Mycorrhiza (2009) 20:1–11
DOI 10.1007/s00572-009-0260-3

ORIGINAL PAPER

Nitrogen form influences the response of *Deschampsia antarctica* to dark septate root endophytes

Rebecca Upson · David J. Read · Kevin K. Newsham

Received: 26 March 2009 / Accepted: 20 May 2009 / Published online: 3 June 2009

Abstract Fungi with dematiaceous septate hyphae, termed dark septate endophytes (DSE), are common in plant roots, particularly in cold-stressed habitats, but their effects on their host plants remain obscure. Here, we report a study that assessed the effects of six DSE on the growth and nutrient balance of *Deschampsia antarctica* when plants were supplied with the same amount of nitrogen in organic (casein hydrolysate) or inorganic (ammonium sulphate) form under controlled conditions. After 60 days, the DSE, that had each been isolated from *D. antarctica* and which analyses of internal transcribed spacer and large subunit regions indicated were similar to members of the Helotiales (*Oculimacula yallundae*, *Mollisia* and *Tapesia* spp.) and unassigned anamorphic ascomycetes, typically had no effect on, or reduced by 33–71%, shoot and root dry weights relative to uninoculated controls when plants had been supplied with nitrogen in inorganic form. In contrast, the DSE usually enhanced shoot and root dry weights by 51–247% when plants had been supplied with organic nitrogen. In the presence of inorganic nitrogen, only sporadic effects of DSE were recorded on shoot and root nitrogen or phosphorus concentrations, whereas in the presence of organic nitrogen, three to six of the DSE isolates increased shoot and root nitrogen and phosphorus contents. Most of the isolates decreased the phosphorus concentrations of shoots and roots when plants had been supplied with nitrogen in organic form. Our data suggest that DSE are able to mineralise peptides and amino acids in the rhizosphere, making nitrogen more freely available to roots.

Keywords Amino acids · Dark septate fungal endophytes (DSE) · DS fungi · Helotiales · Inorganic and organic nitrogen · Peptides

R. Upson · D. J. Read
Department of Animal and Plant Sciences,
The University of Sheffield,
Sheffield S10 2TN, UK

R. Upson · K. K. Newsham (✉)
British Antarctic Survey, Natural Environment Research Council,
High Cross,
Madingley Road,
Cambridge CB3 0ET, UK
e-mail: kne@bas.ac.uk

Introduction

Fungi with dematiaceous septate hyphae, termed dark septate endophytes (DSE), are frequent colonists of plant roots at high latitudes and altitudes (Read and Haselwandter 1981; Currah and van Dyk 1986; Stoyke and Currah 1991; Laursen et al. 1997; Newsham et al. 2009). These fungi, which often form clusters of rounded cells termed microsclerotia in roots, belong to a wide range of ascomycete taxa, but are often members of the Helotiales, such as *Phialocephala fortinii* and *Leptodontidium orchidicola* (Jumpponen and Trappe 1998; Addy et al. 2005). In cold-stressed habitats, arbuscular mycorrhizal fungi, the typical mutualists of herbaceous plant roots at lower latitudes and altitudes, are either entirely absent or occur only sporadically (Bledsoe et al. 1990; Kohn and Stasovski 1990; Väre et al. 1992; Treu et al. 1996; Upson et al. 2008; Newsham et al. 2009), and in the absence of arbuscular mycorrhizas, it has been suggested that DSE might act as surrogate root mutualists in cold-stressed soils (Bledsoe et al. 1990). This is a rather controversial view: although there is evidence in the literature to indicate that DSE can have positive effects on plant growth and nutrient balance (e.g. Haselwandter and Read 1982; Newsham 1999), other studies indicate neutral or negative effects of these fungi on growth (e.g. Wilcox and Wang 1987; Fernando and Currah 1996). Even in cases where DSE have positive effects on plant

growth, little is understood of the mechanisms by which these fungi enhance the growth of their hosts (Mandyam and Jumpponen 2005).

Nitrogen is usually present in cold-stressed soils in organic form, owing to the inhibition of organic matter decomposition at low temperatures (Kielland 1994). For example, at Signy Island in the South Orkneys, Roberts et al. (2009) analysed the different forms of nitrogen in the rhizospheres of the two native maritime Antarctic vascular plant species, *Deschampsia antarctica* and *Colobanthus quitensis*, and found that the concentrations of dissolved organic nitrogen were almost seven times higher than those of inorganic (ammonium and nitrate) forms of the element. DSE, which have recently been shown to be frequent in the roots of *D. antarctica* and *C. quitensis* (Upson et al. 2008), could thus play important roles in these soils, since they are able to utilise organic forms of nitrogen such as proteins and peptides (Caldwell et al. 2000). Previous research suggests that these fungi might allow plants to access organic forms of nitrogen in the rhizosphere, which would otherwise be unavailable to roots (Newsham 1999; Mandyam and Jumpponen 2005). However, there are few data in the literature to support this view.

In this study, using isolates of DSE from the roots of *D. antarctica*, identified by Upson et al. (2009) as currently unassigned anamorphic ascomycetes or members of the Helotiales, an order of fungi with known beneficial effects on plant growth (Smith and Read 2008), we assess the response of *D. antarctica* to DSE in the presence of inorganic and organic forms of nitrogen.

Materials and methods

Taxonomic affinities of isolates

Six DSE were used in the present study. They were isolated, in the survey described by Upson et al. (2009), from the roots of *D. antarctica* plants sampled from South Georgia in the sub-Antarctic and Signy, Coronation, King George and Anchorage islands in the maritime Antarctic (Table 1). Sequencing of the internal transcribed spacer (ITS) regions of rDNA of three of the isolates, termed Da_PoT1_C7, Da_MaP_I9 and Da_Gry_H4 by Upson et al. (2009) but hereafter referred to as C7, I9 and H4, indicated that the isolates were members of the Helotiales and similar to species of *Mollisia* and *Tapesia* (Table 1). Sequencing of the 28S large subunit region of the rDNA of another isolate, referred to here as H3, similarly indicated close affinities with species of *Mollisia* (Table 1). Sequencing of the ITS regions of a further two isolates, referred to as Da_Bac_I4 and Da_PoC_C4 by Upson et al. (2009) but referred to hereafter as I4 and C4, indicated that they were similar to fungi in the Helotiales with close affinities to *Oculimacula yallundae* and currently unassigned anamorphic ascomycetes (Table 1). Sequences of all six isolates have been deposited in GenBank (Table 1). The isolates were maintained on 1% malt extract agar medium at 15°C until required for the experiment reported here.

