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Utilisation of carbon substrates by multiple genotypes of ericoid mycorrhizal fungal endophytes from eastern Australian Ericaceae

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Abstract The abilities of six genotypes of two putative Helotiales ascomycete ericoid mycorrhizal fungal taxa from *Woollsia pungens* and *Leucopogon parviflorus* (Ericaceae) to utilise glucose, galactose, mannose, cellobiose, carboxymethylcellulose, crystalline cellulose, starch and xylan as sole carbon sources were tested in axenic liquid culture. With the exception of all taxon II isolates on carboxymethylcellulose, all genotypes of both taxa produced measurable biomass on all substrates. Significant intraspecific variation was observed in biomass production on all substrates. While pooled data for all genotypes of each taxon revealed significant interspecific differences in biomass production on carboxymethylcellulose, glucose, cellobiose, and starch, mean biomass production for each taxon on the latter three substrates differed less than threefold, suggesting that the saprotrophic abilities of the two taxa are broadly similar.

Keywords Ericoid mycorrhizal fungi · Saprotrophic growth · Epacrids · *Woollsia pungens* · *Leucopogon parviflorus*

Introduction

Ericoid mycorrhiza-forming Ericaceae are globally widespread and occupy a range of habitats that includes climatically diverse heathlands and forests (Read 1996). In these habitats, soils are characteristically nutrient limited and are subject to various other edaphic stresses. Thus, the ability to form ericoid mycorrhizas is regarded as central to the success of Ericaceae (Read 1996; Cairney and Meharg 2003). Most of what is known about functioning of ericoid mycorrhizas is, however, based on northern hemisphere heathland environments. Comparatively little is known regarding the associations in southern hemisphere Ericaceae such as epacrids (Straker 1996; Cairney and Ashford 2002).

Most information about functional aspects of ericoid mycorrhizal fungi has been derived from the common ericoid mycorrhizal endophyte of northern hemisphere Ericaceae *Hymenoscyphus ericae* (Read) Korf & Kernan. This taxon is known to enhance host nitrogen and phosphorus acquisition by providing access to otherwise unavailable organic sources of the elements via absorption of amino acids, production of protease, chitinase and/or phosphomono- and phosphodieasterase activities (Leake and Read 1997). Although information is more limited, other ericoid mycorrhizal endophytes of northern and southern hemisphere Ericaceae, including epacrids, appear to confer similar benefits on their plant hosts (Xiao and Berch 1999; Cairney and Ashford 2002).

In addition to hydrolysing simple organic forms of nitrogen and phosphorus in soil, it is clear that *H. ericae* can degrade components of the plant cell wall (Leake and Read 1997; Cairney and Burke 1998). While this is clearly important in the context of penetration of the host cell wall during establishment of the symbiosis (Perotto et al. 1995), such an ability may also facilitate access to nitrogen and phosphorus sources sequestered within and/or complexed with moribund plant material (Leake and Read 1997; Cairney and Burke 1998; Read and Perez-Moreno 2003) and permit a degree of saprotrophic growth in soil in the absence of a plant host (Perotto et al. 1993). Degradation of cellulose and hemicelluloses by *H. ericae* is facilitated by an array of carbohydrolases that includes endoxylanase, β-D-xylosidase, α-L-arabinosidase, β-L-arabinosidase, α-D-glucuronidase, endomannanase, β-D-mannosidase, α-D-galactosidase, β-D-galactosidase, 1–4-β-D glucan glucohydrolase, cellobiohydrolase and β-D-glucosidase (Varma and Bonfante 1994; Burke and Cairney 1997a, 1997b). *H. ericae* further produces polyphenol oxidase activities that may be important in depolymerising phenolic compounds and releasing com-

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plexed organic nitrogen and phosphorus compounds and/or detoxifying the soil environment by polymerising phenolic compounds (Bending and Read 1996a, 1996b). Carbohydrate oxidase activity may further release H_2O_2, and it has been proposed that the consequent production of hydroxyl radicals in the presence of Fe may facilitate partial lignin degradation (Burke and Cairney 1998). Although less is known about other northern hemisphere ericoid mycorrhizal fungal taxa, it appears that they also possess a degree of saprotrophic potential (Varma and Bonfante 1994; Perotto et al. 1997; Piercey et al. 2002; Thormann et al. 2002).

