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Fungal root endophytes of the carnivorous plant *Drosera rotundifolia*

Richard S. Quilliam · David L. Jones

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Abstract As carnivorous plants acquire substantial amounts of nutrients from the digestion of their prey, mycorrhizal associations are considered to be redundant; however, fungal root endophytes have rarely been examined. As endophytic fungi can have profound impacts on plant communities, we aim to determine the extent of fungal root colonisation of the carnivorous plant *Drosera rotundifolia* at two points in the growing season (spring and summer). We have used a culture-dependent method to isolate fungal endophytes and diagnostic polymerase chain reaction methods to determine arbuscular mycorrhizal fungi colonisation. All of the roots sampled contained culturable fungal root endophytes; additionally, we have provided molecular evidence that they also host arbuscular mycorrhizal fungi. Colonisation showed seasonal differences: Roots in the spring were colonised by *Articulospora tetracladia*, two isolates of uncultured ectomycorrhizal fungi, an unidentified species of fungal endophyte and *Trichoderma viride*, which was present in every plant sampled. In contrast, roots in the summer were colonised by *Alatospora acuminata*, an uncultured ectomycorrhizal fungus, *Penicillium pinophilum* and an uncultured fungal clone. Although the functional roles of fungal endophytes of *D. rotundifolia* are unknown, colonisation may (a) confer abiotic stress tolerance, (b) facilitate the acquisition of scarce nutrients particularly at the beginning of the growing season or (c) play a role in nutrient signalling between root and shoot.

R. S. Quilliam (✉) · D. L. Jones
School of Environment, Natural Resources & Geography,
Bangor University,
Bangor, Gwynedd LL57 2UW, UK
e-mail: R.S.Quilliam@bangor.ac.uk

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Introduction

For over a century, the roots of carnivorous plants have been classified as non-mycorrhizal (MacDougal 1899; Juniper et al. 1989; Brundrett 2009). Although carnivorous plants may occasionally be colonised by facultative mycorrhizas, it is a common belief that they do not develop mycorrhizal associations due to their mode of nutrient acquisition, e.g. the absorption of animal-derived minerals through their specialised leaf structures, which makes the role of the fungal partner redundant. However, entering into a mycorrhizal relationship bestows benefits to the host plant which often exceed the facilitation of nutrient acquisition, e.g. abiotic and biotic stress tolerance (Auge 2001; Pozo and Azcon-Aguilar 2007), and therefore may provide the host plant with a competitive advantage particularly in high-stress environments.

Drosera rotundifolia (the round-leaved sundew) grows in wet or waterlogged, acidic bog or fen soils that are poor in plant-available nutrients (N, P, K, Ca and Mg; Adamec 1997). Therefore, the modification of the leaves into adhesive traps, together with the secretion of a suite of digestive enzymes, has evolved as a competitive strategy to capture, digest and absorb nutrients from insect prey (and other airborne organic substances, e.g. pollen grains) and has allowed these plants to grow successfully in such nutrient poor environments (Ellison and Gotelli 2009). Yet early laboratory-based studies on *Drosera* demonstrated that exclusive foliar uptake of nutrients from insect prey was insufficient for normal growth, whereas growth could be sustained by root mineral nutrition alone (reviewed by

Adamec 1997). The roots of temperate *Drosera* are usually short and weakly branched, with anatomical adaptations for growth in oxygen deficient soils, and often have extremely long root hairs (Adlassnig et al. 2005; Adamec 2005). Whilst these roots have the ability to obtain nutrients from the soil, uptake is inhibited by low nutrient levels, water-logging and anoxic soils. However, Adamec (2002) demonstrated that root nutrient uptake by several species of *Drosera* is actually stimulated by leaf nutrient absorption from insect prey.

Despite being regarded as non-mycorrhizal, several studies have reported colonisation of *Drosera* roots by mycorrhizal fungi (Crowder et al. 1990). Arbuscular mycorrhizal fungi (AMF) and dark septate endophyte (DSE) fungi sporadically colonise *Drosera intermedia* and *D. rotundifolia* (Fuchs and Haselwandter 2004; Weishampel and Bedford 2006); although the characteristic arbuscules of AMF were not observed, the presence of vesicles and aseptate hyphae were reported. As part of a larger study of root-inhabiting fungi of Australian plants, Chambers et al. (2008) isolated three fungal endophytes from the roots of *Drosera spatulata*, and following restriction fragment length polymorphism and sequence comparisons, one of the isolates was identified as an ericoid mycorrhizal species. Furthermore, a high diversity of endophytic bacteria, including N_2-fixing species, has been isolated from the roots of *Drosera villosa* (Albino et al. 2006), suggesting that carnivorous plants may not have evolved to tolerate harsh environments in isolation. There is even a suggestion that a tuberous species of *Drosera* (this perennating adaptation allows these species to survive the dry tropical heat of summers) is capable of ectomycorrhizal associations (Venugopal and Raseshowri Devi 2007).

