory Heller · Andy F. S. Taylor

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Abstract In northern forest ecosystems, most soil nitrogen (N) is in organic form and forest trees are largely dependent on ectomycorrhizal (ECM) fungi and their degradative abilities for N uptake. The ability of ECM fungi to acquire N from organic substrates should, therefore, be a widespread trait given its ecological importance. However, little is known about the degradative abilities of most ECM fungi as they remain untested due to problems of isolation or extremely slow growth in pure culture. In this paper, we present data on extracellular protease activity of 32 species of ECM fungi, most of which have not previously been cultured. Milk powder plates and zymograms were compared for detecting protease activity in these intractable species. In total, 29/32 of the species produced extracellular protease activity, but detection was method dependent. Growth on milk powder plates detected protease activity in 28 of 32 species, while zymograms only detected proteases in *Amanita muscaria*, *Russula chloroides*, *Lactarius deterrimus* and *Lactarius quieticolor*. The study supports the hypothesis that protease excretion is a widespread physiological trait in ECM fungi and that this ability is of considerable significance for nitrogen uptake in forest ecosystems.

Keywords Ectomycorrhizal fungi · Extracellular proteases · Organic nitrogen · Zymograms · Milk powder plates

C. M. R. Nygren (✉) · M. Elfstrand · G. Heller · A. F. S. Taylor
Department of Forest Mycology and Pathology,
Swedish University of Agricultural Sciences,
P.O. Box 7026, SE-750 07 Uppsala, Sweden
e-mail: Cajsa.Nygren@mykopat.slu.se

J. Edqvist
Department of Plant Biology and Forest Genetics,
Swedish University of Agricultural Sciences,
P.O. Box 7080, SE-750 07 Uppsala, Sweden

Introduction

Nitrogen (N) is the most important macronutrient determining plant growth in the boreal forest (Barbour et al. 1987). The major pools of soil N are, in increasing order of availability, humified material, plant litter, live and dead microbial tissue and mineral nutrients, but with most of the soil N sequestered in organic compounds (Tamm 1991). These organic N compounds range from simple amino acids and amino sugars to complex polypeptides and chitin (Leake and Read 1997). The ability of forest trees to utilise organic N sources is limited to simple amino acids (Näsholm and Persson 2001). However, most boreal forest trees form ectomycorrhizas (ECM) with a wide range of soil fungi, and it is believed that this symbiosis is essential for N uptake in these ecosystems (Smith and Read 1997). Numerous studies have shown that some ECM fungi can utilise a wide range of amino acids as sources of both N and carbon (Plassard et al. 2000) and that a proportion of the assimilated N is transferred to the host plant (Abuzinadah et al. 1986; Finlay et al. 1992; Taylor et al. 2004). In addition, a wide taxonomic range of ECM fungi has also been shown to have the enzymes necessary for the degradation of chitin (Lindahl and Taylor 2004), which could also be an important source of N. However, most studies on the saprotrophic abilities of ECM fungi to mobilise N from organic sources have focused on the production of extracellular proteases (see reviews of Leake and Read 1997 and Lindahl et al. 2005).

Protease activity has been typically determined qualitatively by the ability of pure culture isolates to grow on protein as the sole N source (Finlay et al. 1992; Lilleskov et al. 2002), to develop clear zones on protein-agar plates (Taylor et al. 2000) or to liquefy gelatin (e.g. Maijala et al. 1991). Efforts to quantify protease production per unit

biomass have mostly used a fluorescently labelled protein (fluorescein isothiocyanate-bovine serum albumin, FITC-BSA) as an N source in liquid cultures (e.g. Leake and Read 1989). However, the ecological bearing of these data is probably limited as culture conditions significantly influence both production and enzymatic activity (Leake and Read 1990a,b, 1991). In addition, the soil mycelium produced by many ECM fungal species is hydrophobic over much of its length with only the most distal hyphal tips involved in enzyme release and nutrient uptake (Raidl 1997; Agerer 2001). Thus, liquid culture studies may overestimate protease production in ECM fungi, which produce hydrophobic mycelia on solid substrates.

Cohen (1981) used milk powder plates to examine the extracellular protease production in *Aspergillus*, where the diffusion of extracellular proteases from the mycelial front created a clear zone around the fungal colony. Taylor et al. (2000) used a similar approach to detect protease activity in several slow-growing ECM fungi using agar containing the water-insoluble protein gliadin. These approaches should be suitable for detecting even very low levels of protease production by slow growing ECM fungi.