Reed (1989) reported that ericoid mycorrhizal endophytes from the Australian epacrid *Leucopogon juniperinus* R.Br. grew on water agar containing sterilised plant litter. Ericoid mycorrhizal fungi can, however, produce significant biomass by carbon scavenging on water agar alone (DJ Midgely, SM Chambers, JWG Cairney, unpublished data), rendering this observation somewhat equivocal. In order to assess the saprotrophic potential of ericoid mycorrhizal endophytes of epacrids, we have investigated the abilities of two putative taxa to utilise a range of carbon substrates for growth in axenic culture. Considerable intraspecific physiological variation exists in ericoid mycorrhizal fungi (Cairney et al. 2000) and screening of multiple genetically different isolates of the taxa is required for meaningful interspecific comparison (Cairney 1999). Therefore, multiple genotypes of two sterile Helotiales ascomycete endophyte taxa were utilised in the current work.

Materials and methods

The fungi used in this study were 12 ericoid mycorrhizal endophyte isolates obtained from hair roots of two *Woollsia pungens* Cav. (Muell.) plants and one *Leucopogon parviflorus* (Andr.) Lindl. plant at a mixed dry sclerophyll forest site at Murphy's Glen in the Blue Mountains National Park, NSW, Australia (S 33° 42' E 151° 8'). These isolates have previously been shown to form ericoid mycorrhiza with *W. pungens* and identified as representing six genotypes of two Helotiales ascomycete RFLP-types (= putative taxa) using rDNA ITS-RFLP and ITS sequence analyses and inter-simple sequence PCR (Midgley et al. 2002; DJ Midgely, SM Chambers, JWG Cairney, unpublished data). The genotypes of taxon I were obtained from a single *W. pungens* plant (Midgley et al. 2002) and were identified as RFLP-type I in that paper, while the taxon II genotypes originated from the root systems of a second *W. pungens* plant or the *L. parviflorus* plant (DJ Midgely, SM Chambers, JWG Cairney, unpublished data). Taxon I and II isolates were established in axenic culture during 1999 and 2000, respectively. All isolates are retained in the UWS culture collection and routinely maintained on potato dextrose agar medium (Oxoid) at 23°C in the dark, with subculturing every 12–16 weeks. A preliminary study was conducted to ascertain growth characteristics of the 12 genotypes in a low-carbon and nitrogen version of modified Melin Norkrans liquid medium (low CN MMN) (Marx and Bryan 1975) containing (l^{-1}) KH_2PO_4, 0.30 g; $(NH_4)_2HPO_4$, 0.25 g; $MgSO_4.7H_2O$, 0.14 g; $CaCl_2$, 50 mg; NaCl, 25 mg; $ZnSO_4$, 3.0 mg; ferric EDTA, 12.5 mg; thiamine, 0.13 mg; glucose, 5.0 g (pH adjusted to 5.0–5.5 prior to adding ferric EDTA and autoclaving). Following growth in the dark at 23°C, three replicate cultures of each genotype were harvested every 3 days for 27 days by manually removing mycelia from the media, blotting briefly on absorbent paper and drying overnight at 80°C before biomass was determined. Growth curves were then constructed by plotting mean biomass production against time.

All genotypes were grown on low CN MMN agar medium from which glucose had been omitted, and 13 g l^{-1} agar added, for 21 days prior to commencement of the carbon utilisation experiment. For the experimental treatments, glucose-free low CN MMN liquid medium was supplemented with either sodium carboxymethyl cellulose (CMC) (Sigma), Avicel PH 101 cellulose (Sigma), D-cellobiose (Sigma), D-glucose (Sigma), D-galactose (Sigma), D-mannose (Sigma), starch (Fisons) or birchwood xylan (Roth) to a final concentration of 2 g C l^{-1}. A glucose-free treatment was included and used to determine growth from carbon stored in plugs of fungal inoculum. For all treatments, two plugs of inoculum (5 mm diameter) were excised from the leading edge of actively growing colonies on agar medium and inoculated into 9-cm diameter Petri dishes containing 25 ml liquid medium. All treatments were replicated five times and cultures were incubated in the dark at 23°C for 12 days and harvested as outlined above.