Growth experiment

Perlite was selected as the substrate for the experiment because of its low cation exchange capacity and greater porosity than vermiculite or sand. It was acid-washed by placing it into fine mesh cotton bags, which were submerged in 1% HCl for 48 h. The bags were then submerged in distilled water for 12-h periods until the pH of the perlite reached a value of 5, the mean pH value of the soils that *D. antarctica* inhabits (Upson et al. 2008; Roberts et al. 2009). The perlite was then drained, oven-dried (145°C) and stored at room temperature until required for the experiment.

Seeds of *D. antarctica* for use in the experiment were collected from Grytviken on South Georgia during the austral summer and were kept over desiccant in the dark at 5°C. The seeds were removed from their husks using forceps and were surface-sterilised for 5 min in calcium hypochlorite solution (1:28, *w*/*v*) on a vortexer. Under a sterile hood, the sterilant was removed by filtering through

Tables 1 Origins and identities of DSE isolates used in the study

DSE isolate	Origin of DSE isolate	Taxonomic identity of DSE isolate[a]	GenBank accession number
C7	Point Thomas, King George Island (62°09′ S, 58°29′ W)	*Tapesia* sp. (Helotiales)	FN178469
H3	Anchorage Island, Léonie Islands (67°36′ S, 68°12′ W)	*Mollisia* sp. (Helotiales)	FN178470
I9	Mansfield Point, Coronation Island (60°39′ S, 45°44′ W)	*Tapesia* sp. (Helotiales)	FN178471
H4	Grytviken, South Georgia (54°16′ S, 36°30′ W)	*Mollisia* sp. (Helotiales)	FN178472
C4	Potter Cove, King George Island (62°14′ S, 58°41′ W)	*incertae sedis*, anamorphic ascomycete	FN178473
I4	Backslope, Signy Island (60°42′ S, 45°35′ W)	*Oculimacula yallundae* (Helotiales)	FM207641

[a] Upson et al. (2009)

filter paper, on which the seeds were repeatedly washed with sterile water (500 mL). Using sterile technique, the seeds were then transferred to Petri dishes containing 1.5% plant agar (Duchefa Biochemie, Amsterdam, The Netherlands). Ten seeds were transferred to each dish. The dishes were sealed with Parafilm and kept in the dark at 5°C for 15 days to stratify (Holtom and Greene 1967) and were then incubated at room temperature. Any seeds with bacterial or fungal contaminants were removed, along with surrounding agar medium, using sterile technique. Germination of seeds free from contaminants, of which there were approximately 90, occurred in 2–4 weeks.

Perlite (9 g) and nutrient solution (20 mL), also acidified to pH 5, were added to 90 glass conical flasks (100-mL capacity). Half of the flasks received one fifth Rorison's nutrient solution (Hewitt 1966), with N, in the form of ammonium sulphate, at a concentration of 11.2 mg L^{-1}. The remaining flasks received casein hydrolysate added to a basal medium of N-free one fifth Rorison's nutrient solution so that N was also present at a concentration of 11.2 mg L^{-1}. Casein hydrolysate was used as the organic N source because it contains a range of peptides and monomeric amino acids and so covers a range of different organic N forms. At the start of the growing season at Signy Island, the mean total dissolved nitrogen concentration in soil under *D. antarctica* and *C. quitensis* is approximately 8 mg L^{-1} (Roberts et al. 2009).

The necks of the 90 flasks were stoppered with non-absorbent cotton wool, which was covered with aluminium foil, and the flasks were then sterilised at 121°C for 15 min and cooled to room temperature. An individual seedling at the one-leaf stage was then aseptically transferred to each flask under a sterile hood. An inoculate, made by macerating either uncolonised or colonised pieces of 1.2% water agar medium (approximately 20×10×6 mm) in sterile distilled water (15 mL) for 1 min, was then added to the perlite in each flask. The colonised pieces of agar medium had been cut under sterile conditions from the growing margin of 2-week-old colonies of DSE, grown at 18°C in the dark. Eleven to 12 flasks were prepared for each of the six DSE isolates and the uninoculated control, with five or six of these flasks each containing either an inorganic or organic N source. The flasks were then placed into a growth room set to a 16-h cycle of light (300 μmol m^{-2} s^{-1}) at 6°C and 8 h of darkness (4°C). At 30 days after the start of the experiment, sterile distilled water (10 mL) was added to each flask, again under a sterile hood.

Sampling

A single sampling was made of the plants at 60 days. We chose not to make more than one sampling in order to retain five to six replicates for each level of N form and DSE isolate in subsequent statistical analyses. Each individual plant was removed from its flask and split into shoot and root material, and the roots were washed in water and cleaned of debris. Depending on the size of the root system, between ten and 25 segments of root (10-mm length) were randomly selected and removed, leaving 90% fresh weight of roots. This remaining material was dried, along with shoots, at 80°C for 48 h to predict the dry weight of the entire root system. The selected root segments were either surface-sterilised, in order to re-isolate DSE, or were used for microscopy analyses, as described below.

Re-isolation of DSE

Using sterile technique, root segments were agitated briefly in 5% calcium hypochlorite solution and were then serially washed in sterile dH_2O. They were then washed under sterile conditions in batches of three for 5 min, with ten changes of 10 mL sterile dH_2O in 25 mL 'Universal' screw-capped bottles, using a wrist-action flask shaker. Between each wash, root segments were transferred from one bottle to the next with fine forceps using sterile technique. Root segments were then blotted briefly on sterile filter paper and cut into 1- to 2-mm segments. Five root segments were placed equidistantly into 1% malt extract agar medium in 90-mm Petri dishes. Approximately 30 segments of root were plated from each plant. The dishes were incubated at 18°C, close to the temperature optimum for Antarctic soil fungi (Kerry 1990; Azmi and Seppelt 1997), for 12 weeks.