Zymograms from SDS-polyacrylamide gel electrophoresis, using a protein as a substrate, also permit visualisation of protease production by fungi (Roche and Ryan 1986; Lopez-Llorca et al. 2002). Using this technique, Maijala et al. (1991) provided evidence for the production of two proteases by *Amanita regalis* and Nehls et al. (2001) found that two aspartic proteases with molecular weights of ca. 45 and 90 kDa are produced by the ECM fungus *Amanita muscaria*.

To date, the potential for extracellular protease production has been examined in only a small number (ca. 50) of ECM fungal species (Leake and Read 1997; Lindahl et al. 2005). The majority of species examined are easily grown in pure culture (e.g. species of *Hebeloma*, *Laccaria*, *Paxillus*, *Suillus*, *Thelephora*) and tend not to be the dominant components of mature forest ECM communities (Horton and Bruns 2001). The most species-rich and perhaps the most ecologically important genera such as *Cortinarius*, *Lactarius*, *Russula*, *Tomentella* and *Tricholoma* are considered to be extremely difficult to isolate into pure culture (Smith and Read 1997). Consequently, the enzymatic capabilities of these fungi are largely unknown. Despite repeated calls for more investigations on these fungi (e.g. Read and Perez-Moreno 2003), there has been little progress in this area.

Given the ecological importance of organic N sources in many forests and the inability of the trees to utilise this resource, we hypothesis that the ability to break down protein as an organic source of N is a widespread trait amongst ECM fungi. To test this, we used both milk powder plates and zymograms to evaluate extracellular protease activity in a broad taxonomic range of ECM fungi, most of which belong to genera generally considered to be unculturable.

Materials and methods

Isolates

Cultures were obtained from fresh sporocarp material, except *Piloderma* spp. and Piceirhiza bicolorata, which were isolated from mycorrhizal root tips. About 5–50 explants were taken from each sporocarp and plated onto modified Melin–Norkrans (MMN) media (Marx 1969). Stock cultures were maintained on MMN in darkness at 20°C. To confirm the identities of the isolates, ribosomal DNA (ITS region) was sequenced (Genbank accession numbers in Table 1) and compared with UNITE (Kõljalg et al. 2005) and GenBank (Benson et al. 2005). For identification within the Russulaceae and the genus *Tricholoma*, the personal databases of Ursula Eberhardt (Dept of Forest Mycology and Pathology, SLU, Uppsala, Sweden) and Rasmus Kjøller (Dept. Microbiology, Inst. Biology, University of Copenhagen, Denmark) were used, respectively.

Forty-one isolates of ectomycorrhizal fungi, representing 32 different species (Table 1) were screened for protease production. For comparative purposes, a single isolate of the ericoid mycobiont, *Hymenoscyphus ericae* (the type culture, used in Leake and Read 1990a), was also included. For detecting extracellular proteases using zymograms, cultures were grown on liquid Norkrans media (Norkrans 1949) modified to contain 1 g l^{-1} of casein hydrolysate as sole nitrogen source at a pH of 4.5 (Leake and Read 1989). A 5×5 mm plug, cut from the actively growing edge of a culture was inoculated into each flask with 25 ml of media. Some very slow growing mycelia were transferred repeatedly into new media after periods of 1–2 months to maximise biomass production. This was done to increase the possibility of detecting protease activity in the media.

Milk powder plates

The milk powder plates consisted of two layers of agar. The lower layer was modified Norkrans media (mentioned above) containing 15 g l^{-1} agar and the top layer consisted of 6 ml of milk powder agar. The milk powder agar was made by preparing the agar and fat-free milk powder (Semper) separately in distilled water. After autoclaving and cooling to 60°C, the separate solutions were mixed to give a final concentration of 15 g l^{-1} agar and 75 g l^{-1} milk powder. Circular plugs with a diameter of 5 mm were cut from the actively growing edge of a culture, transferred to a new

Table 1 Details of ectomycorrhizal fungal isolates used and protease activity detected in an investigation of extracellular protease production