Mean data for each genotype from the glucose-free treatments were subtracted from raw data obtained for all treatments prior to data analyses. Data analyses were conducted using the Minitab v. 12.23. All data were analysed for normal distribution and, where required, were normalised using logarithmic transformations. Data were analysed using one-way ANOVA and significant differences between genotypes on individual substrates and between treatments for each genotype were determined using Fisher's LSD procedure. Significant differences between pooled mean biomass yields for taxa I and II were analysed using a two-sample t-test.

Results

Growth curves for the 12 genotypes fitted expected patterns of fungal growth in batch culture (Fig. 1.) (Meletiadis et al. 2001) and all were harvested after 12 days growth (during the exponential phase) for the carbon utilisation experiments.

With the exception the six genotypes of taxon II on CMC, all genotypes of the two putative taxa produced measurable biomass on all carbon sources tested (Fig. 2, Fig. 3). The Avicel cellulose substrate was insoluble and adhered to hyphae at the time of harvesting, increasing the apparent biomass yield of all genotypes on this substrate (Fig. 3). For this reason, growth on cellulose was not compared to other substrates. From visual observation of this treatment, however, it was clear that mycelial growth for all genotypes was considerably greater than in the no carbon treatment, indicating that all genotypes of both taxa utilised cellulose. Significant variation in biomass yield was observed between genotypes 1–12 on all other carbon substrates (Fig. 2, Fig. 3). Comparison of individual genotypes of either taxon I or II, indicated significant intraspecific variation in biomass production on all substrates (Fig. 2, Fig. 3).

Biomass production by all genotypes on glucose and mannose was significantly ($P<0.05$) higher than on the remaining substrates. Although all genotypes utilised galactose as a carbon source (Fig. 2), biomass production on this substrate was significantly ($P<0.05$) lower than on either glucose or mannose for all genotypes. Only genotypes 1–6 (taxon I) produced measurable biomass on CMC; however, significant variation was observed between these genotypes with, for example, genotype 3

