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ORIGINAL ARTICLE

Differences in carbon source utilisation by orchid mycorrhizal fungi from common and endangered species of *Caladenia* (Orchidaceae)

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Abstract Terrestrial orchids depend on orchid mycorrhizal fungi (OMF) as symbionts for their survival, growth and nutrition. The ability of OMF from endangered orchid species to compete for available resources with OMF from common species may affect the distribution, abundance and therefore conservation status of their orchid hosts. Eight symbiotically effective OMF from endangered and more common *Caladenia* species were tested for their ability to utilise complex insoluble and simple soluble carbon sources produced during litter degradation by growth with different carbon sources in liquid medium to measure the degree of OMF variation with host conservation status or taxonomy. On simple carbon sources, fungal growth was assessed by biomass. On insoluble substrates, ergosterol content was assessed using ultra-performance liquid chromatography (UPLC). The OMF grew on all natural materials and complex carbon sources, but produced the greatest biomass on xylan and starch and the least on bark and chitin. On simple carbon sources, the greatest OMF biomass was measured on most hexoses and disaccharides and the least on galactose and arabinose. Only some OMF used sucrose, the most common sugar in green plants, with possible implications for symbiosis. OMF from common orchids produced more ergosterol and biomass than those from endangered orchids in the Dilatata and Reticulata groups but not in the Patersonii and Finger orchids. This suggests that differences in carbon source utilisation may contribute to differences in the distribution of some orchids, if these differences are retained on site.

Keywords *Caladenia* · Mycorrhiza · Orchid · Sebacinales · Carbon nutrition · Conservation

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Introduction

Orchids and their symbiotic fungi

Terrestrial orchids are among the most vulnerable components of major plant communities worldwide (Batty et al. 2002). Orchid seeds are dust-like and have negligible amounts of food reserves, making them highly dependent on external food sources. During early stages of development, all orchids depend on limited invasion by compatible orchid mycorrhizal fungi (OMF) to provide carbon sources for their nutrition and to stimulate germination and further development (Rasmussen 1995; Smith and Read 2008). Even as mature plants, natural abundance studies ($\delta^{13}C$ and $\delta^{15}N$) (pioneered by Gebauer and Meyer 2003) have shown that many green orchids depend on their OMF as a significant source of carbon and nitrogen (reviewed by Hynson et al. 2013) and are thus partially mycoheterotrophic (Merckx 2013).

Caladenia species are terrestrial orchids, about 95 % of which are endemic to Australia (Backhouse and Jeanes 1995; Dixon and Hopper 2009; Dixon and Tremblay 2009). In SE Australia, 40 % of *Caladenia* species are endemic (Jeanes and Backhouse 2006). The species' conservation status ranges from common and widespread to vulnerable and

nationally endangered (Australian Government 1999; DEWLP Department of Environment, Water, Land and Planning 2016). In the genus *Caladenia*, the spider orchids and the finger orchids together comprise about 70 % of the species (Hopper and Brown 2004; Hopper 2009). The spider orchids have a much greater proportion of nationally endangered species (E) (32 %) than the finger orchids (0 % E) (Australian Government 1999). Jeanes and Backhouse (2006) identified four morphological groups within the spider orchids in the Australian State of Victoria (SE Australia): Dilatata, Reticulata, Patersonii and Cardiochila. These groups differ in their conservation status, where E = nationally endangered (Australian Government 1999) and T = threatened (DEWLP Department of Environment, Water, Land and Planning 2016): Dilatata (20 % of species E, 30 % of species T), Reticulata (35 % E, 59 % T), Patersonii (25 % E, 83 % T) and Cardiochila (0 % E, 0 % T). The reasons for these differences are not clear, as endangered and common species of *Caladenia*, even from the same morphological group, can occur together at the same site.

Caladenia species have no true roots, making them especially dependent on their OMF for nutrition (Smith 1966; Harley and Smith 1983). The ability of an OMF to compete for and use insoluble and soluble carbon compounds from sources external to the orchid may affect the ability of its host orchid to survive and thrive. The most common OMF isolated from *Caladenia* species belong to the Sebacinales (Agaricomycetes, Basidiomycota) (Warcup 1981; Bougoure and Cairney 2005; Shefferson et al. 2005). In particular, the '*Sebacina vermifera*' complex associated with *Caladenia* species falls into group B of Weiß et al. (2004). This has now been proposed formally as a new family (the Serendipitaceae) within the Sebacinales (Weiß et al. 2016) based on the genus *Serendipita* (Roberts 1993). Most OMF associated with the largely green Orchidoideae (to which *Caladenia* belongs) are believed to be saprophytic basidiomycetes that acquire their carbon nutrition by degrading the surrounding litter (dead organic matter) in the orchid's natural habitat (Curtis 1939; Kristiansen et al. 2001; Bidartondo et al. 2004; Rasmussen and Rasmussen 2007; Sommer et al. 2012). However, this has not been shown experimentally and both Hynson et al. (2013) and Weiß et al. (2016) have called for further attention to be paid to the saprotrophic abilities of Sebacinales and OMF in general. Endangered *Caladenia* species have much narrower specificity than common species (Wright et al. 2010) and the saprotrophic abilities of their OMF are particularly critical when both OMF and their orchid hosts are co-located on the same site.

OMF and litter

In their natural habitats, orchids are frequently surrounded by litter, largely comprising wood, bark and leaves. The relative abilities of OMF to degrade these and grow on the degradation products may have ecological implications for their orchid hosts. Large amounts of litter are in the form of plant cell wall components (Perotto et al. 1997); 90 % of plant cell wall components consist of the three major polysaccharides: cellulose, hemicellulose (mainly substituted xyloglucans) and pectin (mainly arabinogalactans, xylogalacturonans and rhamnogalacturonans) (De Vries and Visser 2001). Leaf litter of the dominant *Eucalyptus* species comprises on average 178 g kg^{-1} lignin, 127 g kg^{-1} cellulose and 165 g kg^{-1} hemicelluloses with a total of 530 g kg^{-1} C and a C:N ratio of 38:1 (Guo et al. 2002; Harrop-Archibald et al. 2016). These values are within the ranges for boreal forest litter (Berg and McClaugherty 2014). Cell wall degradation results ultimately in the release of the pentoses arabinose and xylose, the hexoses galactose, glucose and mannose, the 6-deoxy hexose rhamnose and transitionally the disaccharide cellobiose (which is broken down into glucose).

Despite the common assumption that *Caladenia* OMF obtain nutrition for their host orchids by digesting litter components, there has been no systematic, critical investigation of their ability to grow on litter components. Previous studies on northern hemisphere OMF from different orchid genera found that the complex carbon sources pectin, xylan, cellulose, starch and tannic acid were well utilised, as were the sugars xylose, arabinose, cellobiose, mannose, maltose, glucose and galactose (Burgeff 1909; Holländer 1932; Hadley and Perombel 1963; Smith 1966; Nieuwdorp 1972; Barroso et al. 1985; Rasmussen 1995; Zelmer et al. 1996). Their applicability to OMF from *Caladenia* spp. is unknown. Furthermore, some used solid media despite the ability of OMF to grow on water agar (Midgley et al. 2006). By contrast, there have been only a few investigations on the ability of OMF from Australian orchids to grow on complex carbon compounds likely to be available in litter (Midgley et al. 2006; Wright et al. 2011; Nurfadilah et al. 2013). These investigated the ability of OMF from nine orchids (including two common species of *Caladenia*) to utilise 12 carbon sources. Qualitatively, the profile of carbon sources utilised well or poorly was similar: cellulose, xylan, starch, cellobiose, maltose, glucose, mannose and fructose were utilised well; pectin, chitin, carboxymethylcellulose (CMC), sucrose, galactose and xylose were variable; and arabinose and tannic acid were poorly utilised. This implies that Australian orchid OMF effectively utilise some, but not all, of the soluble products produced during degradation of complex substrates present in the environment to simpler soluble forms from which they derive energy. Quantitatively, however, there were significant differences. Nurfadilah et al. (2013) concluded that the final biomass of the OMF from the nationally endangered *Drakaea elastica* was so much smaller than the biomass of OMF from the common *Pterostylis recurva*, *Caladenia flava* and *Diuris corymbosa* that its slow, uncompetitive growth

could at least partially explain the nationally endangered conservation status of its orchid host.

