Role of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* in arsenate resistance of *Calluna vulgaris* at contaminated mine sites.

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A thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy (Ph.D.)
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
Declaration

I hereby certify that the submission of this thesis is my own work undertaken at University of Western Sydney, Nepean, Australia and Institute of Terrestrial Ecology, Monkswood, United Kingdom. No part of the work has been submitted to any other institutions for a degree.

Signature: [Redacted]

Jade M. Sharple
With love to my Dad,

who passed away before the completion of this thesis.

(18/6/1999)

This one’s for you!
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Abstract

*Calluna vulgaris* L. Hull readily colonises arsenate (AsO$_4^{3-}$) contaminated mine spoil soils in south-west (SW) England. At these sites, it forms mycorrhizal association with the ericoid ascomycete fungus *Hymenoscyphus ericae*. The initial aim of this study was to investigate the physiological response of *H. ericae* populations to AsO$_4^{3-}$. Populations of *H. ericae* (identified by ITS-RFLP analysis) were isolated from roots of *C. vulgaris* colonising two disused As/Cu mine sites and one natural heathland site in SW England. The three populations were screened for their resistance to AsO$_4^{3-}$. Mine site populations were found to have evolved AsO$_4^{3-}$ resistance in comparison to the heathland population. In contrast, *H. ericae* demonstrated constitutive resistance to Cu.

In order to investigate the mechanism of AsO$_4^{3-}$ resistance in these *H. ericae* populations, uptake kinetics of AsO$_4^{3-}$, arsenite (AsO$_2^{3-}$) and phosphate (PO$_4^{3-}$) were determined for an isolate from a mine site and an uncontaminated heathland site. The uptake kinetics of AsO$_4^{3-}$, AsO$_2^{3-}$ and PO$_4^{3-}$ in both mine and heathland isolates were similar. Mine site *H. ericae* had an enhanced As efflux mechanism in comparison to heathland *H. ericae*. Arsenic lost from fungal hyphae was in the form of AsO$_2^{3-}$. It is suggested that this enhanced AsO$_2^{3-}$ efflux from mine site *H. ericae* enables the fungus to colonise AsO$_4^{3-}$ contaminated soils.

The response of *C. vulgaris* to AsO$_4^{3-}$ was also investigated. *C. vulgaris* from the mine site demonstrated a slightly increased resistance in comparison to heathland populations, however both AsO$_4^{3-}$ and PO$_4^{3-}$ uptake kinetics in non-mycorrhizal plants from each site were similar. Mycorrhizal association with AsO$_4^{3-}$ resistant mine site *H. ericae* significantly reduced
the uptake of $\text{AsO}_4^{3-}$ in heathland *C. vulgaris*, demonstrating the ability of *H. ericae* to confer enhanced $\text{AsO}_4^{3-}$ resistance to its host.

The present study demonstrates the evolution of $\text{AsO}_4^{3-}$ resistance through an enhanced $\text{AsO}_2^-$ efflux in populations of *H. ericae* from As contaminated mine spoil soils. *H. ericae* also confers $\text{AsO}_4^{3-}$ resistance to *C. vulgaris*. The ecological significance of these findings is discussed.
Chapter 1

Introduction

1.1. Arsenic levels in the environment

Arsenic (As) is a natural component of the earth’s crust, with low levels being found throughout the environment. Arsenic concentrations in air can range from 1-100 ng m$^{-3}$ (Peirson et al., 1974; Romo-Kroger & Llona, 1993). Concentrations in water are usually less than 10 μg l$^{-1}$ (Maher, 1985), although higher levels often occur near mineral deposits or man-made sources. The majority of As in the environment, however, exists in soil.