Asymptomatic inter- and intracellular fungal hyphae found colonising root tissue are often endophytic species, which colonise living tissue without any apparent negative effects, e.g. DSE fungi (Mandyam and Jumpponen 2005). As endophytic fungi are impossible to identify by morphology alone, it is probable that in some previous investigations, fungal colonisation of roots has been misidentified as mycorrhizal. Whilst their function is not known, it is becoming apparent that fungal endophytes are ubiquitous and abundant (Rodriguez et al. 2009) with the vast majority yet to be characterised. In this study, we aim to determine the extent of root colonisation by fungi of the carnivorous plant *D. rotundifolia* throughout the growing season. We hypothesise that despite their leaf adaptations, which allow them to acquire additional sources of nutrients, carnivorous plants do host mycorrhizal and endophytic fungi. To test this hypothesis, we have used a culture-dependent method to isolate fungal endophytes and diagnostic polymerase chain reaction (PCR) methods to determine AMF colonisation.

Materials and methods

Plant and soil collection

Fourteen individual *D. rotundifolia* (28 plants in total) plants were collected on both 15 May 2008 (spring samples) and 24 July 2008 (summer samples) over an area of ca. 100 m^2, from the Cwm Idwal area of the Snowdonia National Park, Gwynedd, UK (altitude ca. 350 m above sea level; annual rainfall, 3 m; soil type, humic podzol; dominant vascular plant species, *Nardus stricta*, *Festuca ovina*, *Erica cinerea*, subject to free-range grazing of sheep during the summer, together with a significant cover of bryophytes). Whole plants, including the substrate surrounding the roots, were transferred to the lab in sealed plastic bags and stored at 4°C overnight before processing. Plants from the spring sample were very small, often with only two or three true leaves, whilst plants from the summer samples were fully grown and had started to flower. Additionally, substrate samples from the root zone were collected for chemical analyses.

Isolation of fungal endophytes

For each sampling point, roots of seven individual plants were held under running tap water until all of the soil and detritus had been removed. Healthy roots (approximately 30–40 mm per plant) were then cut into three sections of roughly equal length (approximately 10 mm) and surface-sterilised by vigorous shaking in 10% household bleach solution containing 1% Triton-X for 5 min (Wilberforce et al. 2003). Roots were serially rinsed in three washes of 10 ml sterile water, being shaken vigorously for about 2 min for each rinse. The three root sections from each replicate plant were then blotted dry on sterile paper towel and plated out on 10% modified Melin–Norkrans (MMN) agar in 90 mm Petri dishes. As a positive control, all root segments were briefly plated onto the surface of MMN agar medium for several minutes before being transferred to a second plate; this was to ensure that all isolated fungi were endophytic rather than just spores or hyphae on the root surface that had managed to survive the sterilisation procedure. All plates were incubated in the dark at 18°C. After 7, 14 and 21 days, fungal colonies were isolated by sub-culturing them onto 10% MMN.

Molecular characterisation of fungal isolates

A sterile scalpel was used to carefully scrape a small amount of mycelium (without agar contamination) from single isolates of 3-week-old fungal cultures, which was then frozen in liquid nitrogen. Whilst still frozen, the mycelium was ground to a fine powder with a micropestle (Anachem Ltd., Luton, UK), and DNA was extracted with the

DNeasy Plant Mini kit (Qiagen Ltd., Crawley, UK) following the manufacturer's protocol. PCR amplifications were carried out with the fungal-specific primer ITS1F (Gardes and Bruns 1993) and the universal eukaryotic primer ITS4 (White et al. 1990) in 25 µl reactions containing 2 µl template DNA with the following conditions: initial denaturing step of 95°C for 2 min, followed by 30 cycles of 95°C for 60 s, 54°C for 60 s, 72°C for 70 s and a final extension step of 68°C for 10 min. PCR products were purified (QiaQuick PCR purification kit; Qiagen), and target rDNA nucleotide sequence data were obtained by DNA sequencing (Macrogen, Seoul, South Korea) employing the same primers (ITS1F and ITS4). Sequences obtained from both forward and reverse primers were aligned (approximately 550 bp) using BIOEDIT ver. 7.0.9.0 (Hall 1999), initially with the CLUSTALW option (Thompson et al. 1994) and thereafter manually by visual inspection. Sequences were searched against those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm, and in every case, the top hit was recorded.