In Australian orchid habitats, which are primarily woodlands dominated by *Eucalyptus* species, OMF face competition from several microorganisms in their natural habitats, which include competitors that can degrade litter. These include other OMF, ericoid mycorrhizal fungi (ERM) and ectomycorrhizal fungi (ECM) as well as saprophytes. OMF from four *Pterostylis* species (expected to belong to *Ceratobasidium*) were more competitive than ERM isolates from the Australian *Woollsia pungens* on all except chitin and tannic acid (Midgley et al. 2006). ECM are the dominant mycorrhiza on most woody shrubs and may also compete for litter components (Midgley et al. 2006), as may saprophytic fungi. Bacteria may also be competitive for the xylans and their degradation products (Burges 1958; Garrett 1963; Moore et al. 2011).

Aim

Endangered and common *Caladenia* species co-occur in heathland and woodland vegetation communities. If OMF associated with endangered *Caladenia* species are out-competed by OMF from endangered *Caladenia* species in a common niche, this may contribute to their threatened status. This study compared the ability of OMF from eight *Caladenia* species to utilise a broad range of carbon sources usually found in their habitats: natural materials, complex (mostly insoluble) and simple (soluble) compounds. The aim was to determine if the ability of the OMF to utilise carbon compounds potentially available in litter varied with orchid conservation status and/or morphological group as defined by Jeanes and Backhouse (2006).

Materials and methods

OMF isolates and *Caladenia* species

Fungi and orchid hosts were chosen to study the effects of fungus, plant conservation status and plant group on carbon source utilisation. Symbiotically effective OMF from four matched pairs of endangered and more common *Caladenia* species in Victoria were obtained from four traditional groups of *Caladenia* species (Table 1). The Patersonii group has few common species (none in Victoria) and so *Caladenia venusta* (rare) was used (Jeanes and Backhouse 2006). By contrast, the finger orchids include few nationally endangered species and so *Caladenia maritima* (designated as endangered only in Victoria) was used (Jones 1999; DEWLP Department of Environment, Water, Land and Planning 2016).

Six OMF cultures were obtained from culture collections and two by isolating the fungi afresh from orchids (*C. fulva* and *Caladenia carnea* var. *carnea*) (Table 1). For fresh isolates, the stem-collar region was surface-sterilised with 0.5 % NaOCl for 3 min, dissected longitudinally and single pelotons extracted, rinsed with seven changes of sterile Milli-Q water (Raleigh 2005) and plated on to fungal isolation medium (FIM) with 0.05 % streptomycin sulphate (Clements et al. 1986). Fungi that grew were assayed for symbiotic effectiveness on seed of the host species and effective isolates selected on the basis of their continued support of orchid growth and development. All cultures were maintained routinely on Oxoid malt extract agar (MEA).

Insoluble and soluble carbon sources investigated for OMF utilisation

Eleven complex and 12 soluble carbon sources were investigated as likely to be found in the litter in the orchids' environments (roots, bark and leaves from mixed species of eucalypts and shrubs), their constituent insoluble components (e.g. cellulose) and their breakdown products (e.g. disaccharides and monosaccharides) (Table 2). In particular, branchlets (shoots) of casuarina [*Allocasuarina verticillata* (Lam.) L. A. S. Johnson] were chosen because they are commonly favoured as a mulch by native orchid growers [Richard Thompson, Australian Native Orchid Society (ANOS), pers. comm.]. All solid natural materials were cleaned in running water, air-dried and both they and complex insoluble components were ground to a fine powder before use. All pure chemicals were at least AnalaR or equivalent.

Fungal growth experiments

Liquid low carbon-nitrogen Modified Melin-Norkrans medium (CN MMN) (Marx and Bryan 1975) was used to assay the ability of the eight OMF to use the complex and soluble carbon sources. Low CN MMN was adjusted to pH 5.5, sterilised by autoclaving at 121 °C for 20 min and dispensed at 30 mL per plate into deep Petri-plates. All carbon sources were added at a concentration of 2 g L^{-1}. Insoluble carbon sources were added before autoclaving, whereas soluble carbon sources were added after autoclaving by filter-sterilising concentrated aqueous solutions into sterile medium. The negative control had no carbon source added. There were four replicate plates per treatment, each of which was extracted and analysed separately.

To inoculate media, fungi were initially raised on oatmeal agar (OMA) at 25 °C and fungal cubes (5 mm × 5 mm) were excised and transferred randomly to liquid media (three cubes per plate). Plates were randomised for incubation. All plates were incubated statically at 25 ± 2 °C in the dark (covered with aluminium foil) for 4 weeks.

For complex carbon sources, the intermingled fungi and remains of carbon sources were harvested by vacuum filtration through GF/C (glass fibre) filters (ADVANTEC®, GC-50, 25 mm), rinsed with 20 mL volumes of sterile Milli-Q water, freeze-dried in a Dynavac freeze-drier (Model FD12) and

Table 1 Details of taxonomic group, conservation status, location and accession numbers for orchid mycorrhizal fungi isolated from *Caladenia* species

Group[a]	Conservation status			*Caladenia* species	Strain	Location in Victoria	Isolated by	Accession date	Obtained from	Accession number
	FFG[b] (1988)	EPBC[c] (1999)	DEPI[d] (2014)							
Dilatata	L	EN	e	*C. amoena* D. L. Jones	CAMR36	Wattle Glen	Ruth Raleigh	11/6/2006	RBG Melbourne	060848, 061393
	–	–	–	*C. phaeoclavia* D. L. Jones	KPU1D	Kinglake National Park	Tien Huynh	15/10/2010	Tien Huynh	N/A
Reticulata	L	EN	e	*C. robinsonii* G. W. Carr	CARO31	N/A	RBG Sydney	N/A	RBG Melbourne	CARO31
	–	–	–	*C. clavigera* A. Cunn. ex Lindl.	R12	Dodd Street, St Andrews	Ruth Raleigh	6/11/2006	RBG Melbourne	N/A
Patersonii	L	EN	e	*C. fulva* G. W. Carr	B110	Deep Lead Flora and Fauna Reserve, Stawell	Shalika Mehra	22/9/2008	Ann Lawrie	N/A
	X	–	r	*C. venusta* G. W. Carr	R9	Victoria Forest Road, Anglesea	Ruth Raleigh	11/6/2006	RBG Melbourne	61403
Finger (Carnea)	–	–	e	*C. maritima* D. L. Jones	CMA2	Victoria Forest Road, Anglesea	Richard Thomson	12/31/2007	RBG Melbourne	71226
	–	–	–	*C. carnea* var. *carnea* R. Br.	CC1	Kinglake National Park (Mt Everard Block)	Shalika Mehra	22/9/2010	Ann Lawrie	N/A

Where no category is shown, the species is considered secure in that jurisdiction by that source

N/A not available, RBG Royal Botanic Gardens

[a] According to: Jeanes and Backhouse (2006)

[b] Victorian Flora and Fauna Guarantee Act (Victorian Government 1988), *L* listed, *X* nominated but rejected for listing as threatened; taxon ineligible

[c] Environment Protection and Biodiversity Conservation Act (Australian Government 1999), *EN* nationally endangered

[d] Department of Environment and Primary Industries (DEWLP Department of Environment, Water, Land and Planning 2016), *e* endangered (Victoria), *r* rare. *RBG* Royal Botanic Gardens

stored at −20 °C for ergosterol extraction. For soluble carbon sources, mycelia were harvested by vacuum filtration and rinsed as before but then oven-dried overnight at 80 °C and weighed. The final pH of the culture fluid was also measured.