Microscopy analyses

Root segments, cleared in 10% KOH and stained with 0.05% aniline blue using the cold-staining method of Grace and Stribley (1991), were mounted on glass slides in lactoglycerol and scored at 400× magnification for the percentage of root length colonised by hyphae and microsclerotia using the method of McGonigle et al. (1990). Approximately 30 intersections were scored per slide.

Nitrogen and phosphorus analyses

Dried shoots and roots (typically 2.5–3.5 mg) were each digested at 370°C for 6 h in 1 mL of a mixture of salicylic acid dissolved in concentrated sulphuric acid (33 g L^{-1}) with lithium sulphate and copper sulphate (500 mg L^{-1}). The digests were diluted to 10 mL with UHP grade reverse osmosis water, and total N and P were determined using a modification of the Kjeldahl method (Allen 1989). Root N and P contents were corrected for the material removed for re-isolation of DSE and microscopy analyses.

Statistical analyses

One- and two-way analyses of variance were used to determine the main and interactive effects of N form and DSE inoculation on the dry weights and the N and P contents and concentrations of shoots and roots. Tukey's pairwise comparisons test was used to compare individual means. Pearson's correlations were used to determine associations between root lengths colonised by DSE and shoot or root dry weights, nutrient contents or concentrations. Data from uninoculated plants were excluded from the latter analyses. All errors reported below are standard errors.

Results

Colonisation of roots by DSE

Hyphae or microsclerotia were not observed in the roots of uninoculated control plants (Table 2). All isolates except H3 colonised between 91% and 98% of root length (Table 2). The roots of inoculated plants were typically colonised by hyaline hyphae, although dematiaceous hyphae were formed in the roots of all inoculated plants. Hyphae or microsclerotia were only observed in the epidermis or cortex: colonisation of the stele was not observed. Microsclerotia were observed in the roots of plants inoculated with isolates C7, I9 and H4. The mean percentages of root length colonised by the microsclerotia formed by these isolates were 10.42 (±2.64)%, 8.00 (±2.3)% and 0.36 (±0.33)%, respectively. Those formed by isolates C7 and H4 were dematiaceous, whereas those formed by I9 included dematiaceous, stained and unstained hyaline microsclerotia. Individual cells in the microsclerotia formed by each of the three isolates measured approximately 10×5 μm.

Table 2 Percentage of root length of plants grown with inorganic and organic N sources colonised by hyphae of DSE isolates

DSE isolate	N form	
	Inorganic	Organic
None (control)	0.0 (±0.0)	0.0 (±0.0)
C7	91.2 (±3.1)	94.2 (±1.4)
H3	69.4 (±6.4)	87.8 (±3.3)
I9	96.7 (±1.4)	97.8 (±1.1)
H4	93.0 (±1.6)	94.0 (±1.5)
C4	92.0 (±1.8)	95.4 (±1.9)
I4	94.2 (±2.4)	95.3 (±2.0)

Values are means of five or six replicates (±SEM). See Table 1 for taxonomic identities of isolates

Re-isolation of DSE

DSE were re-isolated from the roots of all plants except those inoculated with the C7 and H3 isolates. Contaminants were not isolated from the roots of any plants.

Biomass

N form had a highly significant main effect on shoot dry weights (Table 3): the mean dry weight of shoots grown with an organic N supply was 18% lower than that of shoots supplied with inorganic N (2.367±0.10 vs. 2.883± 0.11 mg; Fig. 1). Similarly, there was a highly significant main effect of N form on root dry weight (Table 3): the roots grown with an organic N supply were 20% lighter than those of plants grown with inorganic N (3.929±0.22 vs. 3.149±0.18 mg; Fig. 1). DSE isolate also had highly significant main effects on shoot and root dry weights (Table 3), with a wide range of responses being recorded to the six different DSE isolates (Fig. 1). Moreover, highly significant interactive effects of N form and DSE isolate were also recorded on shoot and root dry weights (Table 3). The shoot dry weights of plants supplied with N in inorganic form and inoculated with the I9 and H4 isolates were reduced by 42% and 45%, relative to uninoculated control plants, respectively (Fig. 1). In contrast, all DSE isolates except H4 increased the shoot dry weights of plants supplied with organic N, by between 51% and 178%, relative to uninoculated controls (Fig. 1). Similar responses were recorded for root dry weights: all isolates except C7 and H3 reduced root dry weights by between 33% and 71%, relative to uninoculated controls, when plants had been supplied with inorganic N. In contrast, when N had been supplied in organic form, all isolates except H4 and C4 increased root dry weights by 159–247%, relative to control plants (Fig. 1).

Nitrogen

The form of N that plants were supplied with did not influence shoot or root N content (Table 3). However, a main effect of DSE isolate was found on the N contents of shoots and roots (Table 3), with a range of responses recorded to the different isolates (Fig. 2a). Highly significant N form × DSE interactions were also found for shoot and root N contents (Table 3). Relative to uninoculated controls, the DSE isolates had no effect on, or showed a tendency to reduce, the N contents of shoots when N had been supplied to plants in inorganic form (Fig. 2a), but with N supplied in organic form, the C7, H3, I9 and I4

Table 3 Main and interactive effects of N form and DSE on the biomasses, N and P contents and concentrations of shoots and roots of *D. antarctica* plants

Response		Factor	*F* ratio	*P* value
Biomass	Shoot dry weight	N form	30.35	**<0.001**
		DSE	22.61	**<0.001**
		N form × DSE	11.25	**<0.001**
	Root dry weight	N form	29.53	**<0.001**
		DSE	38.49	**<0.001**
		N form × DSE	17.92	**<0.001**
Nitrogen	Shoot N content	N form	0.30	0.587
		DSE	11.05	**<0.001**
		N form × DSE	6.42	**<0.001**
	Root N content	N form	3.67	0.060
		DSE	11.37	**<0.001**
		N form × DSE	4.74	**<0.001**
	Shoot N concentration	N form	18.91	**<0.001**
		DSE	5.24	**<0.001**
		N form × SE	4.57	**0.001**
	Root N concentration	N form	4.99	**0.029**
		DSE	18.36	**<0.001**
		N form × DSE	6.32	**<0.001**
Phosphorus	Shoot P content	N form	15.09	**<0.001**
		DSE	10.94	**<0.001**
		N form × DSE	6.65	**<0.001**
	Root P content	N form	17.48	**<0.001**
		DSE	17.48	**<0.001**
		N form × DSE	8.57	**<0.001**
	Shoot P concentration	N form	3.22	0.078
		DSE	4.74	**<0.001**
		N form × DSE	5.55	**<0.001**
	Root P concentration	N form	2.33	0.132
		DSE	5.33	**<0.001**
		N form × DSE	5.04	**<0.001**

Significant *P* values are marked in bold. Error *df* in all analyses were 63–65

isolates each enhanced shoot N contents by between 52% and 123% compared with uninoculated controls (Fig. 2a). Similarly, root N content was unaffected by DSE isolates when N was supplied in inorganic form, but all DSE isolates except H4 enhanced root N contents by 55–104% when N was supplied in organic form, relative to uninoculated controls (Fig. 2a).