Assessment of AMF colonisation by PCR

The roots of four individual plants from each time point were cleaned under running tap water and sterilised as described above. Roots were then frozen in liquid nitrogen, ground to a fine powder with a micropestle and DNA extracted with the DNeasy Plant Mini kit (Qiagen); total DNA was eluted in 100 µl elution buffer. PCR reactions were carried out with the AMF-specific primers AML1 and AML2 (Lee et al. 2008) in 25 µl reactions containing 2 µl template DNA with the following conditions: initial denaturing step of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 40 s, 72°C for 55 s and a final extension step of 72°C for 5 min. Positive (DNA extracted from leek roots colonised with *Glomus mosseae*) and no DNA negative control reactions were run at the same time. PCR products were visualised by gel electrophoresis, and the presence of a band of ca.795 bp deemed to signify root colonisation by at least one AMF species.

Chemical analyses of soil solution

Soil samples from the root zone were collected as either the surface organic horizon which was mainly composed of decaying *Sphagnum* and surface water or the underlying soil which consisted of waterlogged peat to a depth of approximately 5 cm. Surface samples were centrifuged (4,000×*g*, 10 min, 20°C) to obtain a solution suitable for chemical analyses. Soil solution was extracted from the soil samples by gently shaking 5 g of soil with 25 ml of distilled water, centrifuging as above and recovering the supernatant. All solutions were passed through Whatman 42 filter paper and frozen at −20°C prior to chemical analysis. Nitrate was determined colorimetrically by the vanadium reduction and acidic Griess reaction (Miranda et al. 2001) and ammonium by the salicylate–nitroprusside hypochlorite method (Mulvaney 1996); limit of detection was 0.1 mg N l^{-1}. A measure of the soluble phosphate concentration was determined by the colorimetric method of Murphy and Riley (1962). Soil solution pH was either measured directly or in a 1:1 (*w*/*v*) soil-deionised water extract with standard electrodes. Moisture content was determined by drying soil samples overnight at 105°C, and soil organic matter was calculated by measuring loss on ignition at 450°C.

Results

Characterisation of fungal endophytes

All *D. rotundifolia* plants sampled contained culturable fungal root endophytes, with 19 of 42 (45.24%) root segments showing evidence of fungal colonisation (Table 1). A total of eight morphotypes were distinguished (five from spring samples and four from summer samples) of which only one (morphotype III) was common to both sampling points. Of the 14 plants sampled, only three of them harboured more than one morphotype in their root system, and all of these were from the spring samples. Agar plates used as a positive control remained microbe-free, confirming the endophytic nature of the fungal cultures isolated.

PCR products of ca. 600–1,000 bp were obtained from all of the 21 isolates, and DNA sequence analysis identified eight different species of fungi. A single morphotype (I) was isolated from all seven of the plants sampled in the spring (Table 1), and following sequencing was identified as *Trichoderma viride* (DQ846665 or FJ872073; Table 2). *T. viride* was either the sole coloniser or was isolated together with *Articulospora tetracladia* (EU998926), two species of uncultured ectomycorrhizal fungal clones (DQ233873 and FJ554196) or an unidentified fungal endophyte (EU68189). Four morphotypes were isolated from the summer samples (Table 1) with the most common morphotype being identified as *Alatospora acuminata* (AY204589; Table 2). Single isolates of an uncultured ectomycorrhizal fungal clone (DQ233873), *Penicillium pinophilum* (AB369480) and an uncultured fungal clone (EF434082) were also isolated from the summer root samples.

