

ROOT SURVEY AND ISOLATION OF FUNGI FROM ALPINE EPACRIDS (ERICACEAE)

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Abstract

The roots of five species of epacrids, *Epacris paludosa*, *E. microphylla*, *E. petrophila*, *Leucopogon montanus* and *Richea continentis* were collected in January 2002 from Mount Kosciuszko National Park, NSW, Australia (36°27'S, 148°15'E, elevation 2228 m). Root samples including hair roots were cleared and stained and examined for mycorrhizal structures. Typical ericoid mycorrhizal structures (TEMS) were present in all roots examined. Percentage colonisation ranged from 10%–90%; however, mean colonisation was 46% which was similar to that previously measured in hair roots from epacrids collected at sea level. The roots of all species, especially *L. montanus* (89%) and *E. petrophila* (90%), also contained dark septate hyphae (DSH). The high percentages of DSH were greater than that measured previously in epacrid roots collected from lower altitudes but approximated amounts measured in roots of northern hemisphere Ericaceae. Also present in hair roots collected from epacrids from Mount Kosciuszko were hyphae and vesicles typical of arbuscular mycorrhizal fungi (AMF). Two hundred and ten sterile fungi were isolated from a second set of root samples. Cultures were divided into ten morphological groups according to culture colour and growth rate. In general, the alpine isolates were similar to those collected from epacrids growing at lower altitudes although a higher number of the isolate cultures were light in colour. Two isolates were introduced to roots of axenic seedlings of *Vaccinium corymbosum* (Ericaceae) grown on water agar. One isolate formed TEMS in epidermal cells of hair roots (mean colonisation of 75%) while hyphae from the other isolate entered the hair root cells and formed thick snake-like hyphal coils. Results suggest that the relationship between alpine epacrids and their fungal endophytes (TEMS, DSE and AMF) is more complicated than that found in ericoid associations at lower altitudes and therefore warrants further investigation.

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Introduction

Australian Ericaceae, formally known as Epacridaceae (epacrids), growing on the summit of Mount Kosciuszko are a major component of alpine bog ecosystems. Several epacrids species grow only in alpine areas (*Epacris petrophila* Hook. f., *Leucopogon montanus* (R. Br.) J.H. Willis and *Richea continentis* B.L. Burtt.) and any disturbance to the environment could lead to their loss, and hence, an overall decline in biodiversity. Members of the Ericaceae are reliant on a mycorrhizal fungal partner for nutrient uptake and ultimately for survival (Smith & Read 1997). The roots of epacrids, unlike other genera of Australian plants, are thought to form only one mycorrhizal type (ericoid) and this association is characterised by hyphal coils formed within epidermal cells of the 'hair roots' (Cairney & Ashford 2002, Read 1996).

Previous studies of epacrids collected in Victoria have shown that while there is some regional variation in hyphal thickness (McLean & Lawrie 1996), all epacrids examined formed typical ericoid mycorrhizal structures (TEMS, Hutton 1994, Hutton *et al.* 1996, McLean 1999, Reed 1987, 1989, Steinke *et al.* 1996) with the occasional observation of arbuscular mycorrhizal fungi (AMF) (Bellgard 1991, Khan 1978, McGee 1986, McLean & Lawrie 1996). Fungi isolated from epacrid roots are consistently described as dark, sterile and slow-growing (Hutton 1994, Hutton *et al.* 1996, Lui *et al.* 1998, McLean 1999, Reed 1987, 1989, Steinke *et al.* 1996). Molecular studies of the fungal partners have shown that a well defined group of fungi can be isolated from the roots of epacrid plants and can form TEMS when reintroduced to axenically produced micro-cuttings of epacrid

(McLean *et al.* 1999) or blueberry seedlings (Chambers *et al.* 2000, Lui *et al.* 1998). To date, it is generally accepted that epacrids maintain similar mycorrhizal partners and structures across geographical and ecologically diverse habitats.