Significant main effects of N form on shoot and root N concentrations were recorded (Table 3): the N concentrations of shoots grown with inorganic and organic N supplies were 11.34 (±0.44) and 13.48 (±0.48) μg mg^{-1}, and those of roots were 7.34 (±0.39) and 8.04 (±0.31) μg mg^{-1}, respectively. DSE isolate similarly had highly significant main effects on shoot and root N concentrations (Table 3). Highly significant interactions of N form and DSE isolate was also recorded on shoot and root N concentrations (Table 3). The only isolate to influence shoot N concentration when plants had been grown with an inorganic N supply was I9, which increased the concentration of N in shoots by 53% compared with control plants (Fig. 2b). In contrast, the H3 isolate decreased shoot N concentration by 32%, relative to uninoculated controls, when plants had been grown with an organic N source (Fig. 2b). Root N concentrations showed similar effects: relative to uninoculated controls, when plants had been supplied with inorganic N, the I9 and H4 isolates increased root N concentration by 50% and 152%, respectively, but when plants had been grown with an organic N supply, the H3 isolate decreased root N concentration by 42% (Fig. 2b).

Phosphorus

Main effects of N form on shoot and root P contents were recorded (Table 3): plants grown with an inorganic N supply had a higher mean shoot P content than those

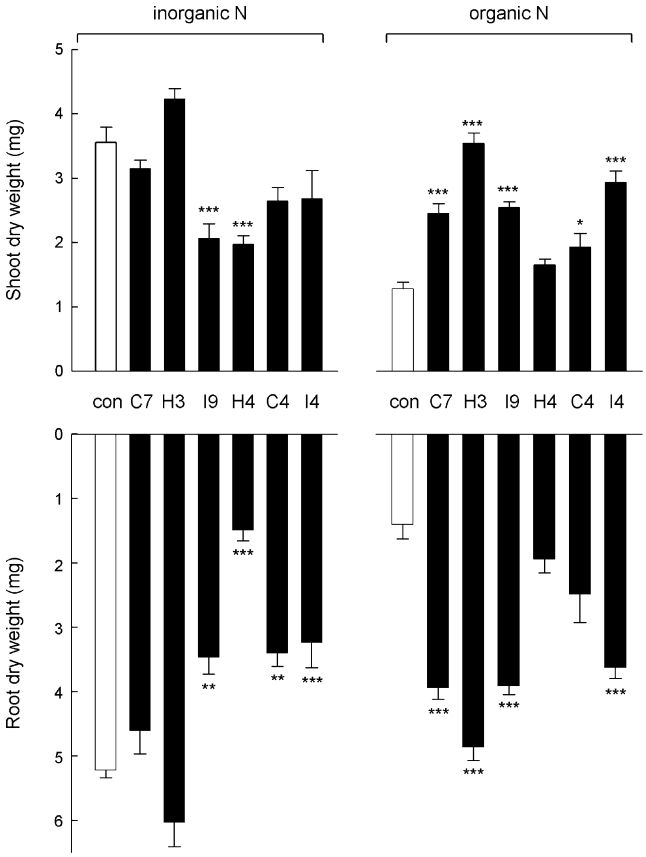


Fig. 1 Shoot and root dry weights of uninoculated control plants (‘*con*’, *open bars*) and of plants inoculated with six DSE isolates (*filled bars*) supplied with inorganic (*left-hand block of bars*) or organic (*right-hand block of bars*) nitrogen. Values are means of five to six replicates + SEM. Values for inoculated plants differing from those of uninoculated control plants within each group of seven values are denoted by **P*<0.05, ***P*<0.01 and ****P*<0.001

grown with organic N (10.63±0.22 vs. 9.51±0.34 μg) and a higher mean root P content (10.01±0.45 vs. 8.46± 0.34 μg). DSE isolate also had highly significant main effects on shoot and root P contents (Table 3), with a range of responses to each of the six isolates (Fig. 3a). Moreover, there were highly significant N form × DSE interaction terms for shoot and root P contents (Table 3): compared with uninoculated controls, isolates H3 and H4, increased and decreased shoot P content by 20% and 19% when N had been supplied in inorganic form, respectively (Fig. 3a), but the H3, I9 and I4 isolates each increased the shoot P contents of plants grown with an organic N supply by 47–65% compared with uninoculated controls (Fig. 3a). Root P contents showed similar effects: relative to uninoculated controls, only the H3 and H4 isolates affected root P content when N was supplied in inorganic form, increasing and decreasing P content by 38% and 51%, respectively (Fig. 3a), whereas the C7, H3, I9 and I4 isolates each increased the root P content of plants supplied with organic N by 90–106% (Fig. 3a).

Main effects of N form were not recorded on shoot or root P concentrations (Table 3). However, highly significant main effects of DSE isolate were found on the P concentrations of shoots and roots (Table 3). There were also highly significant N form × DSE interactions for shoot and root P concentrations (Table 3). Relative to uninoculated controls, the I9 isolate increased shoot P concentration by 70% when plants had been supplied with inorganic N (Fig. 3b), but all isolates except H4 decreased shoot P concentration by 26–41% when N had been supplied in organic form (Fig. 3b). Similarly, when plants had been supplied with inorganic N, only the I9 and H4 isolates affected P concentration in roots, increasing P concentration by 45% and 77%, respectively (Fig. 3b), but when plants had been grown with an organic N supply, the C7, H3, I9 and I4 isolates each decreased shoot P concentration by 29–50% compared with uninoculated controls (Fig. 3b).