Species	Collection code[a]	GenBank accession no.[b]	Host and origin	Cleared zone on milk plate (mm)[c]	Mycelial growth on milk plate (mm)[d]	Zymogram[e]
Amanita muscaria (L.: Fr.) Hook	UP500	DQ658859	*Pinus sylvestris*, Riddarhyttan, Sweden	6	7	1 band (90 kDa)
A. muscaria	UP501	DQ658860	Mixed forest, Uppsala, Sweden	1	3	2 bands (45+90 kDa)
A. spissa (Fr.) Kumm	UP502	DQ658858	Mixed forest, Gusum, S. Sweden	–	1	–
Boletus luridus Schaeff.: Fr.	UP12	DQ658866	*Tilia cordata,* Uppsala, Sweden	3	6	na[g]
Cenococcum geophilum Fr.	UP162	DQ658892	*Picea abies*, Vedby, S. Sweden	–	7	–
Cortinarius glaucopus (Schaeff.: Fr.) Fr.	UP21	DQ658854	*P. abies*, Flakaliden, Sweden	–	8	–
C. purpurascens Fr.	UP534	DQ658852	Mixed forest, Uppsala, Sweden	–	–	–
Hydnum rufescens Schaeff.: Fr.	UP504	DQ658890	*P. abies*, *P. sylvestris*, Uppsala, Sweden	2	3	–
Hymenoscyphus ericae (Read) Korf & Kernan	UP505	DQ658887	Highly acidic stagnohumic gley, North York Moors, UK	–	11	–
Laccaria cf. *bicolor*	UP506	DQ658853	*P. sylvestris, P. abies*, forest nursery, central Lithuania	17	17	–
Lactarius acerrimus Britzelm.	UP507	DQ658885	*Quercus* sp., Belleme, Provence, France	17	6	–
L. auriolla Kytöv	UP537	DQ658886	*P. abies*, Rammsele, Sweden	2	3	na
L. chrysorrheus Fr.	UP510	DQ658873	*Quercus* sp. Trento, Italy	18	7	–
L. controversus Pers.: Fr.	UP508	DQ658881	Mixed, Sparreholm, Flen, Sweden	14	3	–
L. controversus	UP511	DQ658879	*Populus* sp., Monclus S. France.	8	–	–
L. controversus	UP512	DQ658880	*Salix repens*, Newborough Warren, N. Wales.	22	3	na
L. deliciosus (L.: Fr.) Gray	UP513	DQ658870	*P. sylvestris*, Riddarhyttan, Sweden	2	13	–
L. deterrimus Gröger	UP514	DQ658869	*P. abies*, *P. sylvestris* Steinenberg, Tübingen, Germany	13	9	–
L. deterrimus	UP515	DQ658871	*P. abies*, Aberdeen, Scotland.	5	11	1 band (ca 150 kDa)
L. evosmus Kühner and Romagn	UP536	DQ658882	*Q. robur*, Stockholm, Sweden	–	7	
L. pubescens Fr.	UP516	DQ658884	*Betula* sp., Uppsala, Sweden	2	7	–
L. quieticolor Romagn.	UP517	DQ658867	*P. sylvestris*, *P. abies*, Schlossberg, Tübingen, Germany.	9	15	1 band (45 kDa) or 2 bands (45+90 kDa)
L. quieticolor	UP518	DQ658868	Mixed forest, Uppsala, Sweden.	8	9	na
L. quietus (Fr.: Fr.) Fr.	UP519	DQ658876	*Quercus* sp., Belleme, Provence, France	–	2	–
L. quietus	UP520	DQ658877	*Quercus* sp., Aberdeen, Scotland	–	4	–
L. rufus (Scop.: Fr.) Fr.	UP521	DQ658875	Mixed forest, Uppsala, Sweden	–	1	–
L. semisanguifluus R. Heim & Leclair	UP522	DQ658872	*P. sylvestris*, Schlossberg, Tübingen, Germany	9	11	–
L. subdulcis (Pers.: Fr.) Gray	UP523	DQ658874	*Fagus sylvatica*, Lahnberge, Marburg, Germany	–	–	–
L. subumbonatus Lindgr.	UP524	DQ658878	*F. sylvatica*, *Q. robur*, Schlossberg, Tübingen, Germany	–	–	–
L. zonarius(Bull.) Fr.	UP525	DQ658883	*Quercus* sp., Belleme, Provence, France	14	8	–

Table 1 (continued)