Northern hemisphere studies of alpine Ericaceae have revealed a diversity of fungal endophytes and isolates in the alpine environment (Hambleton & Currah 1997, Read & Haselwandter 1981, Stoyke & Currah 1990, Stoyke *et al.* 1992, Urcelay 2002, Vrålstad 2002). There are also reports of the presence of dark septate endophytes (DSE) forming wefts of hyphae across roots, entering root cells and forming coils and microsclerotia (Jumpponen 2001, Jumpponen & Trappe 1998). These fungi are thought not to be mycorrhizal with northern hemisphere Ericaceae (Jumpponen 2001) but in the nutrient poor soils and extreme climates of alpine bogs, Read & Haselwandter (1981) suggest the potential for some functional importance for DSE.

All previous studies of root endophytes of Australian epacrids have focussed on plants growing at or close to sea level on sandy, well-drained soils. The aim of this study was to investigate the diversity of endophytes associated with epacrids growing in alpine conditions at Mount Kosciuszko by surveying hair roots and isolating fungi from them.

Materials and Methods

Plant material

The roots of five species of epacrids; *Epacris microphylla* R. Br. (two plants), *E. paludosa* R. Br. (3 plants), *E. petrophila* (three plants), *Leucopogon montanus* (two plants) and *Richea continentis* (two plants) were collected from the summit of Mount Kosciuszko in January 2002 by digging around and under selected plants and tracing the roots back to the main tap root (Table 1). Care was taken to ensure that the hair roots were not damaged. Half of the root sample of each plant was washed in the field and hair roots removed (about 10 cm lengths) and immediately placed into vials containing 50% ethanol. The remaining half of each root sample was wrapped in wet paper towel and stored at 4°C for 24 hrs for further processing.

Table 1. Percentage colonisation of hyphal coils typical of ericoid mycorrhizal associations, arbuscular mycorrhizal structures and dark septate hyphae in epidermal cells of hair roots of five alpine epacrid species collected from Mount Kosciuszko National Park, Australia. Numbers shown are mean percentage colonisation \pm standard error.

Species	Ericoid mycorrhizal hyphal coils (% epidermal cells with coils)	Dark septate hyphae (% colonised root length)	Arbuscular mycorrhizal structures (% colonised root length)
<i>Epacris microphylla</i>	90 \pm 2	50 \pm 2	0
<i>Epacris paludosa</i>	20 \pm 1	25 \pm 1	5 \pm 0
<i>Epacris petrophila</i>	70 \pm 2	100 \pm 2	10 \pm 1
<i>Leucopogon montanus</i>	45 \pm 2	89 \pm 2	0
<i>Richea continentis</i>	25 \pm 1	74 \pm 1	7 \pm 1

Root endophyte morphology

In the laboratory, hair root samples were removed from the ethanol, and cleared using 10% KOH solution held at 90°C for 30 minutes then stained with 1% acid fuchsin solution (Smith & Dickson 1997) held at 90°C for a further 30 minutes. Roots were mounted on glass slides and examined using a Nikon Eclipse E400 light microscope for the presence of fungal endophyte structures. Percentage cell colonisation by TEMS was assessed by classifying (as colonised or not colonised) 50 adjacent epidermal cells from several randomly chosen hair roots from five replicates for each species (total count of 250 cells per species, method modified from Dalpé, 1986). Percentage colonisation by DSE and AMF structures was determined using the line intersect method (Giovannetti & Mosse 1980).

Fungal isolation

In the laboratory, root samples were unwrapped from the paper towel, washed in running water to remove soil and the hair roots teased apart. Hair roots were surface sterilised with 0.2% sodium hypochlorite for two minutes, washed three times in sterile distilled water and cut into 1 mm length pieces. Each root piece was placed into a separate well of a micro-titre plate containing bovine serum albumin and antibiotics as described by Steinke *et al.* (1996) and incubated in the dark at 25°C. Plates were checked daily for non-mycorrhizal fungal contamination indicated by fast growing hyphae. Contaminated root pieces were killed by the addition of a crystal of copper sulphate to affected wells. After 10 days, the remaining root pieces were transferred to plates containing full-strength solid potato dextrose agar (Oxoid) and grown in the dark at 20°C. Isolates were labelled according to the plant and site location from which they were isolated and numbered according to the morphological group to which they had been assigned (*i.e.* LMK1-29-8 = *Leucopogon montanus* Mount Kosciuszko, plant number 1, isolate number 29, morphological group VIII). Fungi were grouped according to cultural morphology after six weeks of growth (Table 2).