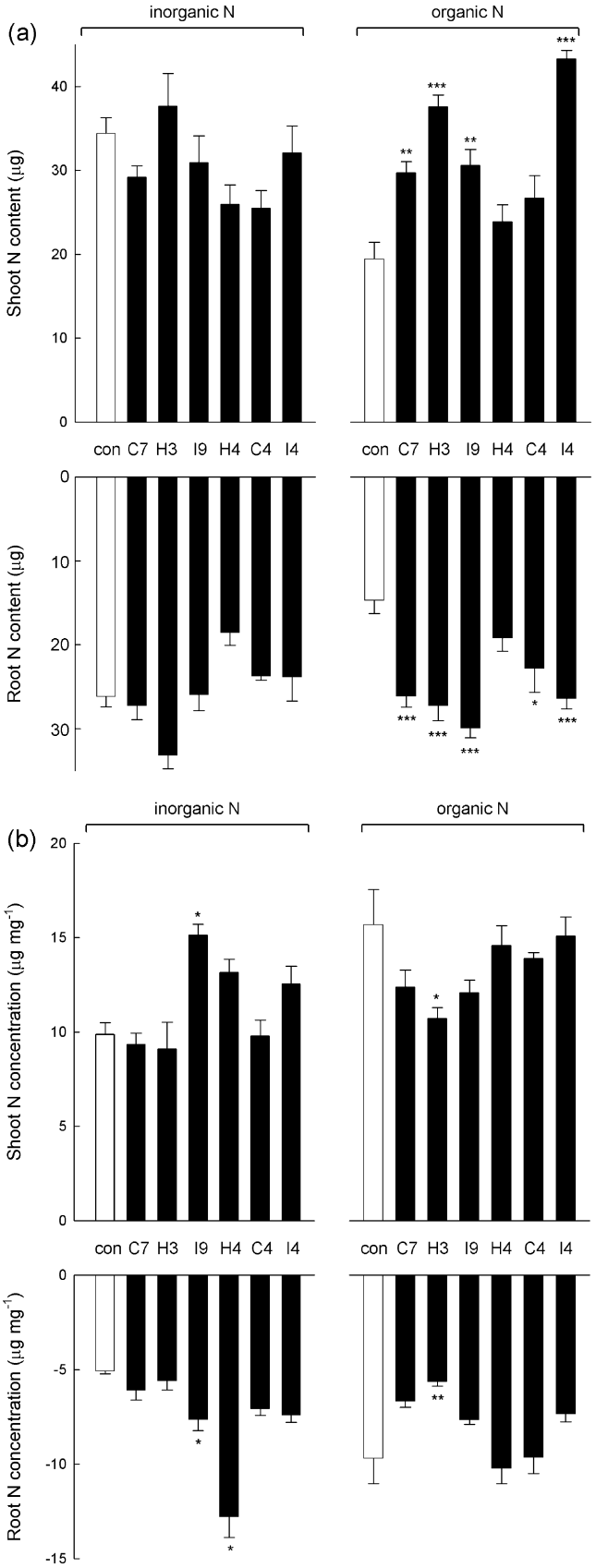
Correlative analyses

When N had been supplied in inorganic form, significant negative associations were found between the percentage of root length colonised by DSE hyphae and shoot and root dry weights, root N content and shoot and root P contents (Table 4). In contrast, when organic N was supplied to plants, no associations were found between the percentage of root length colonised by DSE hyphae and any plant performance parameters ($P>0.05$; Table 4). Plant performance also did not correlate with the percentage of root length colonised by microsclerotia, either when N had been applied in inorganic or organic form ($P>0.05$; data not shown).

Discussion

Koch's postulates (Koch 1882) were satisfied for most of the DSE used in our study, with four of the six fungi being re-isolated from the roots of *D. antarctica* onto which they were inoculated and contaminants not being isolated from any roots. Although the C7 and H3 isolates were not recovered from the roots, C7 formed microsclerotia and both isolates formed dematiaceous hyphae in roots, both of which were similar to those recorded in roots sampled from maritime and sub-Antarctica (Upson et al. 2008). We are thus confident that the effects observed in our study were attributable to the inoculated DSE which are each present in the roots of *D. antarctica* in the natural environment (Upson et al. 2009).

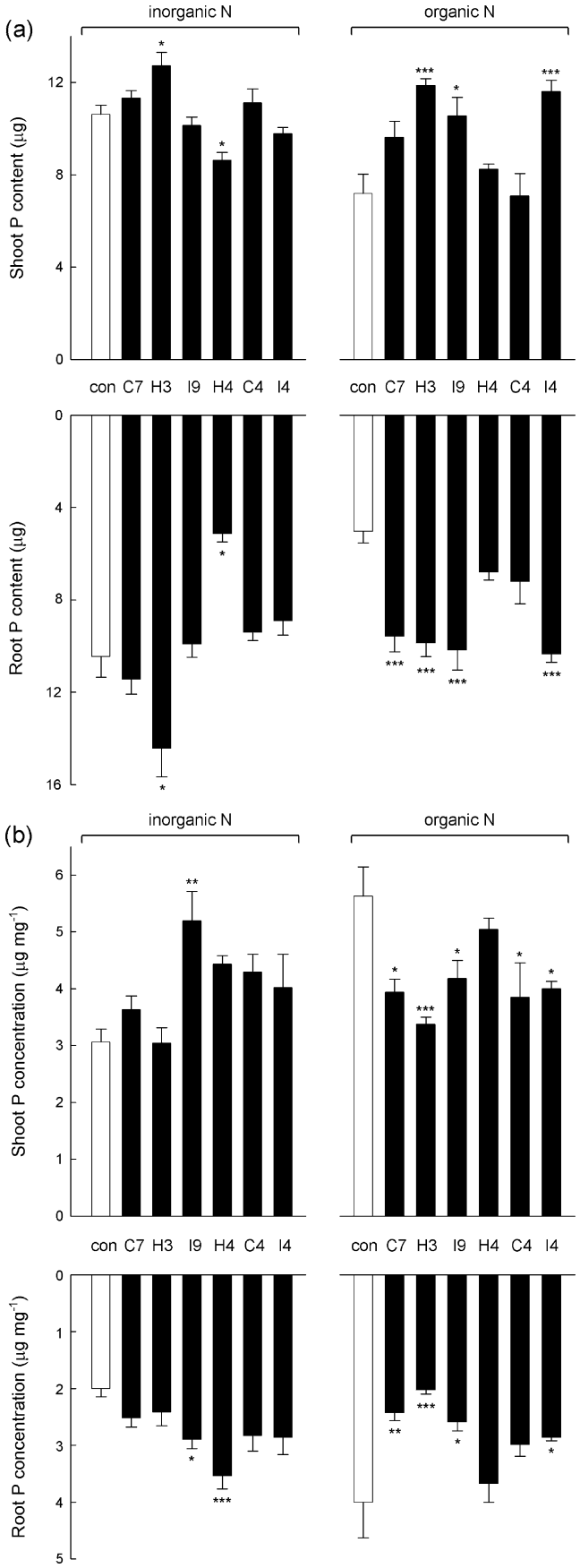
Our study recorded consistent interactions between nitrogen form and DSE isolates on plant biomass and nutrient status, indicating that the form in which nitrogen