Ergosterol quantification for complex carbon sources

For complex carbon sources, ergosterol content was measured to solve the problems previously encountered of measuring OMF growth on insoluble substrates such as crystalline cellulose and chitin (Midgley et al. 2006; Wright et al. 2011; Nurfadilah et al. 2013). Ergosterol is an essential sterol specific for fungi, some microscopic algae and protozoa (Mille-Lindblom et al. 2004) and is measured after extraction by high performance liquid chromatography (HPLC) using its single ultraviolet absorbance peak at 282 nm (Seitz et al. 1979). UPLC has been reported as superior to HPLC (Swartz 2005) and was tested here for the first time with OMF.

The sterol extraction method was adapted from various sources (Arthington-Skaggs et al. 1999; Charcosset and Chauvet 2001; Pinto et al. 2011). Samples were kept in the dark as far as possible to prevent destruction of the photolabile ergosterol. The freeze-dried filters with adhering materials (fungi and remaining carbon sources) were extracted with 3 mL of 25 % alcoholic potassium hydroxide (25 g KOH + 35 ml of sterile distilled water brought to 100 mL with 100 % ethanol), vortexed vigorously for 1 min, incubated at 85 °C for 60 min and cooled to room temperature overnight. Sterile distilled water (1 mL) was added along with 3 mL of n-heptane, followed by vigorous vortexing for 3 min. Suspensions were left to partition overnight and the organic phase (n-heptane) was transferred to small glass vials for evaporation to dryness under nitrogen. Extracted sterols were re-dissolved

Table 2 Complex carbon sources investigated for orchid mycorrhizal fungi utilisation

Treatment	Abbreviation	Insoluble carbon source	Where collected/ purchased
1	NC	None	N/A
2	CS	*Allocasuarina verticillata* branchlets	RMIT University, Bundoora Campus, VIC
3	RT	Roots (*Eucalyptus*, *Allocasuarina*, *Acacia*, assorted shrubs, grasses)	Cardinia Dam (opposite Aura Vale Lake Park), Wellington Road, VIC
4	BK	Bark (*Allocasuarina verticillata*)	RMIT University, Bundoora Campus, VIC
5	LF	Litter (*Eucalyptus leucoxylon*)	RMIT University, Bundoora Campus, VIC
6	KT	Tissue paper	Kleenex tissue
7	FP	Filter paper	Whatman No. 1
8	XB	Xylan	From beechwood (Sigma Aldrich)
9	CM	Cellulose	Microcrystalline cellulose (Sigma Aldrich)
10	PS	Starch	Potato starch (BDH)
11	PE	Pectin	Apple pectin (Sigma Aldrich)
12	CH	Chitin	(Poly-1-4)-β-N-acetyl-D-glucosamine (from crab shells) (Sigma Aldrich)

in 1 mL of dichloromethane/isopropanol (100/1) (Sigma-Aldrich Chromasolv® Plus for HPLC, ≥ 99.9 %) and filtered through 0.22 μm Teflon (PTFE) syringe filters (13 mm) (Labquip) into 2 mL amber screw-top glass vials for quantification by UPLC and HPLC.

Ergosterol in the samples was analysed by UPLC on a Waters Acquity UPLC system using a reverse-phase C18 column (1.7 μm particle size, 2.1 mm × 50 mm). Samples of 1 μL were injected using an auto-sampler. Methanol was used as the mobile phase at a flow rate of 0.65 mL min^{-1}. Ergosterol was detected by absorbance at 282 nm with a retention time of 0.55 min. Standards were run on the UPLC before each batch of samples to produce a standard curve. A stock solution of the ergosterol standard (Sigma ≥95.0 %) was prepared at a concentration of 1 g L^{-1} in dichloromethane:isopropanol (99.5:0.5, *v*/*v*) (Tardieu et al. 2007). The linear relationship between peak area (y) and ergosterol concentration (x) was determined using four replicate 1-μL injections of five standards (0.5–20 mg L^{-1}) for each run. Four replicate samples were injected for each fungal strain grown on each of the complex carbon sources as well as for the negative control treatment. Peak area was integrated for the samples and standards and ergosterol concentration was calculated for OMF growth. The identity of the ergosterol peaks was also confirmed by (1) spiking the samples with ergosterol standards and (2) ultraviolet (UV) spectroscopy. All chemicals used were of high-performance liquid chromatography (HPLC) grade (Sigma-Aldrich). Growth on xylan was analysed by both d.wt (biomass) and ergosterol content to obtain a calibration of ergosterol: d.wt of fungi (Nylund and Wallander 1992).

Ergosterol in selected samples was also analysed by high-performance liquid chromatography (HPLC) on an Agilent 1100 using a reverse-phase C18 column (5 μm, 4.6 mm × 50 mm). Samples of 10 μL were injected using an auto-sampler. Methanol was used as the mobile phase at a flow rate of 1.5 mL min^{-1}. Ergosterol was detected by absorbance at 282 nm with the expected retention time of 3.3 min.

Statistical analysis

Data were first tested for normality and then square-root transformed to normality prior to statistical analysis using Minitab Version 16 (Minitab Inc., www.minitab.com). Analysis of variance was used to test for effects of carbon source, fungal species, orchid taxonomic grouping and conservation status on the ergosterol content, with post-hoc Tukey's family error tests to define differences between treatments. $p < 0.05$ was considered to be statistically significant. For complex carbon sources, linear regression was used to relate the ergosterol content and fungal dry mass of each isolate with the only soluble source, xylan. Linearity was tested by visual inspections of plots and R^2 values. For the combined data for utilisation of insoluble and soluble carbon sources, two multivariate analyses were used to group fungi: principal components analysis (PCA) and hierarchical cluster analysis (HCA) (standardised variables, complete linkage, squared Euclidean distance, similarity level 0.05). The ergosterol content or biomass of controls was subtracted from all treatments before analysis.