1.1.1. Arsenic in soil

Naturally occurring As levels in soils are usually between 1-40 mg kg$^{-1}$ soil d wt (Porter & Peterson, 1977), however these levels can be increased due to mineralisation, contamination from industrial activity (especially Cu smelters) and the use of As-based pesticides (Table 1.1). Arsenate (AsO$_4^{3-}$) is the dominant species of As in aerobic soils, with small quantities of arsenite (AsO$_2^-$) and methanearsonic acid (MMA) being present in mineralised areas. In anaerobic soils, AsO$_2^-$ is the major soluble form of As, while, in strongly reducing environments, elemental As and arsine can exist (anon., 1981). Arsenic present within soil is transported mainly through leaching. However, because many As compounds tend to adsorb to soil particles, leaching usually results in transportation over only short distances. (Moore et al., 1988; Welch et al., 1988). Soil microorganisms can assist in the transport of As. Species of Aspergillus and Scopulariopsis have the ability to oxidize AsO$_2^-$ to volatile As-compounds, which therefore increases the movement of As throughout the soil (Hiroki & Yoshiwara, 1993).
### Table 1.1 Concentrations of As in soil from different locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Site description</th>
<th>Soil As concentration $\text{mg kg}^{-1} \text{ soil d wt}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annapolis Valley, USA</td>
<td>Non-orchard</td>
<td>0.79</td>
<td>Bishop &amp; Chisholm (1962)</td>
</tr>
<tr>
<td></td>
<td>Orchard soil applied with arsenicals</td>
<td>9.8 to 124.4</td>
<td>Bishop &amp; Chisholm (1962)</td>
</tr>
<tr>
<td>New York, USA</td>
<td>Non-orchard</td>
<td>1.8 to 3.0</td>
<td>Merwin et al., (1994)</td>
</tr>
<tr>
<td></td>
<td>Orchard soil previously applied with lead arsenate</td>
<td>1.6 to 141</td>
<td>Merwin et al., (1994)</td>
</tr>
<tr>
<td>Tasmania, Australia</td>
<td>Uncontaminated</td>
<td>0.6</td>
<td>Merry et al., (1983)</td>
</tr>
<tr>
<td></td>
<td>Orchard soil</td>
<td>29</td>
<td>Merry et al., (1983)</td>
</tr>
<tr>
<td>Southern Ontario, Canada</td>
<td>Urban area</td>
<td>9.8</td>
<td>Temple et al., (1977)</td>
</tr>
<tr>
<td></td>
<td>&lt;700 m from secondary Pb smelter</td>
<td>107</td>
<td>Temple et al., (1977)</td>
</tr>
<tr>
<td>SW England</td>
<td>Past mining activity</td>
<td>322</td>
<td>Porter &amp; Peterson (1977)</td>
</tr>
<tr>
<td></td>
<td>Contaminated with mine and smelter waste</td>
<td>8510 to 26530</td>
<td>Porter &amp; Peterson (1977)</td>
</tr>
</tbody>
</table>
Arsenic levels in soil can be increased due to the use of As based pesticides. For hundreds of years the phytotoxic effects of As compounds made them attractive as herbicides. Due to the concern over the build up of As residues in soils and lake sediments, however, their popularity has since declined. As a consequence, other pesticides have replaced lead arsenate (commonly used in orchards to control insect pests) and sodium arsenite, which were used extensively as a herbicide to clear aquatic weeds and defoliate potatoes (anon., 1981). Phosphate (PO$_4^{3-}$) fertilizers are also a potential source of As. Arsenic and P share many chemical similarities, however, the chemistry of As is much more complex as it has the ability to exist in more than one oxidation state in soil compounds (anon., 1981). Arsenic may exist in three different oxidation states, namely as the metalloid (0 oxidation state), as AsO$_2^-$ (+3 oxidation state), and as AsO$_4^{3-}$ (+5 oxidation state) compounds (anon., 1981). Arsenic also forms complexes with not only metals (inorganic As compounds), but also with carbon, hydrogen and oxygen to form organic As compounds (anon., 1981). On the other hand, between 20-70% of total P in soil solution is organically bound PO$_4^{3-}$ (Marschner, 1995).