◀ **Fig. 2** Shoot and root nitrogen contents (**a**) and concentrations (**b**) of uninoculated control plants ('*con*', *open bars*) and of plants inoculated with six DSE isolates (*filled bars*) supplied with inorganic (*left-hand block of bars*) or organic (*right-hand block of bars*) nitrogen. Replication and notation as in Fig. 1

was supplied to plants altered their responses to the different DSE isolates. The clearest responses were observed for plant biomass: in the presence of inorganic nitrogen, shoot or root dry weights were either unaffected by inoculation with DSE or were reduced by 33–71% compared with uninoculated control plants. These data were confirmed by correlative analyses, which showed close negative associations between the percentage of root length colonised by DSE hyphae and biomass when plants had been supplied with ammonium sulphate. In the presence of organic nitrogen, however, the DSE usually increased plant biomass by 51–274%, relative to uninoculated controls. In the absence of the DSE, *D. antarctica* was apparently unable to access nitrogen present in organic form: the differences between the shoot and root weights of uninoculated and inoculated plants supplied with casein hydrolysate were apparently owing to the inability of the former plants to access organic nitrogen. This was similar to the response of *Pinus contorta* to *P. fortinii* recorded by Jumpponen et al. (1998) who found that inoculation with the DSE significantly increased the biomass of the host plant species, but only in the presence of urea. Similarly, Usuki and Narisawa (2007) found that the dry weights of *Brassica campestris* plants supplied with four amino acids as sole nitrogen sources were increased when plants were grown with the DSE *Heteroconium chaetospira*, relative to inoculated control plants. As has been done for *H. chaetospira* (Usuki and Narisawa 2007), it would be useful for future studies to assess whether other DSE affect plant growth differently in the presence of individual amino acids or peptides.

Interactive effects of DSE inoculation were also observed on the nitrogen and phosphorus contents and concentrations of plants: in broad terms, the inoculation of plants with DSE in the presence of inorganic nitrogen had only sporadic effects on the nutrient status of plant parts. In contrast, in the presence of organic nitrogen, between three and five of the six DSE isolates increased the nitrogen and phosphorus contents of shoots and roots relative to uninoculated controls. When plants had been supplied with organic nitrogen, few effects of DSE inoculation were found on the nitrogen concentrations of plant parts, but all but one or two of the isolates decreased phosphorus concentrations in shoots and roots. This suggests that most of the isolates were able to maintain an adequate supply of nitrogen, but not of phosphorus, to roots to support plant growth in the presence of an organic nitrogen source. Five of the six isolates used in our study were members of the



◀ **Fig. 3** Shoot and root phosphorus contents (**a**) and concentrations (**b**) of uninoculated control plants (‘*con*’, *open bars*) and of plants inoculated with six DSE isolates (*filled bars*) supplied with inorganic (*left-hand block of bars*) or organic (*right-hand block of bars*) nitrogen. Replication and notation as in Fig. 1

Helotiales, an order of fungi known to contain mutualistic fungi, notably *Rhizoscyphus ericae*, which are capable of enhancing the flow of nitrogen into roots from the acidic soils that their host plants inhabit (Smith and Read 2008). From the data shown here, it is apparent that the same might be true for the Helotialian fungi similar to *O. yallundae*, *Mollisia* and *Tapesia* spp. that occur in the roots of *D. antarctica* (Upson et al. 2009). However, the ability to enhance plant growth in the presence of an organic nitrogen source is not restricted to members of the Helotiales, since isolate C4, which could not be confidently assigned to any order but was tentatively ascribed to the Dothideales by Upson et al. (2009), was also found to increase the growth of *D. antarctica* in the present study.

Previous reports similarly indicate that DSE in the Helotiales can enhance plant growth. In a study on Northern European *Deschampsia flexuosa*, three DSE isolates belonging to the Helotiales were found to enhance the shoot nitrogen content of seedlings (Zijlstra et al. 2005). *L. orchidicola*, which has close taxonomic affinities with *O. yallundae* (Upson et al. 2009), also has positive effects on the shoot and root biomass of *Picea glauca*, but has mildly pathogenic effects on *Potentilla fruticosa* and *Dryas octopetala* (Fernando and Currah 1996). DSE bearing close rDNA sequence homologies to the reference (ex-type) strain of *L. orchidicola* have also been shown to have positive effects on the shoot biomass and height of the alpine plant species *Saussurea involucrata* (Wu and Guo 2008) and to be associated with the early-season uptake of soil nitrogen by the alpine snow buttercup, *Ranunculus adoneus* (Mullen et al. 1998; Schadt et al. 2001).