Species	Collection code[a]	GenBank accession no.[b]	Host and origin	Cleared zone on milk plate (mm)[c]	Mycelial growth on milk plate (mm)[d]	Zymogram[e]
Piceirhiza bicolorata[f]	UP526	DQ658891	Isolated from a Piceirhiza bicolorata mycorrhizal tip on *P. abies*, Uppsala, Sweden	3	14	–
Piloderma fallax (Lib.) Stalpers	UP527	DQ658864	*P. sylvestris, P. abies*, forest nursery, central Lithuania	2	1	–
Piloderma byssinum (P. Karst.) Jülich	UP535	DQ658865	*P. sylvestris, P. abies*, forest nursery, central Lithuania	2	1	na
Russula chloroides (Krombh.)	UP528	DQ658888	Mixed forest, Uppsala, Sweden	–	1	1 band (ca 45 kDa)
R. sanguinea (Bull.) Fr	UP529	DQ658889	Mixed forest, Uppsala, Sweden	–	5	–
Suillus luteus(L.: Fr.) Roussel	UP530	DQ658861	*P. sylvestris*, near Abisko, N. Sweden	3	13	–
S. luteus	UP531	DQ658862	*P. sylvestris*, near Abisko, N. Sweden	5	17	–
S. variegatus (Sw.: Fr.) O. Kuntze	UP532	DQ658863	*P. sylvestris*, near Abisko, N. Sweden	8	15	–
Tricholoma cf. *equestre* (L.: Fr.) Kummer	UP533	DQ658856	*P. sylvestris*, Riddarhyttan, Sweden	4	1	–
T. fulvum (DC.: Fr.) Sacc.	UP88	DQ658855	Mixed forest, Uppsala, Sweden	–	6	–
T. scalpturatum (Fr.) Quél.	UP93	DQ658857	*Betula pendula*, Uppsala, Sweden	6	4	–

[a] Cultures held in the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden
[b] Partial rDNA sequence (ITS region)
[c] Width (mm) of cleared zone surrounding mycelia on milk plates
[d] Radial mycelial growth (mm) on milk plates
[e] Detection of protease activity using 1D gel electrophoresis with gelatin as a substrate
[f] Isolate G8 in Vrålstad et al. (2002)
[g] *na* data not available

MMN plate for 3–5 days to allow regrowth of severed hyphal ends and then placed on milk powder plates. There were three replicates of each isolate. Plates were incubated in darkness at 20°C for 1 month. The growth of the mycelia was determined by taking the mean of two measurements from the plug to the edge of the mycelia. The width of any cleared zones extending beyond the edge of the mycelial front was also determined as the mean of two measurements.

Zymograms

For the zymograms, 2 ml of culture filtrate was extracted and concentrated with centricons (Millipore, Y-10) centrifuged for 30 min at 4°C at 4,000×*g*. Two different kinds of zymogram gels, with gelatin as a substrate, were used; Invitrogen (EC6175) and Biorad (161-1167). The gels were used according to the manufacturer's instructions with some exceptions; the loading buffer contained 8 M Urea, 2.5% glycerol, 0.01% bromophenol blue and 2.5 mM Tris–HCl adjusted to pH 6.8. A mixture of 20 µl of concentrated sample and 20 µl loading buffer was loaded into each well. The gels were run for 90 min at the recommended voltage and then renatured for 2×30 min in 2.5% Triton X-100 (Sigma). After equilibrating for an additional 30 min in development buffer (Biorad, 161-0766), they were transferred to new development buffer and incubated overnight at 37°C. A range of pH values (pH 2, 3, 4, 4.5, 5, 6, 7, 7.5 and 8) were used for the development buffer. A pH of 7.5 was found to give strongest bands for all the species that produced bands. A Seeblue plus2 pre-stained ladder was used (Invitrogen, LC5925) to estimate the size of the proteases.

Results and discussion

Despite repeated attempts to obtain isolates from fresh sporocarp material, only a small percentage of species from some genera produced cultures. In particular, species within *Cortinarius* and *Russula* were the most intractable. In most cases where cultures were obtained, the mycelium was usually derived from a single explant, even when ca. 50 were plated out. Surprisingly, once growth was initiated, the *Cortinarius* isolates had a relatively rapid growth rate (5 mm wk^{-1}). *Lactarius* species, even though they were amongst the slowest growing, were the most amenable to pure culture isolation.

Once in culture, *Lactarius*, *Hydnum*, *Piloderma*, *Russula* and *Tricholoma* showed measurable but very slow growth on agar, ca. 1–15 mm $month^{-1}$. This slow growth rate dictates that any analysis of enzymatic capabilities should not be dependent on a large biomass production.