Mycorrhizal status

Seeds of *Vaccinium corymbosum* L. (blueberry) were removed from 10 berries, surface sterilised for five minutes using 2.0% sodium hypochlorite, rinsed in sterile deionised water and germinated on water agar (15 gL⁻¹ agar (Oxoid)) under light at 30 $\mu\text{Em}^2\text{s}^{-1}$ ('Sylvania' fluorescent tubes) and at a constant temperature of 25°C. Two fungal isolates, EMK2-23-8 and EPK2-17-10, were selected for this trial on the basis of database searches of their rDNA ITS sequences (C. McLean unpublished data). EMK2-23-8 closely matched the sequence (AF072298) of a mycorrhizal isolate C28 from the epacrid *Woollisia pungens* (Cav.) F. Muell. (Chambers *et al.* 2000) and EPK2-17-10 matched the sequence of the DSE *Phialocephala fortinii* (AY078152F). Three small squares of actively growing mycelia (5 mm³) were taken from the potato dextrose agar cultures of EMK2-23-8 and EPK2-17-10 (Table 1) and were introduced next to the roots of single blueberry seedlings. Seedlings were grown in well-lit conditions (30 $\mu\text{Em}^2\text{s}^{-1}$ with 12 hours light:12 hours dark) at 25°C. Hair roots of blueberry seedlings were excised after six weeks of growth, cleared with 10% KOH and stained with 1% acid fuchsin as above and examined microscopically. Percentage cell infection was assessed as before.

Results

Root endophyte morphology

Hyphal coils representing typical ericoid mycorrhizal structures (Fig. 1A) were found in hair roots from all plants of each of the five species examined. Percentage colonisation by TEMS ranged from 20% in *Epacris paludosa* to 90% in *E. petrophila* (Table 1), with mean percentage colonisation across all five species being 46%. Relatively low percentage colonisation by AMF hyphae and vesicles (Fig. 1B) were measured in all species except *E. microphylla* and *Leucopogon montanus* in which no AMF structures were found. A high percentage of colonisation by DSE (Figs 1C & 1D) was observed in all five species with the highest levels of colonisation measured in *E. petrophila* (100%), *L. montanus* (89%) and *R. continentis* (75%, Table 1).

Fungal isolation

Two hundred and ten fungi were isolated from the hair roots of the five species of alpine epacrids collected. All isolates were sterile and slow-growing. Fungal isolates were divided into groups on the basis of their morphological appearance including culture colour, reverse colour (colour of the fungal isolate when viewed from the underside of the plate), colony margin colour, presence/absence and colour of exudates, growth rate (mm/week) and hyphal texture (Table 2). One hundred and forty isolates were dark in colour varying from dark brown through green-brown to dark grey, and the remainder (70 isolates) varied from white to cream. The spread of isolates across morphological groupings was fairly even with five groups containing 25 or more isolates (I, II, VI, VIII and IX) and only one group (X) with less than 10 isolates.

Mycorrhizal status

Typical ericoid mycorrhizal structures were readily observed in all hair roots excised from axenic blueberry seedlings inoculated with the fungal isolate EMK2-23-8 (mean infection 76 \pm 5%, range 62–95%, Fig. 1E). The isolate, EPK2-17-10 did not form TEMS in hair roots from axenic blueberry seedlings but rather colonised epidermal cells with large snake-like coils (Fig. 1F).