Results

Ergosterol analysis by UPLC

UPLC had a shorter retention time (0.55 min) than HPLC (3.3 min). Calibration curves of the peak area against the concentration of the ergosterol standard at 282 nm gave good linear responses with correlation coefficients (R^2) of 0.974–0.999. UV spectra of the extracts showed only one peak with a maximum at 282 nm. With xylan, there was a positive linear relationship between ergosterol and dry weight ($R^2 = 0.720$, $p < 0.001$) that resulted in a conversion factor of ergosterol (μg $plate^{-1}$): d.wt (mg

plate^{-1}) of 4.70 ± 0.64 (R^2 = 70.46 %, p < 0.001). Regression analysis with Minitab against dry weight and conservation status (using conservation status common/secure as the default) gave different conversion factors by conservation status in the equation:

$$\text{Ergosterol}\ (\mu\text{g}) = 27.8 + 1.39\ \text{d.wt}\ (\text{mg}) - 17.5\ \text{ConstatusE} + 26.4\ \text{ConstatusS} - 31.6\ \text{ConstatusR} \quad (R^2 = 73.5\%)\ (\text{ANOVA},\ F = 22.54,\ p < 0.001) \quad (1)$$

where ConstatusE = nationally endangered, ConstatusS = state-endangered and ConstatusR = state-rare. All factors were significant (p < 0.001–0.013). This implied that OMF from the orchids that were common and state-endangered had more ergosterol in their membranes than those from nationally endangered and state-rare ones.

Effect of carbon source

Fungi grew on all insoluble and soluble carbon sources, with a decrease in pH in proportion to the amount of ergosterol and fungal biomass produced (Figs. 1 and 2). The pectin treatment repeatedly became contaminated and results were omitted from the analysis. Two-way ANOVA with the factors carbon source and fungal isolate alone explained 90 % of the variation in the data (carbon source F = 189.39, p < .0.001; fungal isolate F = 44.18, p < 0.001; interaction F = 7.58, p < 0.001). The significant interaction between fungal isolate and carbon source showed that not all fungi behaved the same with each carbon source (Figs. 1 and 2).

Carbon source alone explained 62 and 66 % of the variation in ergosterol and fungal biomass respectively. The two complex carbon sources that resulted in most ergosterol were xylan and starch, followed by Kleenex tissue and casuarina branchlets, and the least were bark and chitin. Fungi produced as much ergosterol with the natural materials casuarina branchlets and leaf litter as with most forms of cellulose. The five soluble carbon sources that resulted in greatest biomass were glucose, fructose, mannose, cellobiose and maltose, and the least were galactose, CMC, arabinose and

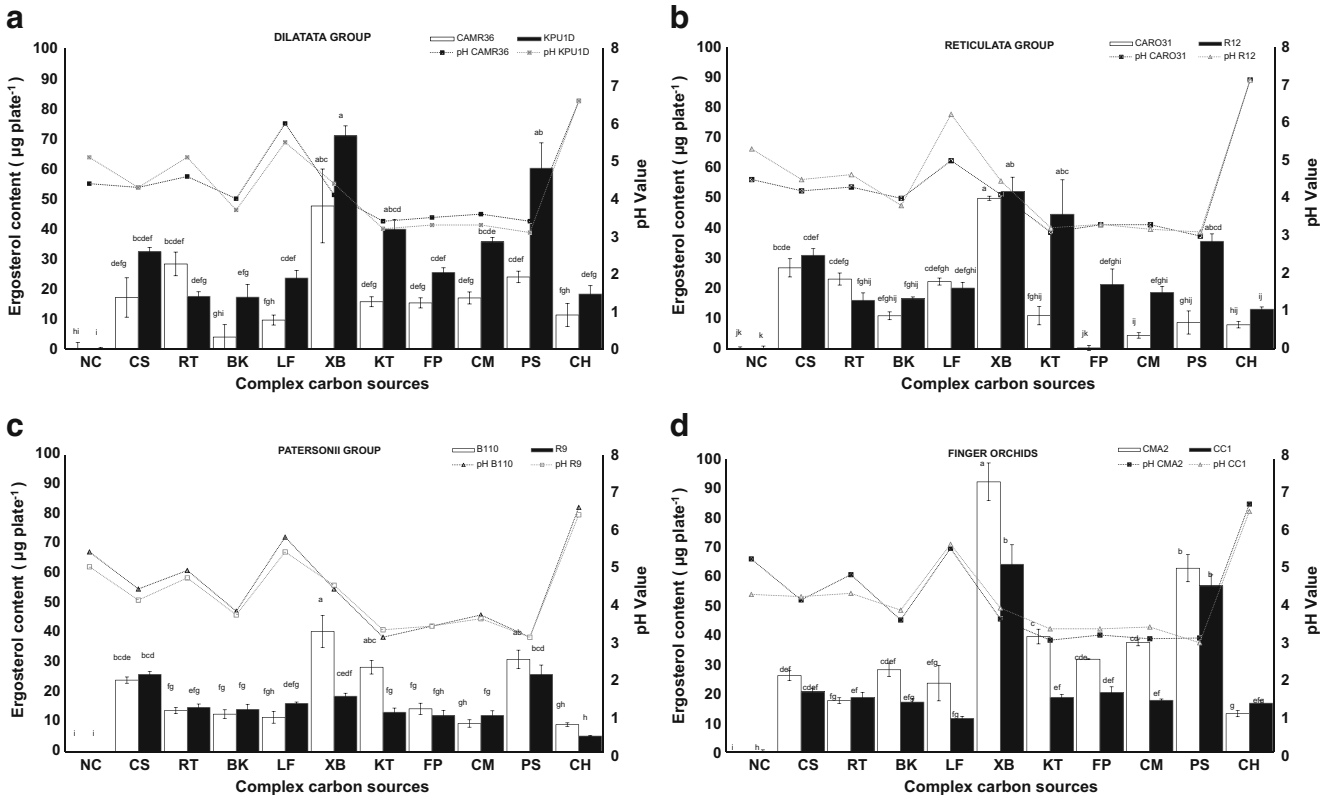

Fig. 1 Mean ergosterol yield (µg plate^{-1}) of eight orchid mycorrhizal fungi from eight *Caladenia* spp. from four *Caladenia* groups grown in liquid low carbon-nitrogen modified Melin-Norkrans media containing 2 g L^{-1} of each complex polysaccharide as the sole carbon source for 4 weeks at 25 °C and final pH of media. *Caladenia* groups: **a** Dilatata, **b** Reticulata, **c** Patersonii, **d** Finger orchids. *Bars* = 2 × standard error. *Columns without letters* in common indicate significant ($p \leq 0.05$) differences between yields of the fungal strains by ANOVA. Key: *NC* negative control, *CS* casuarina branchlets, *RT* roots, *BK* bark, *LF* litter, *KT* Kleenex tissue, *FP* Whatman filter paper, *XB* xylan (beechwood), *CM* microcrystalline cellulose, *PS* potato starch, *CH* chitin

