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Chitinolytic activities of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (*Ericaceae*)

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ARTICLE INFO

Article history:
Received 7 April 2005
Received in revised form
22 August 2005
Accepted 30 September 2005
Published online 27 December 2005
Corresponding Editor: Derek T. Mitchell

Keywords:
Chitinase
Endophytes
Hymenoscyphus ericae
Mycorrhizas

ABSTRACT

Ericoid mycorrhizal endophytes and other root-associated fungi from *Epacris pulchella* (*Ericaceae*) in an eastern Australian sclerophyll forest, along with *Hymenoscyphus ericae*, were tested for their abilities to produce extracellular chitinolytic activities during growth in axenic culture. Two root-associated fungi produced activities that were active against only a monomeric 4-methylumbelliferyl (4-MU) glycoside of *N*-acetylglucosamine, suggesting exo-acting β-*N*-acetylhexosaminidase (EC 3.2.1.52) activity. All ericoid mycorrhizal fungi and two root-associated fungi produced activities against dimeric and trimeric 4-MU glycosides of *N*-acetylglucosamine, suggesting production of chitobiosidase and endo-acting chitinase (EC 3.2.1.14) respectively in addition to β-*N*-acetylhexosaminidase. Specific activities for all ericoid mycorrhizal fungi, including *H. ericae*, were of the same order of magnitude, suggesting that their chitinolytic potential is broadly similar. Chitinase activities were only produced by an ericoid mycorrhizal fungus when chitin was included in the medium, however, no activity was produced if glucose was also present in the medium.

Introduction

Plants in the family *Ericaceae* generally inhabit heathland or forest habitats where soils are acidic and extremely nutrient deficient (Read 1996; Cairney & Meharg 2003). Nitrogen is the major limiting nutrient in such habitats, and the ability of *Ericaceae* to form ericoid mycorrhizal associations, that facilitate host access to organic nitrogen sources, is regarded as critical to their success (Leake & Read 1997; Cairney & Ashford 2002). There is considerable evidence that *Hymenoscyphus ericae* and other ericoid mycorrhizal endophytes of Northern Hemisphere *Ericaceae* can obtain nitrogen from acidic, neutral and basic amino acids, along with releasing acid proteases that mediate utilisation of simple protein (e.g. Bajwa & Read 1986; Leake & Read 1990b; Xiao & Berch 1999). Although proteolytic activities of ericoid mycorrhizal endophytes from Southern Hemisphere *Ericaceae* have yet to be investigated, their abilities to utilise amino acids and protein as sources of nitrogen appear to be broadly similar to *H. ericae* (e.g. Whittaker & Cairney 2001; Midgley *et al.* 2004a).

H. ericae is known to produce enzymes that may also facilitate access to nitrogen (and phosphorus) sources sequestered within and/or complexed with moribund plant material (Leake & Read 1997; Cairney & Burke 1998). Specifically, this mycobiont produces an array of cellulolytic and hemicellulolytic activities that may contribute to degradation of plant cell wall material (Varma & Bonfante 1994; Burke & Cairney 1997a, b). *H. ericae* also produces polyphenol oxidase activities that may be important in depolymerising phenolic compounds and releasing organic nitrogen compounds that are complexed with them (Bending & Read 1996). Carbohydrate oxidase activity may further release H_2O_2, which, via production of hydroxyl radicals in the presence of Fe may

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doi:10.1016/j.mycres.2005.09.015

contribute to partial lignin degradation by this fungus (Burke & Cairney 1998). The demonstrated abilities of other ericoid mycorrhizal endophytes from northern and southern hemisphere Ericaceae hosts to utilise a range of carbohydrates for growth (e.g. Varma & Bonfante 1994; Midgley *et al.* 2004b) implies that production of certain cellulolytic and hemicellulolytic enzymes, at least, is widespread in this group of fungi.

Aside from plant material, a significant proportion of soil biomass is present in the form of living and dead fungal mycelium (e.g. Zvyagintsev 1994). Fungal mycelium represents a major pool of soil nitrogen, much of which is deposited in cell walls as chitin (a polymer of β–1,4 linked *N*-acetylglucosamine units), and can represent up to around 30 % of total soil nitrogen in some Northern Hemisphere heathlands (Kerley & Read 1997). The ericoid mycorrhizal endophytes *H. ericae* and *Oidiodendron griseum* can utilise purified chitin as a sole nitrogen source *via* absorption of the products of chitinolysis (Leake & Read 1990a; Mitchell *et al.* 1992; Kerley & Read 1995). Significantly, some of the nitrogen derived from purified chitin is transferred to the *Ericaceae* host plant (Kerley & Read 1995). Moreover, the likely ecological significance of these chitinolytic capabilities is emphasised by the observation that *H. ericae* can derive nitrogen from fungal necromass and effect its transfer to *Ericaceae* hosts (Kerley & Read 1997). Most current knowledge of chitin utilisation by ericoid mycorrhizal fungal endophytes, however, relates to *H. ericae* and the chitinolytic potential of other endophyte taxa remains unexplored.