Discussion

This study illustrates the diversity of fungi isolated from the roots of alpine epacrids; TEMS, AMF structures and DSE were present in hair roots of one or more of the species examined. As expected, the epidermal cells of hair roots of all individuals from each of the five epacrid species contained TEMS, suggesting a similar role for this mycorrhizal type at high and low altitudes. The TEMS from alpine epacrids were of a similar morphology to those observed in hair roots of epacrids from lower altitudes although percentage infection of hair roots of alpine epacrids was slightly lower (*Epacris paludosa* 20%, *Richea continentis* 25%) than that reported previously (McLean 1999, Reed 1989). Haselwandter (1979) found low levels of colonisation by TEMS in alpine Ericaceae from the northern hemisphere suggesting this was due to low levels of humus in soils at extreme altitudes. Similarly, Read & Haselwandter (1981) suggested that the low levels of AMF colonisation observed in mycorrhizal plants from northern hemisphere alpine environments was due to a combination of factors including low nutrients, low temperatures and a short time period for root growth. Johnson & Ryan (2000) suggested that a similar range of conditions in conjunction with low levels of inoculum due to topsoil disturbance produced low levels of colonisation by AMF in roots of alpine herbs collected from Mount Kosciuszko. All of these factors coupled with severe drought conditions experienced over the last few years in most of eastern Australia may explain the low levels of TEMS and AMF structures observed in this study. An alternative explanation is that as colonisation levels of hair roots vary seasonally and given the transient nature of the epacrid hair root (Bell & Pate 1996, Smith & Read 1997), maximum colonisation of hair roots by TEMS may not have been captured from the single sampling period described here.

Table 2. Morphological classification of fungal isolates from the hair roots of five species of alpine epacrids (*Epacris microphylla*, *E. paludosa*, *E. petrophila*, *Leucopogon montanus*, *Richea continentis*) collected from Mount Kosciuszko when grown on potato dextrose agar, Colour classification according to Henrik (1983).

Group	Culture colour	Reverse plate colour	Culture margin colour	Exudate colour	Culture texture	Growth rate (mm wk ⁻¹)	Number of isolates
I	White	Yellow	-	Clear	Felty	7	25
II	White	White	-	-	Furry flat	5	26
III	White	White	Clear	-	Felty	7	20
IV	Grey 28C1	Black	White	-	Fluffy	10	12
V	Grey brown 8E2	Brown	Clear	-	Flat furry	6	18
VI	Grey brown 30F6	Brown	Dark	-	Flat furry	7	29
VII	Dark grey 30F1	Dark grey	White	Dark	Fluffy	4	14
VIII	Dark brown 8F4	Black	Dark	Dark	Felty	3	27
IX	Dark grey 30F4	White	-	-	Flat furry	3	31
X	Metallic green 30F7	Dark	Green	Black	Flat furry	4	8

Arbuscular mycorrhizal fungi are not usually associated with members of the Ericaceae (Cairney & Ashford 2002) although vesicles have been recorded in Ericaceae from Hawaii (Koske *et al.* 1990) and in Australian epacrids from low altitudes (Bellgard 1991, Khan 1978, McGee 1986, McLean & Lawrie 1996). Occasionally arbuscules have been reported in Australian epacrids (Bellgard 1991, Kahn 1978, McGee 1986) but their functional significance has not yet been determined. The relatively low levels of AMF colonisation in alpine epacrids investigated in this study supports the accepted convention that the AMF relationship is not as important to alpine epacrids as the ericoid mycorrhizal association but may also be due to environmental factors suggested by Johnson & Ryan (2000) as discussed above. Although no arbuscules were seen in any of the plants examined in this study, Urcelay (2002) recently reported arbuscules in older roots of *Gaultheria poeppigii* (Ericaceae)