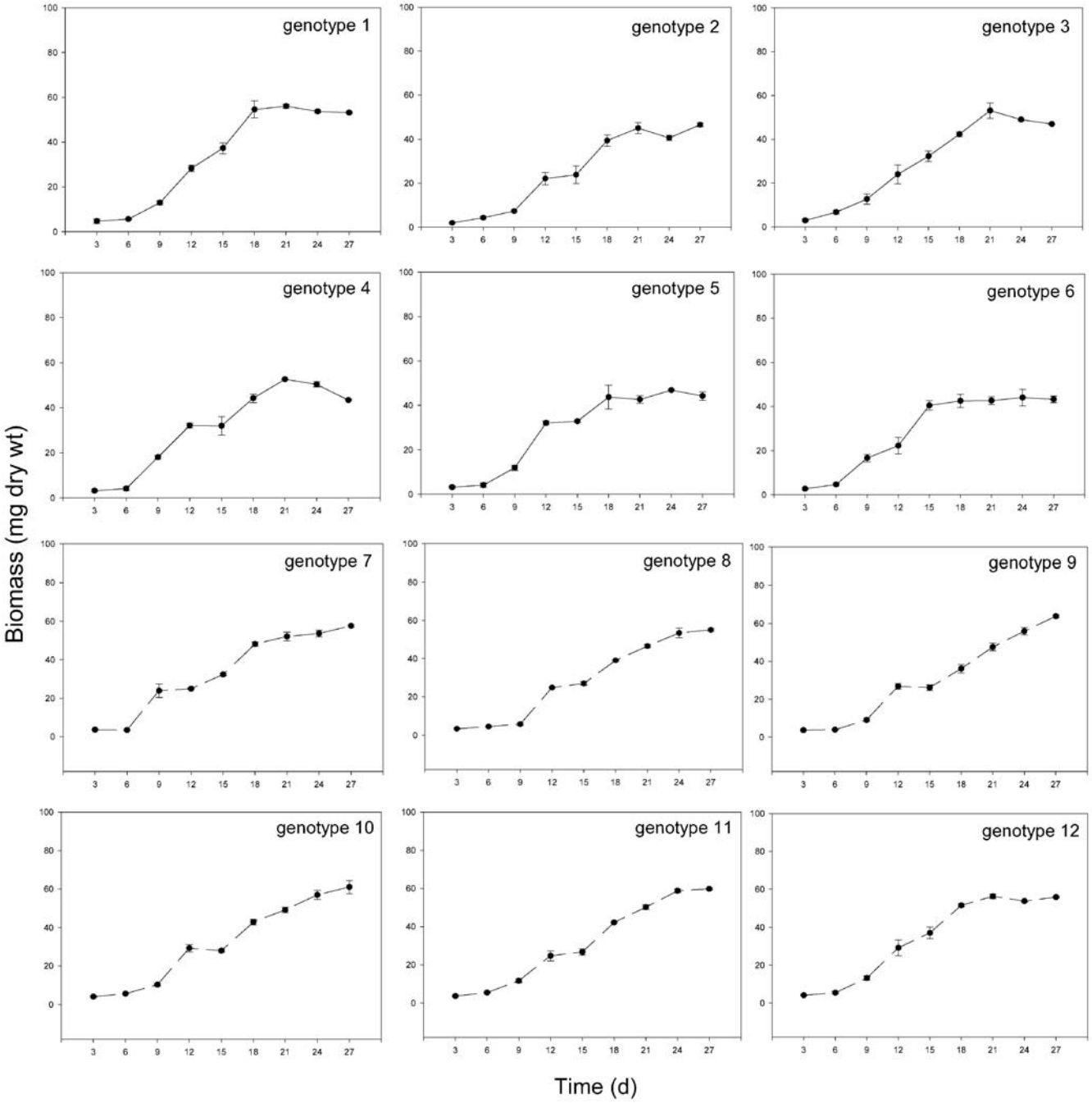

Fig. 1 Mean biomass production (± SE) over time for 12 genotypes of taxon I (1–6) and II (7–12) when grown for 27 days on a low-carbon and nitrogen version of modified Melin Norkrans liquid medium

producing ca. 48 times more biomass than genotype 5 (Fig. 3). Biomass yields for all genotypes on starch and xylan were significantly (P<0.05) lower than on glucose or mannose; however, all genotypes produced measurable biomass on both carbon sources (Fig. 3). Clearing of insoluble xylan was observed for all genotypes at the time of harvesting, further emphasising their ability to utilise xylan as a sole carbon source. Variation in biomass yield between genotypes on starch and xylan was considerable, and in some cases spanned an order of magnitude (Fig. 3).

In order to compare overall carbon substrate utilisation by the two taxa, raw data from genotypes 1–6 (taxon I) and genotypes 7–12 (taxon II) were pooled and mean values determined. Mean values for pooled data indicated that, in addition to CMC, taxon I genotypes produced significantly (P<0.05) higher mean biomass yields on glucose, cellobiose and starch than taxon II genotypes (Table 1). Mean biomass yields did not differ significantly between the two taxa on the other substrates (Table 1).