It is unlikely that the DSE studied here acted as true mycorrhizal symbionts. In the mycorrhizal symbiosis, nutrients are transferred directly through hyphae into plant tissues from beyond the nutrient depletion zones around roots (Smith and Read 2008). In order to effect the transfer of nutrients via hyphae, true mycorrhizal fungi form branched or convoluted fungal structures in roots, such as the highly branched arbuscules formed by arbuscular mycorrhizas or the convoluted coils formed by ericoid mycorrhizas. Such structures were not observed in the roots of *D. antarctica*. In mycorrhizal associations, these structures are enclosed by a host-derived perifungal membrane or interfacial matrix material, which allows the active exchange of nutrients between plant and fungus

Table 4 Correlation coefficients (r) and probability (P) values for associations between percentage of root length colonised by DSE hyphae and the biomasses, N and P contents and concentrations of shoots and roots of *D. antarctica* plants grown with inorganic and organic N sources

Response		N form			
		Inorganic		Organic	
		r	P value	r	P value
Biomass	Shoot dry weight	−0.573	**0.001**	−0.181	0.297
	Root dry weight	−0.526	**0.002**	−0.187	0.282
Nitrogen	Shoot N content	−0.165	0.376	−0.125	0.475
	Root N content	−0.410	**0.020**	−0.053	0.761
	Shoot N concentration	0.343	0.056	0.041	0.614
	Root N concentration	0.303	0.092	0.180	0.302
Phosphorus	Shoot P content	−0.477	**0.006**	−0.168	0.335
	Root P content	−0.496	**0.004**	−0.035	0.841
	Shoot P concentration	0.344	0.054	0.020	0.911
	Root P concentration	0.263	0.146	0.165	0.344

Significant P values are marked in bold. Error *df* in analyses were 30

(Bonfante and Perotto 1995). These structures, which are the hallmarks of active biotrophic fungal associations, are apparently not formed by DSE (Peterson et al. 2008). Recently, Usuki and Narisawa (2007) argued that organic nitrogen could be transferred directly into the roots of *B. campestris* via the hyphae of *H. chaetospira*, which extensively colonise the roots of plants supplied with an amino acid as a sole nitrogen source. However, Peterson et al. (2008) point out that intracellular hyphae of *H. chaetospira* in the roots of *B. campestris* are not enclosed by a perifungal membrane, but instead by fibrillar material, and that host cell cytoplasm has a degraded appearance. Whilst the association between *H. chaetospira* and *B. campestris* might represent a special case, we postulate that the majority of DSE most probably utilise proteolytic enzymes, which they are known to produce (Caldwell et al. 2000), to mineralise organic nitrogen in the rhizosphere, making the nitrogen more freely available to roots in inorganic form. This was put forward as an explanation for the positive growth responses of a temperate grass to the DSE *Phialophora graminicola* (Newsham 1999). Similarly, Mandyam and Jumpponen (2005) suggest that DSE may be able to mobilise nutrients from amino acids, which are abundant in soil. There is a small possibility that respiratory CO_2 release contributed a further fertilising effect upon the plants grown in the presence of decomposable organic N, but since the ambient CO_2 concentration (approximately 380 ppm) to which plants in all treatments were exposed in the growth room was well above that normally considered to be limiting for photosynthesis, this is unlikely to have had a major impact on plant growth.

Other than hyphae, the only structures formed in roots by the fungi studied here were microsclerotia. The possibility that microsclerotia might act as sites of nutrient exchange between fungus and plant (Herrmann 1990) is not supported by our study, since only two of the five DSE isolates that increased the shoot or root biomass of *D. antarctica* in the presence of organic nitrogen formed microsclerotia in roots, and correlations between plant performance parameters and root lengths colonised by microsclerotia were not recorded. Furthermore, it is unlikely that these structures acted as sites of active nutrient exchange between DSE and plant: compared with the coils of ericoid mycorrhizal fungi and arbuscules, these structures have a low surface area in contact with root cells (e.g. Wu and Guo 2008). Fernando and Currah (1996) similarly concluded that microsclerotia were not sites for active nutrient exchange and were most probably resting structures.

The frequencies of DSE hyphae in roots in the current study were higher than are recorded in roots sampled from the Antarctic natural environment: Upson et al. (2008) report that between 3% and 75% of the root length of *D. antarctica* sampled from 14 locations in the sub- and maritime Antarctic is occupied by DSE hyphae. It is hence likely that the current study overestimates the effects of these fungi on the grass in its natural habitat. However, the pH of the substrate used in the experiment reported here was the same as that of the soil in which *D. antarctica* grows, the temperatures at which the experiment was conducted were representative of daytime soil surface temperatures recorded in maritime Antarctic habitats, and the concentration of dissolved organic nitrogen supplied to plants was similar to that available to plants in their natural habitat (Roberts et al. 2009). Hence, given that much of the nitrogen present in maritime Antarctic soils is present in organic form (Roberts et al. 2009), we anticipate that at least some of the positive benefits reported here of DSE on plant growth

might be expected in the field, and that these could have significant impacts on the growth and survival of *D. antarctica* in the natural environment.

Acknowledgements Financial support was provided by a research studentship to RU awarded by the Natural Environment Research Council through the Antarctic Funding Initiative (award no. NER/G/S/2000/00318). Bob Keen analysed N and P concentrations of plant material. Paul Bridge kindly assisted with sequence analyses and two anonymous referees supplied helpful comments. All are gratefully acknowledged.

References

Addy HD, Piercey MM, Currah RS (2005) Microfungal endophytes of roots. Can J Bot 83:1–13. doi:10.1139/b04-171

Allen SE (1989) Chemical analysis of ecological materials. Blackwell Scientific, Oxford, UK

Azmi OR, Seppelt RD (1997) Fungi of the Windmill Islands, continental Antarctica. Effect of temperature, pH and culture media on the growth of selected microfungi. Polar Biol 18:128–134. doi:10.1007/s003000050167

Bledsoe C, Klein P, Bliss LC (1990) A survey of mycorrhizal plants on Truelove Lowland, Devon Island, N.W.T., Canada. Can J Bot 68:1848–1856

Bonfante P, Perotto S (1995) Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol 130:3–21. doi:10.1111/j.1469-8137.1995.tb01810.x

Caldwell BA, Jumpponen A, Trappe JM (2000) Utilization of major detrital substrates by dark-septate root endophytes. Mycologia 92:230–232. doi:10.2307/3761555

Currah RS, van Dyk M (1986) A survey of some perennial vascular plant species native to Alberta for the occurrence of mycorrhizal fungi. Can Field Nat 100:330–342