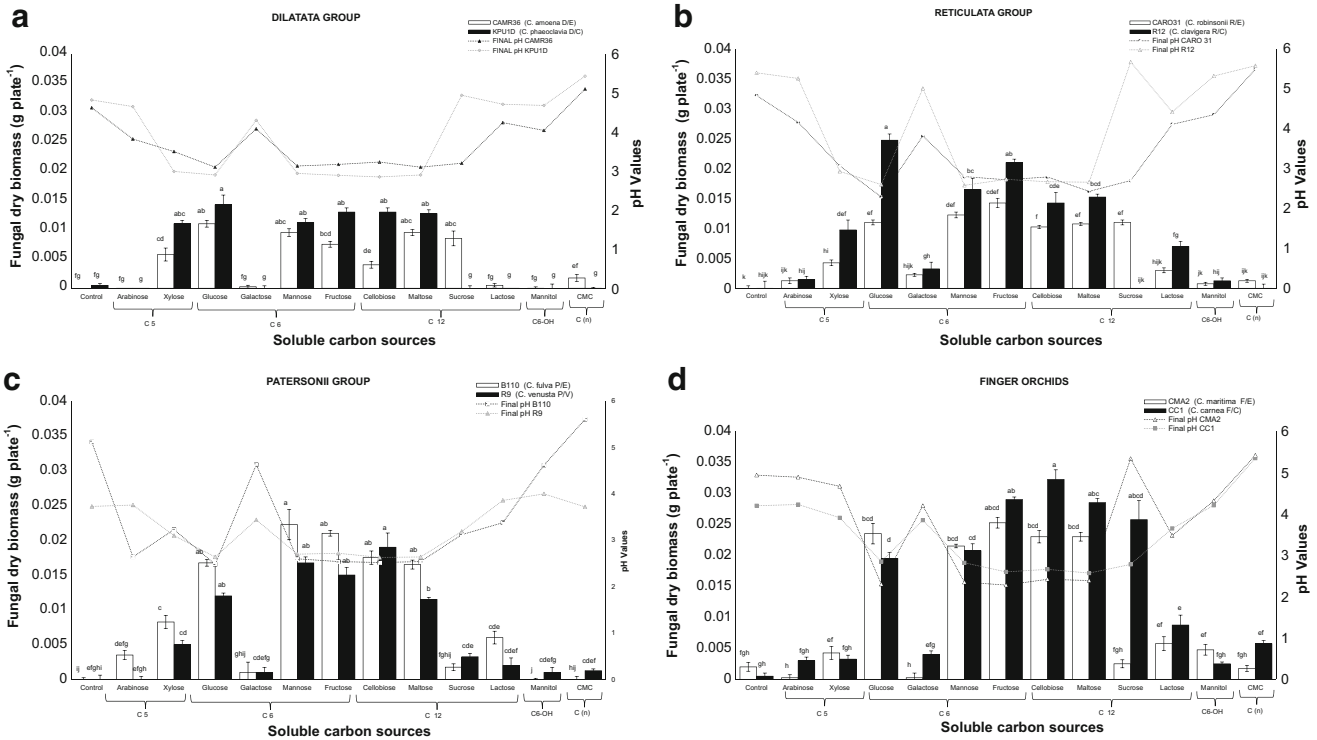

Fig. 2 **a**–**d** Mean fungal dry biomass (g plate^{-1}) of eight orchid mycorrhizal fungi from eight *Caladenia* spp. from four *Caladenia* groups grown in liquid low carbon-nitrogen modified Melin-Norkrans media containing 2 g L^{-1} of each simple soluble carbon source as the sole carbon source for 4 weeks at 25 °C and final pH of media. *Caladenia* groups: **a** Dilatata, **b** Reticulata, **c** Patersonii, **d** Finger orchids. *Bars* = 2 × standard error. *Columns without letters* in common indicate significant ($p \leq 0.05$) differences between yields of the fungal strains by ANOVA

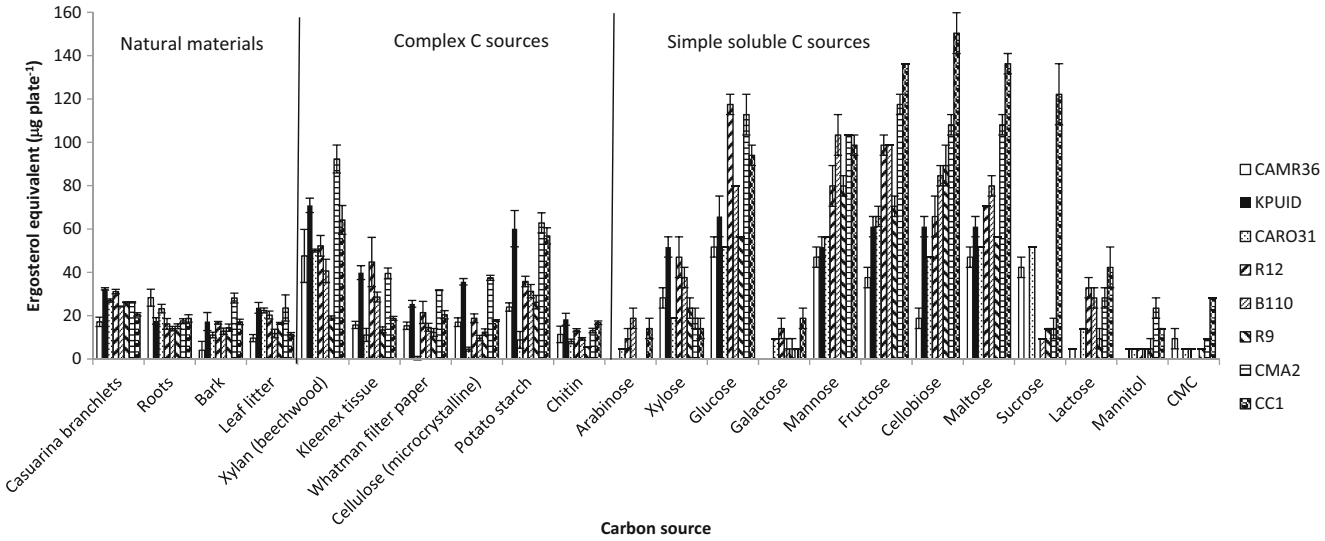

Fig. 3 Mean ergosterol equivalent (µg plate^{-1}) of orchid mycorrhizal fungi from eight *Caladenia* species after growth in liquid low carbon-nitrogen Modified Melin-Norkrans media with each carbon source for 4 weeks at 25 °C. Key to orchid host abbreviations: CAMR36 *C. amoena*, KPUID *C. phaeoclavia*, CARO31 *C. robinsonii*, R12 *C. clavigera*, B110 *C. fulva*, R9 *C. venusta*, CMA2 *C. maritima*, CC1 *C. carnea* var. *carnea*

mannitol. In the pentoses, xylose was relatively well utilised but not arabinose (Fig. 2). In the hexoses, fructose, glucose and mannose were well utilised but not galactose. In the disaccharides, cellobiose and maltose but not lactose were well utilised; sucrose either produced as much biomass as cellobiose and maltose or very little.

Using the conversion factor of 4.70 ± 0.64 μg mg^{-1} biomass (calculated from ergosterol content and biomass for xylan) showed that on a common scale (ergosterol equivalent biomass), growth on natural materials was less than that on their complex compounds, which was less than that on simple soluble carbon sources (Fig. 3).

Effect of fungus

Fungal isolate explained 9 and 14 % of the variation in ergosterol content and fungal biomass respectively. With complex carbon sources, the greatest amount of ergosterol was produced by CMA2 (from *C. maritima*) and was 40 % greater than the least (R9 from *C. venusta*) (Figs. 1 and 2). The greatest amount of ergosterol produced in any fungus-carbon source combination was from CMA2 (from *C. maritima*) with xylan and the least (apart from the negative control) from CAMR36 (from *Caladenia amoena)* with bark. CMA2 (from *C. maritima*) and the isolates from two common orchids (CC1 from *C. carnea* var. *carnea* and KPU1D from *Caladenia phaeoclavia*) produced more ergosterol than those from two endangered and rare orchids (B110 from *C. fulva* and R9 from *C. venusta*—both Patersonii group), with others intermediate. Fungi varied greatly in their production of ergosterol from the various forms of cellulose (tissues, filter paper and microcrystalline cellulose). Fungi also varied by up to 10-fold in ergosterol production from natural resources, especially bark. For soluble carbon sources, more biomass was produced by the OMF from common species in the Dilatata and Reticulata than the OMF from their corresponding nationally endangered species, but this was not the case in the Patersonii and Finger orchids.