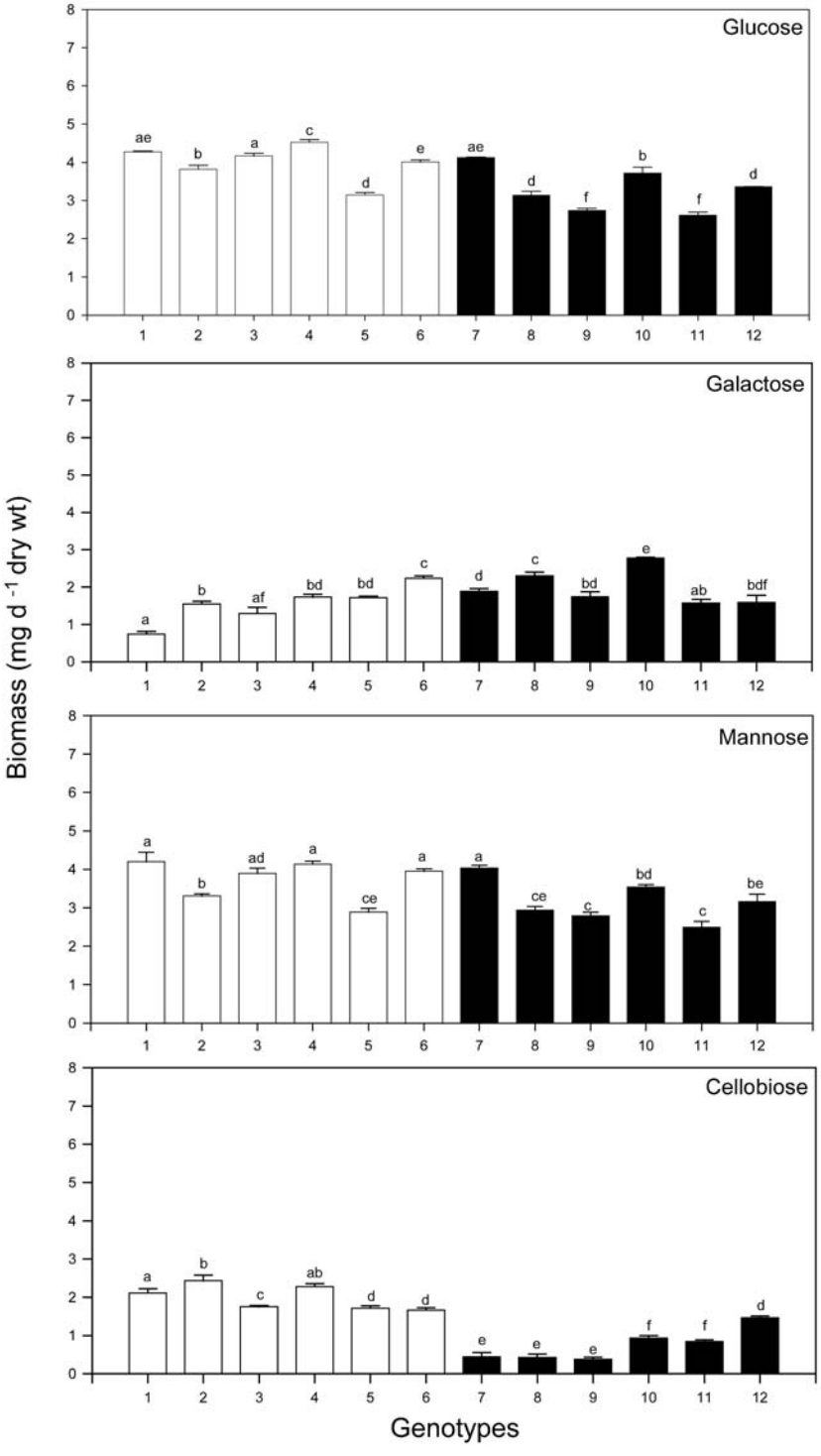

Fig. 2 Mean biomass production (± SE) by 12 genotypes of taxon I (unshaded) and II (shaded) in liquid media containing glucose, galactose, mannose or cellobiose. Letters on columns indicate significant differences (P <0.05) between genotypes of the same taxon within a treatment determined using Fisher's pair-wise comparisons

Table 1 Mean biomass yields (mg dry wt. day $^{-1}$ ± SE) on various carbon sources for each taxon derived from pooled data. Significant differences were determined using the two-sample *t*-test (*ns* not significantly different)

Treatment	Taxon I	Taxon II	Significance
Glucose	3.989 ± 0.06	3.284 ± 0.06	<0.05
Galactose	1.542 ± 0.11	1.974 ± 0.12	ns
Mannose	3.728 ± 0.08	3.158 ± 0.07	ns
Cellobiose	1.992 ± 0.08	0.746 ± 0.06	<0.001
Starch	0.931 ± 0.03	0.304 ± 0.04	<0.05
Xylan	1.469 ± 0.50	0.733 ± 0.06	ns

Discussion

With the exception of the six genotypes of taxon II on CMC, all genotypes produced measurable biomass on all cell wall-related carbohydrates as sole sources of carbon. Biomass production on glucose or mannose was generally higher than on other substrates. In contrast, despite its simple chemical structure, galactose was a relatively poor substrate for growth. In common with other filamentous fungi, some isolates of northern hemisphere ericoid