Assessment of arbuscular mycorrhizal colonisation

PCR products, obtained from DNA extracted from sterilised roots of *D. rotundifolia* and amplified with the AMF-

Table 1 Fungal cultures isolated from root segments of *D. rotundifolia*

	Plant/ plate	Root segment	Fungal isolate	Morphotype
Spring	DrSp001	1	DrSp001_1	I
		2	DrSp001_2	II
		3	–	
	DrSp002	1	–	
		2	DrSp002_2	I
		3	–	
	DrSp003	1	–	
		2	DrSp003_2	I
		3	–	
	DrSp004	1	DrSp004_1	I
		2	–	
		3	–	
	DrSp005	1	DrSp005_1	I
		2	DrSp005_2A	III
			DrSp005_2B	IV
		3	–	
	DrSp006	1	–	
		2	DrSp006_2	I
		3	DrSp006_3	I
	DrSp007	1	–	
		2	DrSp007_2	V
		3	DrSp007_3A	I
			DrSp007_3B	I
Summer	DrSu001	1	DrSum001_1	VI
		2	–	
		3	–	
	DrSu002	1	DrSum002_1	III
		2	DrSum002_2	III
		3	–	
	DrSu003	1	–	
		2	DrSum003_2	VI
		3	–	
	DrSu004	1	–	
		2	DrSum004_2	VI
		3	–	
	DrSu005	1	DrSum005_1	VII
		2	–	
		3	–	
	DrSu006	1	DrSum006_1	VI
		2	–	
		3	–	
	DrSu007	1	DrSum007_1	VIII
		2	–	
		3	–	

specific primers AML1 and AML2, were visualised by gel electrophoresis. For both the spring and the summer samples, four individual plants were analysed for AMF colonisation. A single plant from the spring samples and three of the plants from the summer samples produced a positive band of ca. 795 bp.

Soil properties

Both the soil and surface layer were characterised by their acidic pH and low concentrations of available nutrients (Table 3). Although the level of PO_4^{3-} in the soil remained fairly constant from the spring to summer, it was undetectable in the surface layer at either time. The concentrations of NH_4^+ and NO_3^- decreased in both the soil and surface layer, with NO_3^- becoming undetectable by the summer. Soil organic matter and moisture content were high in both seasons, with the soil remaining waterlogged in the summer.

Discussion

The diversity of fungal endophytes (in both roots and leaves) and the ubiquity of mycorrhizas in a wide range of plants are currently being embraced by both mycologists and plant scientists (Rodriguez et al. 2009; Brundrett 2009). In this study, we have demonstrated that the roots of the carnivorous plant *D. rotundifolia* host at least eight species of fungal endophyte, although as culture-dependent approaches are known to bias the species isolated, e.g. favouring fast-growing species, and the number of replicates was small, the true figure could be much higher. Additionally, we have provided molecular evidence that *D. rotundifolia* does host arbuscular mycorrhizal fungi.

Fungal colonisation of *D. rotundifolia* roots showed seasonal differences, most probably reflecting the life cycle of the fungus or the developmental stage of the host plant together with the dynamics of the abiotic environment. Soil nutrient levels were generally low in both seasons; however, the levels of soluble ammonium and nitrate were slightly higher in the spring most likely due to the process of decomposition restarting more quickly than plant growth following the winter. With the exception of *T. viride* in the spring, none of the fungal endophytes was ubiquitous, and fungal species often colonised the roots of individual plants in isolation.

Identity of the fungi isolated from *D. rotundifolia* roots

T. viride

Whilst every plant sampled in the spring was colonised by *T. viride*, it was absent from the roots of plants sampled in the summer. *T. viride* is found in a wide range of environments as a natural inhabitant of soils and is often isolated as an endophyte (Jaklitsch et al. 2006). In addition

Table 2 Sequence analysis of the ITS region (ITS1–5.8S–ITS2) of fungal endophytes isolated from the roots of D. *rotundifolia*