Effect of orchid taxonomic group and conservation status

Caladenia group alone explained 7 and 13 % of the variation in ergosterol and fungal biomass respectively (Figs. 1 and 2). Differences in carbon source utilisation among the OMF were quantitative rather than qualitative, as all had similar profiles of carbon source utilisation (except for sucrose). The ergosterol equivalent biomass over all carbon sources in the Finger orchid group was more than twice those in the Dilatata, Reticulata and Patersonii groups ($F = 4.33$, $p = 0.006$).

Conservation status of the host orchid explained 9 and 3 % of the variation in ergosterol and fungal biomass respectively (Figs. 1 and 2). There was no consistent difference between OMF from endangered and more common orchids, as the effects of host conservation status varied with orchid group. OMF from endangered species had less ergosterol equivalent biomass than those from common species in the Dilatata and Reticulata, but growth was equivalent in OMF from the Patersonii and Finger orchids ($F = 6.20$, $p = 0.001$) (Fig. 4). This was mainly attributable to differences in growth on complex carbon sources ($F = 12.60$, $p < 0.001$), as there was no difference between endangered and more common groups in growth on natural materials ($F = 2.45$, $p = 0.114$) or simple soluble carbon sources ($F = 2.11$, $p = 0.117$).

Both cluster analysis and principal components analysis on the combined data for utilisation of complex and soluble carbon sources clustered four of the five OMF from endangered and rare orchid hosts separately from OMF from more common hosts, except for CMA2 from *C. maritima* (Fig. 5). The left-hand clade of the cluster analysis dendrogram and the lower left quadrant of the score plot for the principal components analysis contained the OMF from three endangered spider orchids (*C. amoena*, *Caladenia robinsonii* and *C. fulva*) (in the groups Dilatata, Reticulata and Patersonii) along with that from the rare/vulnerable *C. venusta* (Patersonii). In the principal components analysis, OMF from both the state-endangered (*C. maritima*) and common (*C. carnea* var. *carnea*) finger orchids clustered in the lower right quadrant and those from the common spider orchids (*C. phaeoclavia* and *Caladenia clavigera*) in the top left and right quadrants respectively.

Discussion

Terrestrial orchids in the wild depend on their mycorrhizal fungi throughout their life cycle. These OMF depend on their ability to compete for and degrade available carbon sources in litter in their habitat. This poses critical questions about the role of competitive ability of OMF in orchid ecology. This study has shown for the first time that symbiotically effective OMF from eight *Caladenia* species from four traditional groups grew on various components of litter, its component complex carbon sources and their metabolites. The results suggest that litter can play a role in supporting orchid survival.

The OMF did not grow well on all the carbon compounds produced, implying that litter degradation by OMF may provide substrates amenable to foraging by competing microorganisms. Alternatively, OMF may only be able to utilise some in the presence of others, e.g. arabinose in the presence of xylose. The OMF from these *Caladenia* species mainly differed quantitatively rather than qualitatively in their growth on litter and its metabolites. If these results can be extrapolated to the field situation, this suggests that competition between OMF is based on differences in growth rate rather than in intrinsic metabolism. In particular, the growth of OMF from threatened species in two of the four traditional *Caladenia* groups was less than that from common species in the same groups, suggesting that OMF competitiveness could contribute

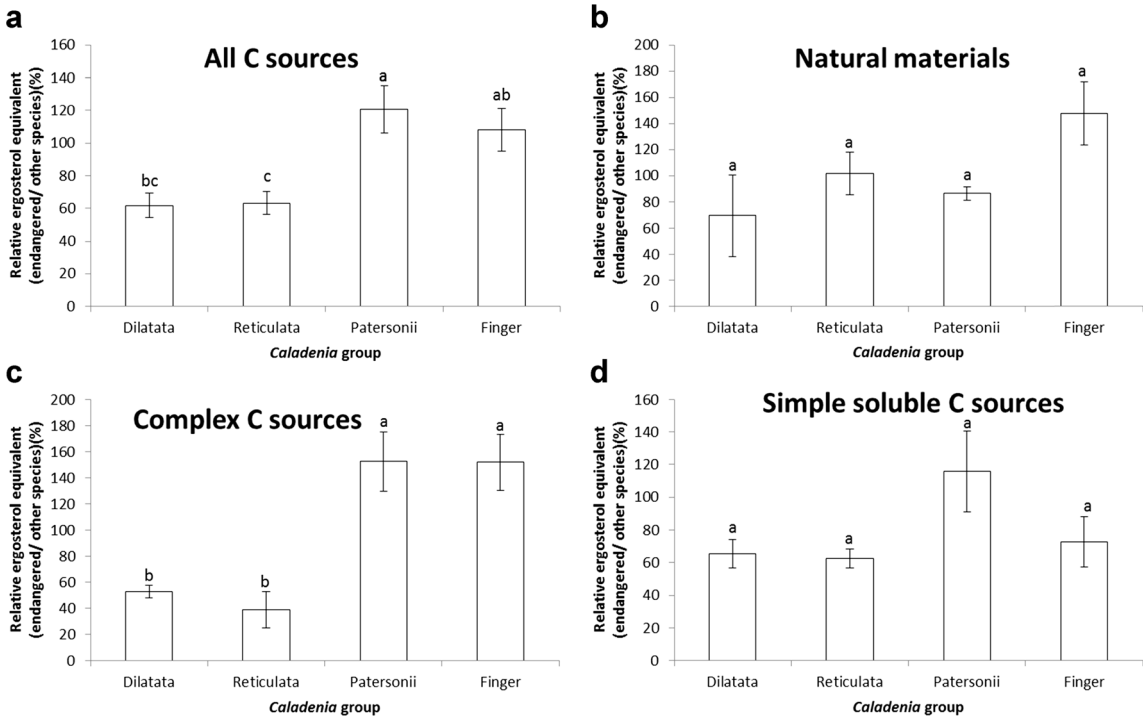

Fig. 4 Percentage growth [ergosterol equivalent (μg plate^{-1})] of orchid mycorrhizal fungi from endangered species relative to those from more common species in four *Caladenia* groups on liquid low carbon-nitrogen modified Melin-Norkrans media for 4 weeks at 25 °C

to their hosts' conservation status, provided that the OMF isolates used are typical. The dichotomy in sucrose utilisation may affect OMF symbiotic effectiveness and isolation. UPLC, used here for the first time with OMF to quantify ergosterol, was as effective as HPLC but was faster and more economical, thus facilitating ergosterol quantification of fungal growth in environmental substrates. These points are discussed further below.