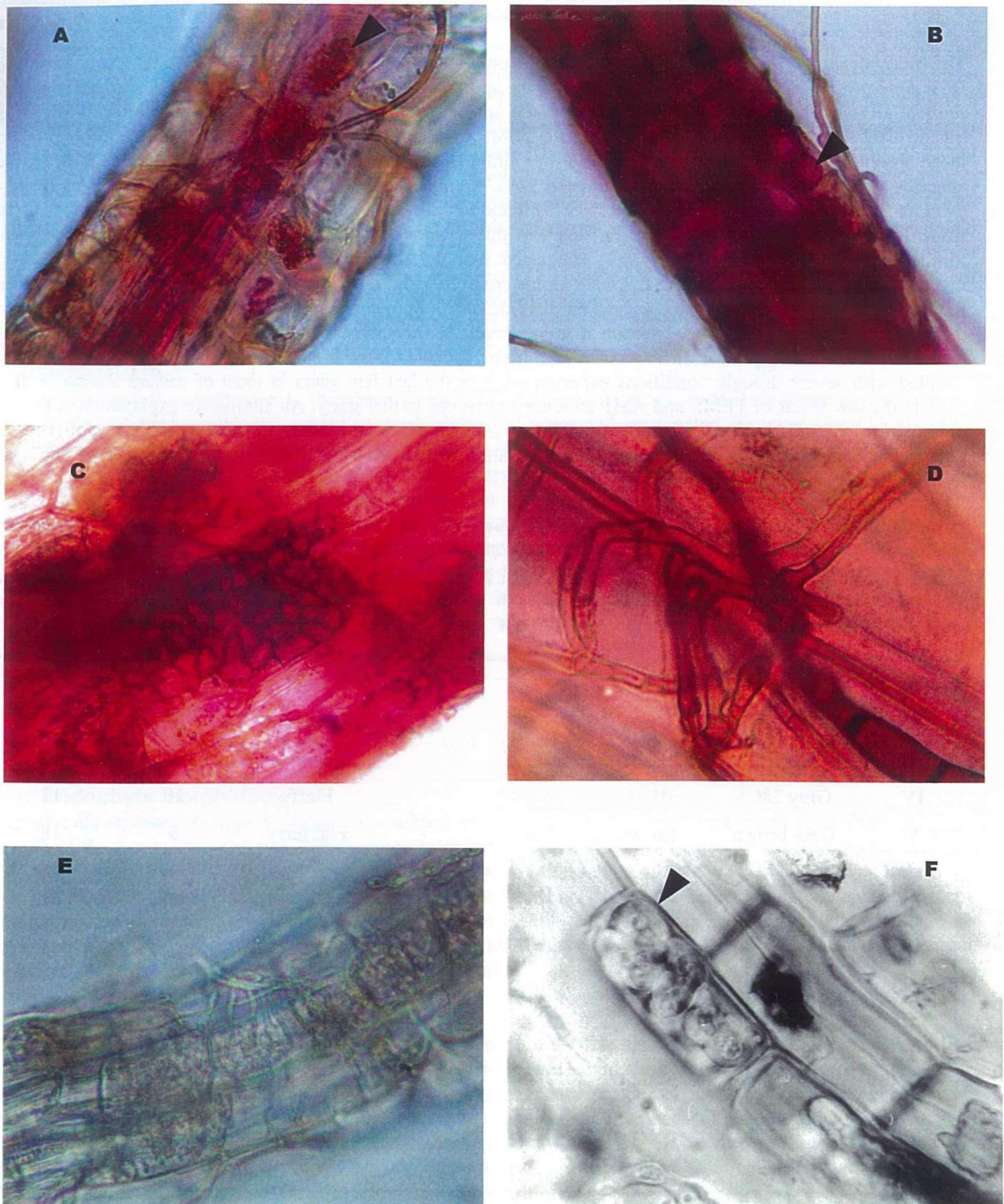


Figure 1 A–F. Light micrographs of ericoid endophytes. **A–D** field-collected hair roots; **E–F** hair roots from axenically grown *Vaccinium corymbosum* (blueberry) seedlings. **A.** Typical ericoid mycorrhizal structures (TEMS, arrow) in epidermal cells of hair roots of *Epacris microphylla*, x500, **B.** Arbuscular mycorrhizal vesicles (arrow) in hair roots of *Epacris paludosa*, x500, **C.** Microsclerotia of the *Phialocephala*-type in epidermal cells of hair roots of *Epacris petrophila*, x1500, **D.** Dark septate hyphae (DSE) growing on the surface of hair roots of *Leucopogon montanus*, x1500. **E.** TEMS (arrow) formed by the isolate LMK2-23-8 in epidermal cells of hair roots of *Vaccinium corymbosum*, x1500. **F.** Hyphal coils of the DSE (arrow) formed by the isolate EPK 2-17-10 in epidermal cells of hair roots of *Vaccinium corymbosum*, x1500.

collected from a similar altitude (1700–2200 m) in Argentina. This suggests that there may be some functional role for arbuscular mycorrhizal fungi in certain alpine Ericaceae. A more extensive seasonal survey of alpine epacrids would help to answer this question and further investigation into the nature and importance of this relationship is required.

This study records the presence of DSE in the hair roots of alpine epacrids for the first time and matches observations for species collected from lower altitudes. The DSE observed in this study formed a loose web of hyphae over the root and internal microsclerotia of the *Phialocephala*-type (Urcelay 2002). DSE have been reported in northern hemisphere relatives of epacrids at both low and high altitudes suggesting the potential for a similar functional role (Jumpponen & Trappe 1998).

Fungal isolation

As expected, all fungi isolated from alpine epacrids were sterile and could not be identified by normal taxonomic means. Classification of isolates by culture characteristics resulted in a similar number of morphological groupings as have been identified in other studies (McLean 1999, McLean *et al.* 1999) suggesting that fungal species involved may be similar at both high and low altitudes. However, in previous studies of epacrid endophytes most of the fungi isolated were dark in colour with only a few light isolates reported (Hutton *et al.