	Isolate	Top BLAST match[a]	Accession	Maximum identity (%)	Sequence length (bp)
Spring	DrSp001_1	*Trichoderma viride*	DQ846665	100	567
	DrSp001_2	*Articulospora tetracladia*	EU998926	99	511
	DrSp002_2	*Trichoderma viride*	FJ872073	100	536
	DrSp003_2	*Trichoderma viride*	DQ846665	100	566
	DrSp004_1	*Trichoderma viride*	DQ846665	100	566
	DrSp005_1	*Trichoderma viride*	DQ846665	100	573
	DrSp005_2A	Uncultured ectomycorrhizal fungus clone	DQ233873	99	516
	DrSp005_2B	Uncultured ectomycorrhiza (Leotiomycetes) clone	FJ554196	98	516
	DrSp006_2	*Trichoderma viride*	DQ846665	100	569
	DrSp006_3	*Trichoderma viride*	DQ846665	100	566
	DrSp007_2	Fungal endophyte sp.	EU686189	98	546
	DrSp007_3A	*Trichoderma viride*	DQ846665	100	569
	DrSp007_3B	*Trichoderma viride*	DQ846665	100	569
Summer	DrSum001_1	*Alatospora acuminata*	AY204589	95	691
	DrSum002_1	Uncultured ectomycorrhizal fungus clone	DQ233873	99	518
	DrSum002_2	Uncultured ectomycorrhizal fungus clone	DQ233873	99	518
	DrSum003_2	*Alatospora acuminata*	AY204589	95	691
	DrSum004_2	*Alatospora acuminata*	AY204589	95	691
	DrSum005_1	*Penicillium pinophilum*	AB369480	99	541
	DrSum006_1	*Alatospora acuminata*	AY204589	95	691
	DrSum007_1	Uncultured fungus clone	EF434082	91	466

[a] The *E* value for all sequences was 0, except for isolate DrSum007_1 (2.00E-176)

to its pathogen suppressive qualities, strains of *T. viride* are able to solubilise tricalcium phosphate in vitro and can significantly improve growth, yield parameters and P uptake in chickpea fertilised with rock phosphate as the sole source of P (Rudresh et al. 2005).

Table 3 Edaphic properties of *D. rotundifolia* habitat

	Spring	Summer
Surface layer		
pH	4.86±0.03	4.87±0.10
Ammonium (mg/l)	1.76±0.33	0.20±0.01
Nitrate (mg/l)	1.25±0.28	BDL
Phosphate (mg/l)	BDL	BDL
Substrate		
pH	5.06±0.10	5.12±0.05
Ammonium (mg/kg)	4.94±0.97	4.22±0.63
Nitrate (mg/kg)	2.12±0.66	BDL
Phosphate (mg/kg)	3.05±0.21	3.85±0.15
Soil moisture (%)	83.2±2.6	78.9±2.9
Organic matter (%)—loss on ignition	33.0±2.4	40.9±7.0

Values are the mean of four replicates±SE

BDL below detection limit

Ingoldian fungi (aquatic hyphomycetes)

Aquatic hyphomycetes commonly occur in running freshwater and play a major role in the breakdown of dead plant material. However, recent evidence supported by molecular data has suggested that some Ingoldian species spend at least part of their life cycle living endophytically in plant tissues (Sokolski et al. 2006; Selosse et al. 2008). The reasons behind this remain elusive although an endophytic stage may facilitate a sexual stage of these otherwise asexual species or may have an important role in propagule dispersal (Selosse et al. 2008). In the present study, two species of Ingoldian fungi were isolated from the roots of *D. rotundifolia*: *A. tetracladia* (isolated once from the spring samples) and *A. acuminata* (isolated four times from the summer samples). Both species have previously been isolated from riparian plant roots (Fisher et al. 1991; Sridhar and Barlocher 1992; Sati and Belwal 2005).

Ectomycorrhizas

Two species of uncultured ectomycorrhizal clones were isolated from the roots of *D. rotundifolia* in the spring samples, and one of them was again isolated from the

summer samples, although none of the roots collected in this study showed the morphology characteristic of ectomycorrhizal colonisation. However, a previous study from India has reported characteristic ectomycorrhizal colonisation of the corms and lateral foliar organs of the tuberous species *Drosera peltata* (Venugopal and Raseshowri Devi 2007). A similar investigation of wild growing members of *Drosera* subgen. *Ergaleium* (Conran 2008) found no such ectomycorrhizal structures; however, branching fungal hyphae of opportunistic saprophytes (presumed to be DSE fungi) was observed within the papery remnants of previous epidermal layers.

P. pinophilum

A single isolate of this species was isolated from the roots of *D. rotundifolia* collected during the summer. The decomposition of organic matter by this saprophytic fungus in cellulose-amended soil has been demonstrated to have a synergistic effect on AMF, e.g. by stimulating sporulation (Gryndler et al. 2002). Recently, it has been proposed that *P. pinophilum* is itself capable of forming arbuscular mycorrhizal associations: Following inoculation of strawberry roots, it was found to increase biomass, N and P content and the photosynthetic rate of strawberry plants (Fan et al. 2008). However, this claim has been questioned by Hempel (2009), who suggested that the sterile soil used by Fan et al. (2008) was probably contaminated by genuine (Glomeromycotan) AMF propagules.