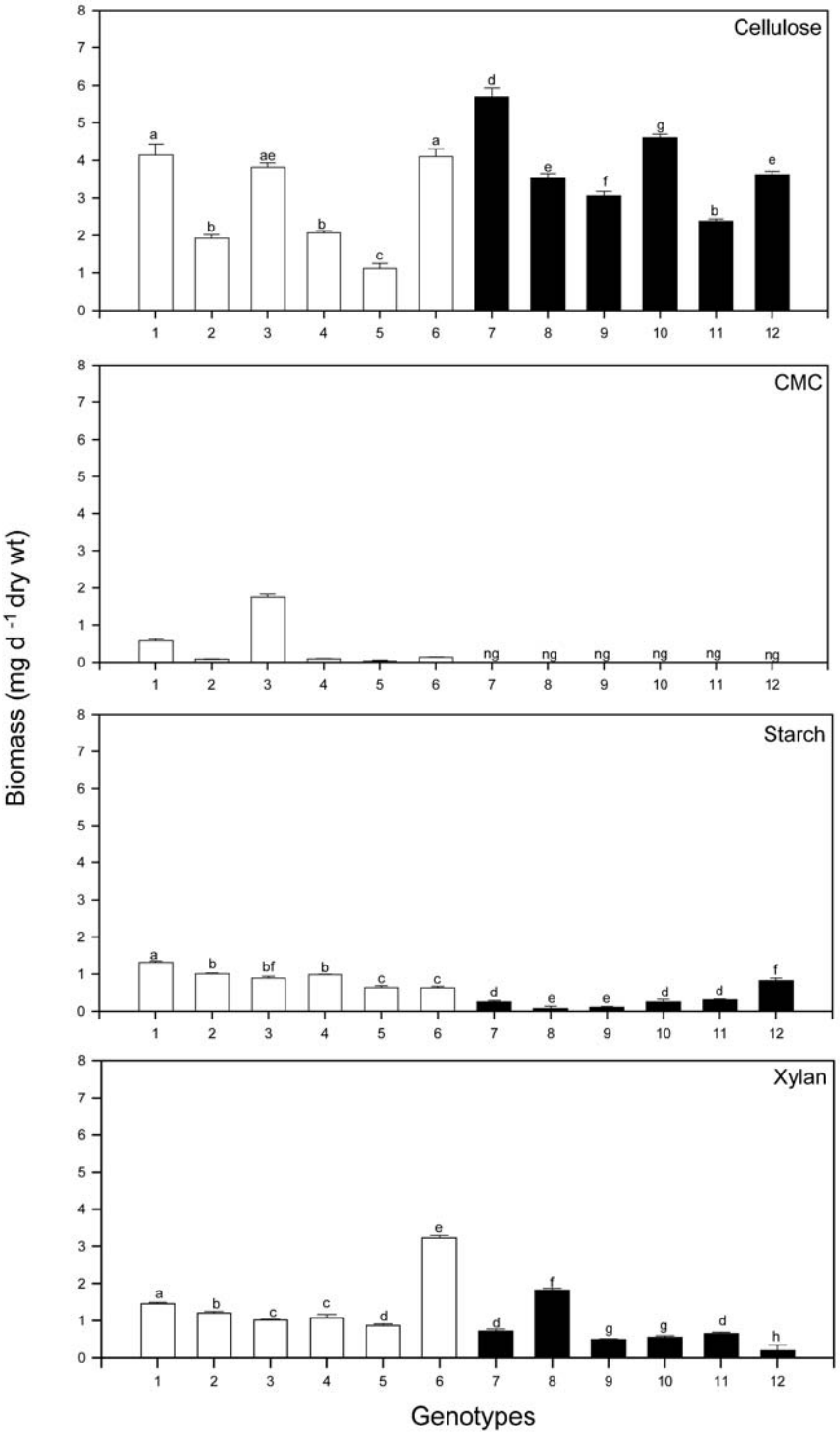

Fig. 3 Mean biomass production (± SE) by 12 genotypes of taxon I (unshaded) and II (shaded) in liquid media containing Avicel (cellulose), carboxymethylcellulose (CMC), starch or birchwood xylan (xylan). Letters on columns indicate significant differences (P <0.05) between genotypes of the same taxon within a treatment determined using Fisher's pair-wise comparisons (*ng* no measurable growth)

mycorrhizal endophytes can use galactose for growth, while others appear to be relatively deficient in this regard (Varma and Bonfante 1994; Jennings 1995). In non-mycorrhizal filamentous fungi, galactose appears to be absorbed via non-specific hexose transporters (Jennings 1995) and it is transported via such a transporter with lower efficiency than either glucose or mannose in the ectomycorrhizal basidiomycete *Amanita muscaria* (L.:Fr)Pers. (Wiese et al. 2000). Thus it seems likely that the hexose transport system(s) of the two ericoid mycorrhizal fungal taxa from the present study have a similar low affinity for galactose.