The ease with which protease activity was detected varied considerable between the two methods employed in this study (Table 1). With only three exceptions, all isolates grew on the milk powder plates and produced a clear zone around or under the mycelia, indicating protease activity. The width of the cleared zone around the mycelial front varied considerably (Table 1) and was not related to the rate of radial extension of the mycelium. Some mycelia (e.g. *Laccaria* cf *bicolor*, and *Lactarius zonarius* [Fig. 1a]) degraded the milk powder proteins over extensive areas, while others (e.g. *Hydnum rufescens* [Fig. 1a]) had narrow cleared zones in the immediate vicinity of the mycelial front. Milk powder plates proved to be a very simple and effective method for detecting extracellular proteases produced by slow-growing ECM fungi.

Even a very thin mycelium was sufficient to produce a transparent or clear zone in the milk agar. The occurrence of these zones around mycelia, which were clearly visible

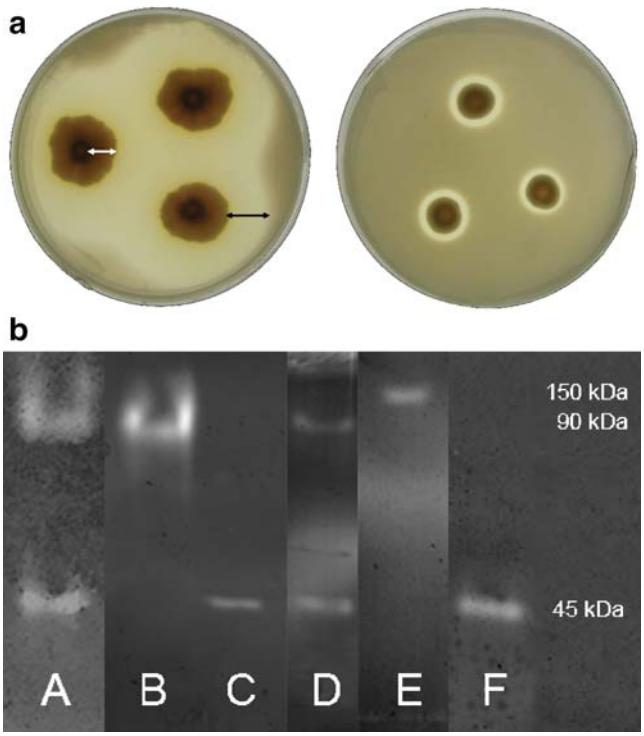

Fig. 1 **a** Growth of two ectomycorrhizal fungi (left: *Lactarius zonarius*; right: *Hydnum rufescens*) after 1 month on media containing insoluble milk powder where cleared zones indicate the activity of secreted proteases. *White arrow* indicates mycelial growth and *black arrow* the cleared zone. **b** Zymograms showing extracellular protease activity in culture filtrate derived from the growth of ectomycorrhizal fungi on liquid medium containing casein hydrolysate as an N source. Lane *A*: *A. muscaria* UP501, two proteases with a molecular weight of around 45 and 90 kDa. Lane *B*: *A. muscaria* UP500, one 90-kDa protease. Lane *C*: *L. quieticolor* one protease, 45 kDa. Lane *D*: Same isolate of *L. quieticolor* at another time point, two proteases 45 and 90 kDa. Lane *E*: *L. deterrimus*, around 150 kDa and Lane *F*: *R. chloroides*, 45 kDa. The bands are from different gels except for *B* and *C*

with most isolates, is dependent upon the diffusion of the proteases out beyond the growing margin of the mycelium. Some isolates failed to produce a clear zone around the mycelium, but in these cases the milk powder had been degraded directly beneath the mycelium. It is possible that in these species the proteases have very low diffusion rates or that they remain wall-bound. In white rot fungi, β-glucosidases, which generate assimilable products, are wall-bound, while glucanase components, which produce the substrates for the β-glucosidases, are released into the environment (Cai et al. 1999). Retaining active catabolic enzymes in close proximity to the hyphae could be expected to greatly enhance the uptake of any assimilable breakdown products produced by these enzymes.