Enzymatic hydrolysis of chitin to *N*-acetylglucosamine requires the action of both endo- and exo-acting activities (Patil *et al.* 2000). Thus the endo-acting chitinases [EC 3.2.1.14 (=1,4-β-poly-*N*-acetylglucosaminidase)] randomly cleave 1,4-β-linkages in chitin macromolecules to release oligomers of *N*-acetylglucosamine of varying length. Exo-acting chitobiosidases (=*N*,*N*′-diacetylchitobiohydrolase) hydrolyse chitin and oligomers of *N*-acetylglucosamine from the non-reducing ends to release chitobiose, while exo-acting β-*N*-acetylhexosaminidases (EC 3.2.1.52) cleave chitin and oligomers of *N*-acetylglucosamine from the non-reducing end to release *N*-acetylglucosamine monomers (Patil *et al.* 2000; Kubicek *et al.* 2001). A number of ectomycorrhizal basidiomycetes produce both endo- and exo-acting chitinolytic activities (Hodge *et al.* 1995), and genes encoding β-*N*-acetylhexosaminidases are known to be widespread in ectomycorrhizal basidiomycetes (Lindahl & Taylor 2004).

Although *H. ericae* has been shown to produce extracellular acid chitinase activity (Mitchell *et al.* 1992; Kerley & Read 1997), further characterisation of chitinolytic activities of ericoid mycorrhizal fungi has not been conducted. We have thus investigated production of extracellular endo- and exo-acting chitinolytic activities by an isolate of *H. ericae* and several ericoid mycorrhizal endophytes from the Australian *Ericaceae* host *Epacris pulchella*. As a first step towards addressing competition for nutrients sequestered in fungal necromass between ericoid mycorrhizal fungi and other root-associated fungi, we have also determined extracellular chitinolytic activities of several non-mycorrhizal fungi associated with hair roots of *E. pulchella*, and compared these with those of the ericoid mycorrhizal fungi.

Materials and methods

Fungal isolates and growth conditions

The fungi used in this work were nine isolates obtained from hair roots of *Epacris pulchella* (*Ericaceae*), a common understorey shrub in south-eastern Australian sclerophyll forests (Table 1), along with an isolate of *Hymenoscyphus ericae* (*Read 101*) (Table 1). The identities of the isolates from *E. pulchella* have been inferred conservatively by ITS rDNA sequence comparisons (Bougoure & Cairney 2005a) (Table 1). Cultures were routinely maintained on 2 % potato dextrose agar (PDA) (Oxoid) and were transferred to modified Melin-Norkrans (MMN) agar medium (Marx & Bryan 1975) for 28 d prior to establishment of the experiments. Due to difficulties in germinating seed from *E. pulchella*, the abilities of the nine isolates from this host to form ericoid mycorrhizas were tested using *Woollsia pungens* (another Australian *Ericaceae* host from the *Ericaceae* subfamily *Styphelioideae*). Mycorrhiza infection was tested under gnotobiotic conditions using the methods described by Bougoure & Cairney (2005b).

For investigation of chitinase activities, all isolates were grown in a basal liquid medium to which 0.5 $g\,l^{-1}$ purified crystalline chitin and 0.25 $g\,l^{-1}$ $(NH_4)_2HPO_4$ were added. The basal medium was based on MMN liquid medium (Marx & Bryan 1975) and contained (l^{-1}): KH_2PO_4, 0.30 g; $MgSO_4 \cdot 7H_2O$, 0.14 g; $CaCl_2$, 50 mg; NaCl, 25 mg; $ZnSO_4$, 3.0 mg; ferric EDTA, 12.5 mg; thiamine, 0.13 mg. To determine the effects of extracellular carbon, nitrogen and phosphorus on chitinase production, RFLP 1, the most commonly-isolated RFLP type from two *E. pulchella* plants (Bougoure & Cairney 2005a), was grown in basal medium containing: (1) 0.5 $g\,l^{-1}$ purified crystalline chitin and 0.25 $g\,l^{-1}$ $(NH_4)_2HPO_4$; (2) 0.5 $g\,l^{-1}$ purified crystalline chitin; (3) 0.25 $g\,l^{-1}$ $(NH_4)_2HPO_4$; (4) 10 $g\,l^{-1}$ D-glucose; or (5) 0.5 $g\,l^{-1}$ purified crystalline chitin and

Table 1 – Details of isolates used for determination of chitinolytic activities

Isolate	Identity[a] (ITS sequence GenBank accession no.)	Ericoid mycorrhizal status	Culture collection accession[b]
RFLP 1	*Helotiales* (AY627804)[1]	yes	BRIP 46109 a
RFLP 2	*Helotiales* (AY627806)[1]	yes	BRIP 46105 a
RFLP 50	*Helotiales* (AY627812)[2]	yes	BRIP 46112 a
RFLP 13	*Oidiodendron* sp. (AY627815)[1]	yes	BRIP 46113 a
RFLP 15	*Oidiodendron* sp. (AY627817)[1]	yes	BRIP 46114 a
RFLP 52	*Dermataceae* (AY627818)[2]	no	BRIP 46115 a
RFLP 56	*Hypocreales* (AY627831)[2]	no	BRIP 46116 a
RFLP 57	*Agaricales* (AY627833)[2]	no	BRIP 46117 a
RFLP 58	*Tremelles* (AY627834)[2]	no	BRIP 46118 a
Read 101	*Hymenoscyphus ericae*	yes	BRIP 46119 a

a Isolates were identified conservatively from ITS sequence matches as described by Bougoure & Cairney (2005a): 1 Bougoure & Cairney (2005a); 2 Bougoure & Cairney, (unpubl).
b Accessions in Plant Pathology Herbarium (BRIP), Queensland Department of Primary Industries.