As has been demonstrated previously for the ericoid mycorrhizal taxa *H. ericae* and *Oidiodendron maius* Barron (Pearson and Read 1975; Thormann et al. 2002), all genotypes of the two putative taxa utilised starch for growth, presumably as a result of amylase activity. All genotypes also grew on xylan as a sole carbon source,

with clarification of the xylan suspension in the medium indicating complete degradation of xylan. An isolate of *H. ericae* has also been shown to degrade xylan in axenic culture and production of xylanolytic enzymes has been studied in detail in this isolate (Burke and Cairney 1997b). This process is mediated by 1-4-β-xylanase and β-D-xylosidase, along with a range of accessory enzyme activities in *H. ericae* (Burke and Cairney 1997a, 1997b) and a similar xylanase complex would be required for complete xylan degradation in the endophytes from *W. pungens* and *L. parviflorus*.

Complete hydrolysis of cellulose by fungi requires the synergistic action of endo-acting β-1,4-glucanohydrolase and cellobiohydrolase activities, along with β-glucosidase activity that hydrolyses the resulting cellobiose to glucose (Tuohy et al. 2002). All three enzyme activities have been demonstrated for *H. ericae* (Burke and Cairney 1997a) and growth of all RFLP-type I genotypes on crystalline cellulose, CMC and cellobiose indicates that this putative taxon also produces a complete complex of cellulolytic enzymes. Similarly, growth of the RFLP-type II genotypes on crystalline cellulose and cellobiose suggests production of a complete cellulase complex; however, the failure of these genotypes to grow on CMC may reflect differences in the cellobiohydrolase activities produced by the two RFLP-types. This appears to be the case in non-mycorrhizal ascomycetes, with cellobiohydrolases of certain taxa being able to hydrolyse crystalline cellulose such as Avicel, but unable to hydrolyse soluble cellulose derivatives such as CMC (Tuohy et al. 2002). While detailed analyses of enzyme activities will be required for confirmation, this may explain the observed difference between the RFLP-type I and VI genotypes.

The two putative taxa used in this investigation were the dominant ericoid mycorrhizal fungi in root systems of individual epacrids at the field site (Midgley et al. 2002; DJ Midgely, SM Chambers, JWG Cairney, unpublished data). Significant intraspecific variation was observed for genotypes of each putative taxon. With the exception of taxon I genotypes on CMC, however, variation in biomass production on the various substrates at the intraspecific level was considerably less than fourfold and is similar to the level of intraspecific variation observed for multiple *H. ericae* isolates on various nitrogen sources (Cairney et al. 2000). Comparison of pooled data for six genotypes of each putative taxon indicated that, although significantly different on certain substrates, mean biomass yields on all substrates differed by a factor of less than ca. three, suggesting that their saprotrophic capabilities may be broadly similar.

The data we have presented imply that the two putative taxa of ericoid mycorrhizal fungi from Australian eparcrids produce a number of cellulolytic and hemicellulolytic enzyme activities that have been reported previously for an isolate of *H. ericae*. Such activities, while undoubtedly important in cell wall penetration during establishment of the ericoid mycorrhizal association with their epacrid hosts (Perotto et al. 1995), may also bestow a degree of saprotrophic ability on these fungi. As such, the data add support to the hypothesis that ericoid mycorrhizal associates of epacrids are broadly functionally analogous to those of northern hemisphere Ericaceae (Cairney and Ashford 2002). Given the apparent seasonality of hair root production and/or ericoid mycorrhizal infection in epacrids (see Cairney and Ashford 2002), aside from contributing to nutrient acquisition, the ability to derive carbon from complex substrates may facilitate saprotrophic persistence of the mycorrhizal endophyte mycelia in soil and form an important source of inoculum during periods of active hair root growth.

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