Detection of proteases using zymograms was very problematic, and positive results were sporadic with no discernable pattern found that could be related to taxonomy. Only three species, *A. muscaria*, *L. deterrimus* and *L. quieticolor*, consistently gave bands and a fourth species, *Russula chloroides*, gave a band on only a single occasion (Fig. 1b). Intraspecific variation was obtained in *A. muscaria* with UP501 secreting two proteases with a size of ca. 45 and 90 kDa, while UP500 only produced a single protease at the higher molecular weight. *L. deterrimus* secreted the extracellular protease with the highest molecular weight in this study, ca. 150 kDa. *L. quieticolor* produced two bands (45 and 90 kDa) after 2 months' growth, but after 7 months, the same culture secreted only the smaller protease. All of the isolates that produced bands on the zymograms grew on the milk plates and all, except *R. chloroides*, produced a cleared zone around the mycelia.

There are a number of possible explanations for the difficulty in obtaining bands in the zymograms. The SDS in the loading buffer may have caused irreversible damage to the protein structure, which prevented renaturation. Support for this comes from the observation that the *H. ericae* isolate did not produce any bands in the zymograms, but is known to have very high extracellular protease activity (Leake and Read 1990a). However, the proteases appeared to be remarkably stable; it was possible to transfer a plug of agar from the cleared zone of one plate onto a new milk powder plate and obtain a new cleared zone.

It is also possible that aggregation of the protease molecules, either with themselves or other proteins, into larger units restricted entry into the zymogram gel. These ECM fungi excrete a range of different proteins into the media when grown in liquid culture (Martino et al. 2002) and in the present study, culture filtrate of *H. ericae* contained at least 36 different proteins with molecular weights from ca. 10–150 kDa (data not shown). There is, therefore, considerable potential for protein aggregation.

Protease production by mycorrhizal fungi has been most extensively studied in the ericoid endophyte *Hymenoscyphus ericae* (Leake and Read 1990a,b, 1991). Using the FITC-BSA assay to measure activity, it was found that high levels of glucose and ammonium in the culture medium repressed protease production, while casein hydrolysate and BSA induced proteolytic activity (Leake and Read 1991). Based on these findings, casein hydrolysate and BSA were both initially used as N sources in the zymogram study, but very few isolates showed growth on BSA (data not shown).

Contrary to the work of Leake and Read (1991) on *H. ericae*, Nehls et al. (2001) found evidence that the presence of glucose and casein hydrolysate reduced expression of the gene coding for the 45-kDa protease in *Amanita muscaria*. However, in the present study, the culture conditions allowed sufficient expression of proteases genes by *Amanita muscaria* to produce one or two bands in the zymogram gel, indicating that the culture conditions were suitable, at least in this species.

In general, the molecular weights of fungal acidic proteases are in the range of 30 to 45 kDa (Rao et al. 1998). The sizes of the proteases in this study ranged from ca. 45 to 150 kDa. One possible explanation for the higher molecular weights of the proteases detected in the present study could be that the 90-kDa protease is a dimer of two 45-kDa protease molecules and that the ca. 150-kDa protease may be a trimer of 45-kDa protease.

Fungi are known to produce a broad range of enzymes involved in the breakdown of organic N compounds (see reviews by Rao et al. 1998; Sinsabaugh 2005). Even single species, e.g. *Aspergillus oryzae*, may produce acid, neutral (metalloproteases) and alkaline extracellular proteases (Gomi et al. 1993; Sandhya et al. 2005; Ogawa et al. 1990). However, the ability of ECM fungi to grow on proteinaceous substrates was only demonstrated relatively recently (Abuzinadah et al. 1986; Leake and Read 1997). To date, protease production has only been conclusively demonstrated in a small fraction of the huge diversity of ECM fungi. The data presented in this paper significantly increase the number of ECM species known to produce proteases. More importantly, most of these species are representatives of ecologically important taxa for which there was little, if any, previous data due to problems related to their culturability. In addition, these results are derived from isolates where the taxonomic identity of the mycelia has been authenticated using molecular identification. This procedure is essential as non-target fungi can be readily isolated from sporocarp or root tip material, which may lead to spurious claims of enzymatic capabilities of ECM fungi.

Information on ecologically important physiological characters in ECM fungi has been limited to a small number of easily culturable species, but this study demonstrates that by using appropriate methods, it is possible to

determine enzymatic capabilities even in slow-growing genera like *Russula* and *Lactarius*. The results show that a broad taxonomic range of ECM fungi are capable of producing extracellular proteases, which supports the hypothesis that this trait has a considerable significance for nitrogen uptake in the boreal forest.

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