Unidentified fungal endophytes

Two isolates remained unidentified: The sequence similarity of the isolate from the spring sample most closely matched a fungal endophyte (EU686189) originally isolated from a liverwort (Davis and Shaw 2008), whilst the isolate from the summer sample most closely matched an 'uncultured fungus' (EF434082) from humic horizon soil (Taylor et al. 2007).

Arbuscular mycorrhizas

Roots from both spring and summer samples amplified PCR products of an appropriate size to suggest colonisation by AMF, with a greater number of plants colonised in the summer (although with such a small number of replicates, this seasonal observation must be interpreted with caution). Other reports have also shown *Drosera* root colonisation by AMF to be seasonal, e.g. higher colonisation in the spring than the autumn of *D. intermedia* (Fuchs and Haselwandter 2004). In another study, the simultaneous colonisation of *D. rotundifolia* roots by AMF and DSE was observed in the late summer (Weishampel and Bedford 2006). In both of these studies, the occurrence of AMF was inferred by the presence of vesicles.

The low nutrient status, low pH and waterlogged habitat of *D. rotundifolia* would result in a particularly stressful environment for rhizosphere microorganisms, and it is clear that some fungal endophytes avoid this stress through plant colonisation. Recently, Rodriguez et al. (2008) have defined this occurrence as a 'habitat-specific, symbiotically-conferred stress tolerance' and suggest that it is responsible for the establishment of plants in high-stress environments. Similar assumptions have been made about DSE fungi, which dominate extreme environments and stressed conditions (Newsham et al. 2009). Every single plant in the current study was colonised by at least one fungal species, raising the question of whether colonisation is essential or at least confers an advantage for *D. rotundifolia* to live in its stressful habitat. For example, in acidic waterlogged soils, the decomposition of organic matter may lead to high levels of toxic H_2S, and a low redox potential can solubilise Fe and Mn to levels toxic to plant roots; additionally, under these conditions, some micronutrients will become unavailable to plants.

The role of fungal endophytes in the roots of carnivorous plants is complicated by the unique mode of nutrition of these plants. As carnivorous plants acquire substantial amounts of their nutrients from the digestion of their prey, a role in facilitating nutrient acquisition by a fungal partner seems unlikely. However, the physiological effect of absorbing nutrients from prey into the leaf is known to stimulate root nutrient uptake (Adamec 2002), although whether this is a result of increased fungal activity in the root clearly needs to be tested. Additionally, the ability of carnivorous plants to efficiently re-utilise nutrients from their senescing leaves and roots (Adamec 2002) may be facilitated by the saprophytic nature of endophytic fungal extracellular enzymes, which would be better suited to utilise and recycle this complex source of nutrients.

This work has also raised questions about interspecies colonisation dynamics of carnivorous plants. In contrast to the pervasive colonisation in the spring, *T. viride* was absent in the summer samples, which could equally be host or endophyte dependent. At the beginning of the growing season, when carbohydrates from photosynthesis are limited and insect prey is scarce (and easily washed away during heavy rain), hosting *T. viride* may be a cost-effective way for *D. rotundifolia* to gain nutrients from the soil. *T. viride* may also suppress colonisation by other fungal species, particularly AMF which are known to demand significant amounts of carbon from their host plants. Alternatively, *T. viride* may be parasitising necrotic endophytic hyphae present from the previous year; hence, its presence only being detected early in the season.

The microbial competition in this high-stress environment is also complicated by the selective fungicidal properties characteristic of the Droseraceae. Many *Drosera* species produce large quantities of the secondary metabolites plumbagin and 7-methyljuglone (water-soluble 1,4-naphthoquinones), which are known for their antifungal activity (Meazza et al. 2003; Babula et al. 2009) particularly against plant pathogens. Interestingly, the production of 7-methyljuglone in *Drosera capensis* grown in vivo was affected by the ratio of nitrate to ammonium, with production decreasing at lower nitrate concentrations (Ziaratnia et al. 2009), although the authors conclude that this result may be an artefact of variations in the pH of the media due to differential uptake of the two ions. In the current study, the nitrate to ammonium ratio of the substrate decreased later in the growing season. Whether this acts as a signal to reduce the production of naphthoquinone and allows colonisation by a functional group of fungal endophytes and AMF more advantageous for the changing abiotic conditions requires further experimental data.

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