Fernando AA, Currah RS (1996) A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (fungi imperfecti) on the growth of some subalpine plants in culture. Can J Bot 74:1071–1078. doi:10.1139/b96-131

Grace C, Stribley DP (1991) A safer procedure for routine staining of vesicular–arbuscular mycorrhizal fungi. Mycol Res 95:1160–1162. doi:10.1016/S0953-7562(09)80005-1

Haselwandter K, Read DJ (1982) The significance of a root-fungus association in two *Carex* species of high-alpine plant communities. Oecologia 53:352–354. doi:10.1007/BF00389012

Herrmann W (1990) 'Microsclerotia' a mycorrhizal structure? In: Reisinger A, Bresinsky A (eds) Abstracts of the 4th International Mycological Congress. University of Regensburg, Regensburg, p 368

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. East Malling, UK

Holtom A, Greene SW (1967) The growth and reproduction of Antarctic flowering plants. Philos Trans R Soc Lond B Biol Sci 252:323–337. doi:10.1098/rstb.1967.0021

Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol 140:295–310. doi:10.1046/j.1469-8137.1998.00265.x

Jumpponen A, Mattson K, Trappe JM (1998) Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. Mycorrhiza 7:261–265. doi:10.1007/s005720050190

Kerry E (1990) Effects of temperature on growth rates of fungi from Subantarctic Macquarie Island and Casey, Antarctica. Polar Biol 10:293–299

Kielland K (1994) Amino acid absorption by Arctic plants: implications for plant nutrition and nitrogen cycling. Ecology 75:2373–2383. doi:10.2307/1940891

Koch R (1882) Über die Milzbrandimpfung. Eine Entgegnung auf den von Pasteur in Genf gehaltenen Vortrag. Fischer, Berlin, Germany

Kohn LM, Stasovski E (1990) The mycorrhizal status of plants at Alexandra Fiord, Ellesmere Island, Canada, a high Arctic site. Mycologia 82:23–35. doi:10.2307/3759959

Laursen GA, Treu R, Seppelt RD, Stephenson SL (1997) Mycorrhizal assessment of vascular plants from subantarctic Macquarie Island. Arct Alp Res 29:483–491. doi:10.2307/1551996

Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. Stud Mycol 53:173–189

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swann JA (1990) A new method which gives an objective measure of colonization of roots by vesicular–arbuscular mycorrhizal fungi. New Phytol 155:495–501. doi:10.1111/j.1469-8137.1990.tb00476.x

Mullen RB, Schmidt SK, Jaeger CH (1998) Nitrogen uptake during snowmelt by the snow buttercup, *Ranunculus adoneus*. Arct Alp Res 30:121–125. doi:10.2307/1552126

Newsham KK (1999) *Phialophora graminicola*, a dark septate fungus, is a beneficial associate of the grass *Vulpia ciliata* ssp. *ambigua*. New Phytol 144:517–524. doi:10.1046/j.1469-8137.1999.00537.x

Newsham KK, Upson R, Read DJ (2009) Mycorrhizas and dark septate endophytes in polar regions. Fungal Ecol 2:10–20. doi:10.1016/j.funeco.2008.10.005

Peterson RL, Wagg C, Pautler M (2008) Associations between microfungal endophytes and roots: do structural features indicate function? Can J Bot 86:445–456

Read DJ, Haselwandter K (1981) Observations on the mycorrhizal status of some alpine plant communities. New Phytol 88:341–352. doi:10.1111/j.1469-8137.1981.tb01729.x

Roberts P, Newsham KK, Bardgett RD, Farrar JF, Jones DL (2009) Vegetation cover regulates the quantity, quality and temporal dynamics of dissolved organic carbon and nitrogen in Antarctic soils. Polar Biol. doi:10.1007/s00300-009-0599-0

Schadt CW, Mullen RB, Schmidt SK (2001) Isolation and phylogenetic identification of a dark-septate fungus associated with the alpine plant *Ranunculus adoneus*. New Phytol 150:747–755. doi:10.1046/j.1469-8137.2001.00132.x

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London

Stoyke G, Currah RS (1991) Endophytic fungi from the mycorrhizae of alpine ericoid plants. Can J Bot 69:347–352. doi:10.1139/b91-047

Treu R, Laursen GA, Stephenson SL, Landolt JC, Densmore R (1996) Mycorrhizae from Denali National Park and Preserve, Alaska. Mycorrhiza 6:21–29. doi:10.1007/s005720050101

Upson R, Newsham KK, Read DJ (2008) Root–fungal associations of *Colobanthus quitensis* and *Deschampsia antarctica* in the maritime and sub-Antarctic. Arct Antarct Alp Res 40:592–599. doi:10.1657/1523-0430(07-057)[UPSON]2.0.CO;2

Upson R, Newsham KK, Bridge PD, Pearce DA, Read DJ (2009) Taxonomic affinities of dark septate root endophytes of *Colobanthus quitensis* and *Deschampsia antarctica*, the two native Antarctic vascular plant species. Fungal Ecol (in press)

Usuki F, Narisawa K (2007) A mutualistic symbiosis between a dark septate endophytic fungus, *Heteroconium chaetospira*, and a

nonmycorrhizal plant, Chinese cabbage. Mycologia 99:175–184. doi:10.3852/mycologia.99.2.175

Väre H, Vestberg M, Eurola S (1992) Mycorrhiza and root associated fungi in Spitsbergen. Mycorrhiza 1:93–104. doi:10.1007/BF00203256

Wilcox HE, Wang CJK (1987) Mycorrhizal and pathological associations of dematiaceous fungi in roots of 7-month-old tree seedlings. Can J Res 17:884–889. doi:10.1139/x87-140

Wu L, Guo S (2008) Interaction between an isolate of dark-septate fungi and its host plant *Saussurea involucrata*. Mycorrhiza 18:79–85. doi:10.1007/s00572-007-0159-9

Zijlstra JD, Van't Hof P, Baar J, Verkley GJM, Summerbell RC, Paradi I, Braakhekke WG, Berendse F (2005) Diversity of symbiotic root endophytes of the Helotiales in ericaceous plants and the grass *Deschampsia flexuosa*. Stud Mycol 53:147–162