Digestion of litter and metabolites

The growth of these symbiotically effective OMF from *Caladenia* species on various components of litter, its component complex carbon sources and their metabolites, suggests that litter (with or without inoculation with suitable OMF) could be added to habitats to support the growth of OMF and their host orchids. This could be either to ameliorate habitats with threatened orchids or to support newly planted seedlings of threatened orchids grown *ex situ*. This suggestion is supported by increased protocorm formation in buried seed of two out of three northern hemisphere orchids after organic amendment (with or without fungi), where wood was more effective than leaves in increasing the abundance of the OMF (*Tulasnella* sp.) (McCormick et al. 2012). The patchy spatial distribution of OMF and their host orchids may be related to the abundance of such accessible sources of carbon (Wardle 1992; McCormick et al. 2012). The greater growth here on casuarina branchlets and leaf litter than roots or bark may reflect their greater nutrient content, especially of nitrogen, as casuarinas are nodulated nitrogen-fixing plants in the area in which the branchlets were collected (Lawrie 1982). Wright et al. (2009) proposed that casuarina branchlets retained moisture and provided an accessible source of nutrition. Its support of OMF growth suggests that its status as a favoured mulch in the *ex-situ* cultivation of Australian terrestrial orchids (Kasomenakis 1989) is justified.

Organic litter is an important and available resource for OMF and other mycorrhizal fungi present on and in the soil (Brundrett and Abbott 1995), for which the OMF must compete not only with one another but also with other mycorrhizal and saprophytic fungi. Although Hynson et al. (2013) commented that the Sebacinales seemed to have relatively poor saprotrophic ability, the OMF tested here seemed to utilise a wider range of carbon sources and possibly to be more competitive than ECM fungi, based on growth rate (Hacskaylo 1973; Hutchison 1990; Maijala et al. 1991; Cao and Crawford 1993; Cairney and Burke 1996; Burke and Cairney 1998; Colpaert et al. 1999; Hughes and Mitchell 1995; Terashita et al. 1995; Terashima 1999).

The large amount of growth on insoluble forms of cellulose and its degradation products cellobiose and glucose suggests that the OMF probably synthesise a complete set of cellulose-degrading enzymes, as previously reported for the main ERM fungus *Rhizoscyphus ericae* (Burke and Cairney 1997a; Midgley et al. 2006). Similarly, Hadley (1969) showed that ball-milled Whatman 'Chromedia' powder increased protocorm development in three symbiotically grown northern

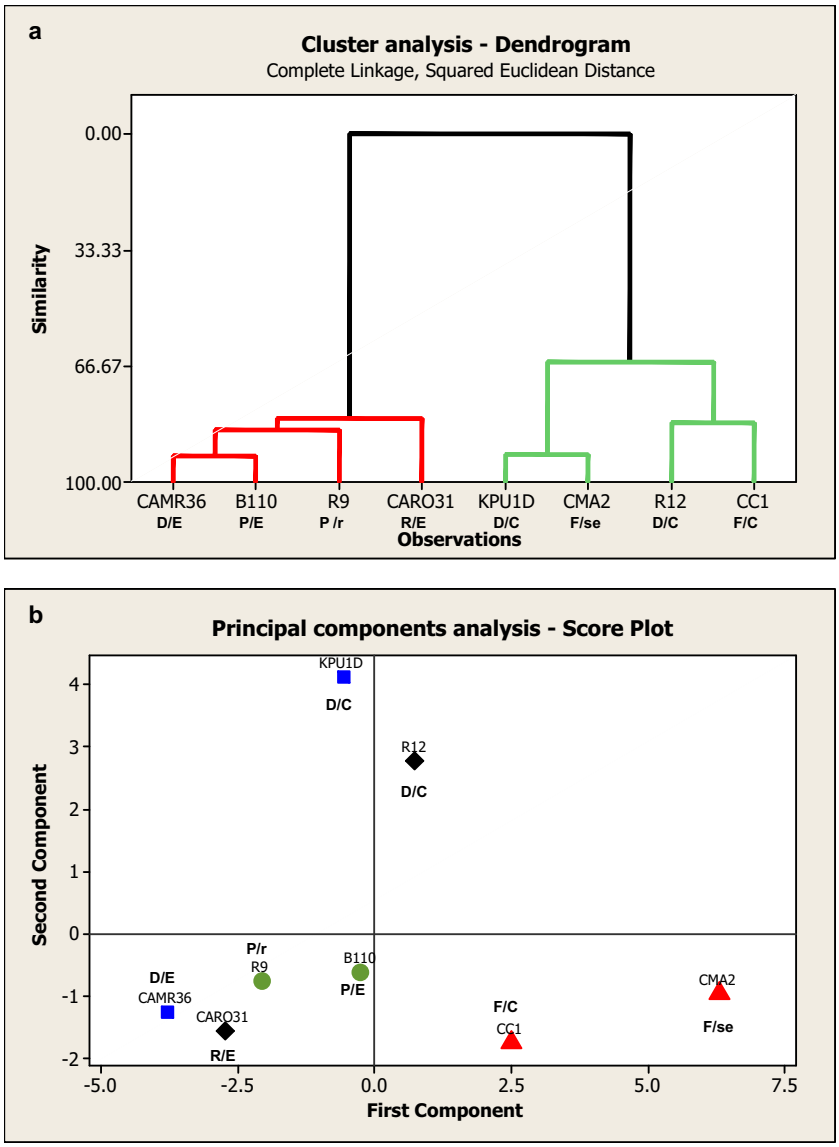

Fig. 5 Multivariate analysis of carbon utilisation of eight fungi isolated from eight *Caladenia* species with ten insoluble (complex) and 12 soluble carbon sources. **a** Hierarchical cluster analysis. **b** Principal components analysis. Key: Taxonomic status [*D* Dilatata group, *R* Reticulata group, *P* Patersonii group, *F* Finger (Carnea) group]; conservation status [*E* nationally endangered, *r* rare, *se* state-endangered (Victoria), *C* common]

hemisphere orchids. There is no directly comparable study in Australian OMF, as other studies used the soluble CMC or estimated growth on insoluble forms of cellulose by eye or subtraction after filtration (Midgley et al. 2004, 2006; Wright et al. 2011; Nurfadilah et al. 2013). CMC appears to be a poor substitute for cellulose, as all these OMF isolates were limited in their utilisation of CMC, as found earlier with OMF (Midgley et al. 2006; Nurfadilah et al. 2013), ERM fungi (Midgley et al. 2004) and non-mycorrhizal ascomycetes (Tuohy et al. 2002).

By contrast, the relatively good growth on the hemicellulose xylan and the relatively poor growth on its degradation products (xylose, arabinose and galactose) suggest that either uptake or metabolism of the constituent sugars is limited in these OMF; relatively poor growth on these sugars was attributed to poor energy yield (Jennings 1995). Similar results were reported for other Australian OMF (Midgley et al. 2006; Wright et al. 2011; Nurfadilah et al. 2013), the main ericoid mycorrhizal (ERM) fungus (*R. ericae*) (Burke and Cairney 1997b) and the basidiomycetous ectomycorrhizal (ECM) fungi *Pisolithus tinctorius* (Cao and Crawford 1993) and *Amanita caesarea* (Daza et al. 2006). Xylans and their breakdown products are utilised well by bacteria (Garrett 1963; Moore et al. 2011) and bacteria may be effective competitors.

OMF did not grow well on chitin (0.06–0.18 mg day^{-1}, calculated using the conversion factor of 4.7 mg ergosterol g^{-1} biomass), which was surprising because chitin is a main constituent of fungal cell walls. Comparably poor chitin utilisation (0.17–0.34 mg fungal d.wt day^{-1}) was reported for six OMF from *Caladenia tentaculata* (Wright et al. 2011). These figures are much less than the 1.65 mg day^{-1} for the ERM fungus *R. ericae* and supports the suggestion that OMF from *Caladenia* species may be less competitive in accessing chitin

than ERM, with which they are commonly associated in their natural habitats (Midgley et al. (2006).