* 1994, Lui *et al.* 1998, McLean *et al.* 1999, Reed 1988, Steinke *et al.* 1996). One third of the fungi isolated in this study were light in colour suggesting a greater diversity of fungi present in the roots of alpine epacrids more similar to levels of diversity found in the northern hemisphere Ericaceae (Hambleton & Currah 1997, Stoyke & Currah 1990, Stoyke *et al.* 1992, Vrålstad 2002).

Mycorrhizal status

Only one of the two isolates introduced to the hair roots of axenically grown blueberry seedlings (EMK2-23-8) formed TEMS. The rDNA ITS sequence of this isolate closely matched (96%) a sequence from a low altitude epacrid mycorrhizal fungus (AF072298, Chambers *et al.* 2000). This sequence match, coupled with the formation of TEMS suggests that the high elevation isolate, EMK2-23-8 has the potential to form a functional ericoid mycorrhiza, although efficacy has yet to be tested. The sequence of the isolate that did not form TEMS in hair roots of axenically grown blueberry seedlings (EPK2-17-10) closely matched (90%) a sequence from *Phialocephala fortinii*, a known dark septate endophyte. This sequence match, along with the thick snake-like coils similar to those produced by *P. fortinii* in other hosts (Jumpponen & Trappe 1998), suggests that EPK2-17-10 is closely related to *P. fortinii*. Various roles have been suggested for DSE in hosts other than Ericaceae ranging from saprophytes to weak parasites through to weak symbionts (Jumpponen 2001). The high percentage of hair roots of alpine epacrids colonised by DSE and the relatively low percentage of colonisation by TEMS may suggest an important functional role for DSE in alpine epacrids. True *et al.* (1996) reported large amounts of DSE in the roots of Alaskan alpine plants and suggested a mutualistic role for these fungi in highly stressed alpine environments. However, Stoyke & Currah (1993) reported a detrimental effect of *P. fortinii* on *Menziesia ferruginea* (Ericaceae). It will therefore be necessary to further research into the diversity of epacrid endophytes across seasons, their mycorrhizal or saprophytic status and their identity for ecological roles and conservation purposes.

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