Starch grains are frequently present inside plant cells in litter and both it and its degradation products (maltose and glucose) supported much growth, suggesting that the OMF produced amylases capable of degrading starch. Similarly, starch and its metabolites were utilised well by other Australian OMF from *Pterostylis* (Midgley et al. 2006; Nurfadilah et al. 2013). Starch grains have also been observed in the orchid cortex (Aybeke et al. 2010) but only in uninfected cells, and in proportion to glucose in the medium (Leroux et al. 1995). It seems likely that this starch is unavailable to the OMF because the pelotons are confined within specific infected cells.

Ergosterol: UPLC vs HPLC

In this study, UPLC had greater resolution, speed and sensitivity than HPLC, which is due to the smaller particle size used in the UPLC column (1.7 μm) than in the HPLC column (5 μm). Ergosterol eluted with a retention time of 0.55 min, compared with the time of 3.3 min recorded here and 4–9 min commonly reported for HPLC (Salmanowicz and Nylund 1988; Martin et al. 1990; Gessner et al. 1991; Antibus and Sinsabaugh 1993; Charcosset and Chauvet 2001; Villares et al. 2012). The smaller retention time of ergosterol in UPLC was also more cost-effective because less solvent was used. UPLC has been reported only once previously for the measurement of ergosterol in fungi (Choon et al. 2012) and this is the first report of its use in OMF with natural and insoluble materials. UPLC quantification of ergosterol in litter could be used, in parallel with 454 pyrosequencing studies of the relative frequencies of fungal species present, to estimate the relative occupation of litter by OMF, as used by Wallander et al. (2010) to show the dominant ECM fungi in *Picea abies* forests in Sweden.

Ergosterol is commonly used as a measure of fungal biomass because it is unique to fungi, but it must be proportional to fungal biomass to be useful (Schnürer 1993). Converting between ergosterol and biomass measurements using this figure allowed the comparison of growth on natural substances, complex insoluble and soluble carbon sources to a common scale. The conversion factor for ergosterol to fungal biomass calculated here was 4.7 mg ergosterol g^{-1} d.wt, which was within the range of 3–6 mg g^{-1} d.wt commonly reported (Salmanowicz and Nylund 1988; Nylund and Wallander 1992; Antibus and Sinsabaugh 1993; Newell 1994; Montgomery et al. 2000).

Effects of conservation status and host taxonomy

The variation in biomass produced by OMF according to orchid group suggests that in a common habitat, the OMF from the Finger orchid group could out-compete those from the Dilatata group, with the other OMF intermediate between the two. However, the effect of taxonomic group may in part be due to the Finger orchids having larger proportions of common orchids than these other groups. Further investigation of more OMF from the same and different groups is needed to see if this trend can be confirmed with other species of *Caladenia*.

The large quantitative differences in average growth of OMF from the endangered and common species in the Dilatata and Reticulata suggest that OMF from these nationally endangered species would be less competitive than OMF from common species in the same *Caladenia* group in a common habitat. Nurfadilah et al. (2013) showed similarly that the OMF from the endangered *Drakaea elastica* had less biomass than OMF from common orchids, e.g. *P. recurva*, on the same carbon sources. Unfortunately, nutritional studies of OMF from Australian terrestrial orchids are limited and there is no other comparable Australian study for comparison. In this context, the greater variety of OMF strains with which common species associate may confer a competitive advantage (Wright et al. 2010), as found also in northern hemisphere orchids (Shefferson et al. 2005).

The differences between OMF from nationally endangered and common orchids in the Dilatata and Reticulata manifested at the intermediary stage rather than at the natural materials stage, suggesting that this is the critical rate-limiting stage in orchid competitiveness. In this context, the differences in calculated ratio of ergosterol: d.wt with conservation status may be important because less ergosterol in the plasma membrane of the threatened OMFs could affect its permeability. OMF from nationally endangered species may be limited in the extrusion of catabolic enzymes or the uptake of intermediates relative to common species in the same group.

The relatively large growth and lack of quantitative differences between OMF from endangered and more common species in the Patersonii and Finger orchids suggest that their relative competitive ability does not contribute significantly to the conservation status of their host orchids. There was less contrast in conservation status between pairs of orchids within these groups because the Patersonii had few common species (none in Victoria) and the Finger orchids had few nationally endangered species (Jeanes and Backhouse 2006). Further investigations should endeavour to use pairs of species with clear conservation status and more than one fungal isolate from each orchid to ensure that they are typical.

Sucrose

The OMF dichotomised in utilisation of sucrose, the main photosynthetic product transported in the phloem by plants to the underground locations of the OMF. Those OMF unable to use sucrose may lack sucrose transporters on the fungal plasma membrane or active sucrose catabolism enzymes such

as invertase, as all OMF grew well on both glucose and fructose (the degradation products of sucrose). Inability to utilise sucrose has also been reported for one of six OMF from *C. tentaculata* (Wright et al. 2011) and the ECM fungi *Amanita muscaria* and *Hebeloma crustuliniforme* (Tagu et al. 2000; Nehls et al. 2001) but not *Laccaria bicolor* (Tagu et al. 2000). *A. muscaria* appears to lack its own sucrose transporters, as the host-plant membrane-bound invertase in the root cortex is not rate-limiting in generating supplies of glucose and fructose to the fungus (Nehls et al. 2001). However, the OMF may have genes for sucrose transporters/catabolic enzymes that are not/poorly active in the free-living state but become active inside the pelotons.

OMF that cannot utilise sucrose may provide more benefits to their host orchids than those that can, because the latter may compete for sucrose with the host plant and so shift symbiotic mutualism to a pathogenic relationship. The ability to use sucrose may also explain the perplexing failures at the 'green leaf' stage in orchid symbioses that start with high seed germination but fail once the plant becomes photosynthetic and presumably starts to supply sucrose to its subterranean parts (Rasmussen 1995; Huynh et al. 2009). If OMF inside the root cortical cells cannot utilise sucrose from the host plant, then this may contribute to peloton lysis followed by the release of nutrients that can be utilised by the orchid, and thus be beneficial to the orchid.

A further consequence from this dichotomy in sucrose utilisation is that the soluble carbon source in the medium used to isolate and grow OMF from pelotons is critical. The main media used are FIM (Clements et al. 1986) (which has sucrose as the carbon source), modified Melin-Norkrans medium (MMN) (Marx and Bryan 1975) (glucose and malt extract) and potato dextrose medium (PDA) (Latalova and Balaz 2010) (starch and glucose). If FIM is used rather than MMN or PDA, the medium is selective for OMF that utilise sucrose (although small amounts of glucose and fructose are generated during autoclaving). With FIM, a large proportion of the OMF isolated commonly fail to stimulate germination and seeds are frequently 'eaten' by the fungi (e.g. Raleigh 2005; Huynh et al. 2009). Also, data of Wright et al. (2011) show an inverse relationship between growth on sucrose and seed germination in *C. tentaculata*. It may be desirable to use an alternative carbon source for isolation and to select OMF on their inability to utilise sucrose. More research is needed to test if OMF that differ in sucrose utilisation also differ significantly in symbiotic effectiveness, especially once the orchid develops a green leaf.

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Performed the experiments: S. Mehra, P. D. Morrison
Analysed the data: S. Mehra, A.C. Lawrie
Wrote the paper: S. Mehra, A.C. Lawrie

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