The largest input of As to soils results from the smelting of Cu, which represents approximately 40% of the total anthropogenic As input. The As in naturally occurring metal arsenides and arsenic sulfides is volatilized and oxidized during the Cu ore roasting process and condenses as arsenic trioxide (As$_2$O$_3$) in mine smelter flues. Arsenic trioxide is the most important commercial As compound and is recovered from Cu smelters as a by-product of Cu production. The combustion of coal is another form of anthropogenic As pollution. Arsenic is released into the atmosphere during the combustion process, and As containing ash is also released into the surrounding soil (anon., 1981).
Contamination of soils by the mining industry tends to be localized. However, because As contamination is often widespread, the area around a mine is also likely to have soils with a naturally high level of As. In SW England 722 km\(^2\) has been described as contaminated on the basis of correlations between soil As concentrations and stream sediment As concentrations (O’Neill, 1990). Crops grown in such areas do not generally have unusually high levels of As, though grasses and other plants growing on the mine spoil soils have been shown to have increased As concentrations (Porter & Peterson, 1977).

1.1.2. Arsenic in plants

In general, AsO\(_4^{3-}\) is less toxic to plants than AsO\(_2^-\). Arsenite interferes with the pentose phosphate pathway and therefore inhibits light activation of ATP (Marques & Anderson, 1986). The first indication of AsO\(_2^-\) toxicity in plants is wilting caused by loss of turgor. In contrast, AsO\(_4^{3-}\) is known to uncouple phosphorylation by replacing PO\(_4^{3-}\), blocking the coupled phosphorylation of adenosine diphosphate (ADP) and therefore resulting in the energy of adenosine triphosphate (ATP) not being available to the plant. Under AsO\(_4^{3-}\) stress, the plant suffers chlorosis but no loss of turgor.

Injury symptoms on crop plants resulting from toxic quantities of As in soils were noted in the 1930s, when it was found that young trees planted in old orchard soils which had been treated with organic As compounds grew slowly and were stunted (Snyder, 1935). Trappe et al. (1973) reported that apple trees growing in soil contaminated from the earlier use of PbAsO\(_4\) insecticide (300 mg kg\(^{-1}\) soil d wt) were stunted and showed sparsely mycorrhizal fine roots. In contrast, roots of trees growing in the same area in soil at < 50 mg kg\(^{-1}\) soil d wt showed healthy growth and were intensely mycorrhizal (Trappe et al., 1973).
Porter and Peterson (1977) demonstrated that As/Cu contaminated (mine and smelter waste) areas of SW England supported an impoverished and restricted flora. Experiments showed that selected flora from these sites were, however, resistant to As. Similarly, Pollard (1980) reported that Plantago lanceolata L. growing in As contaminated soil in southeastern USA showed significant tolerance to As when compared with plants from a control site. Paliouris and Hutchinson (1991) compared a population of Silene vulgaris (Moench) Garcke collected near mine tailings (soil As concentrations ranged from 2.1 to 6.9 mg kg\(^{-1}\) soil d wt) with a population at an uncontaminated site (0.02 mg kg\(^{-1}\) soil d wt). Seedlings from the mine tailings were resistant to increased levels of As, Co and Ni compared with plants from the uncontaminated sites. Paliouris and Hutchinson (1991) also reported that some As resistant plants occurred at the uncontaminated site. This finding may indicate that genetic diversity exists within the S. vulgaris population, allowing populations of plants to develop As resistance at contaminated sites.

The majority of higher plants that grow on highly metal contaminated soils are mycorrhizal (Meharg & Cairney, 2000). Mycorrhizas can exhibit both constitutive and adaptive resistances to metals. A study by Bradley et al. (1982) showed that the heathland ericaceous species, Calluna vulgaris, exhibited an increased resistance to Cu when in mycorrhizal association. This study has led to suggestions that the ability of plants to colonise metal contaminated soils may be dependent on their ability to form mycorrhizal associations. In SW England, the mining of Cu and As ore (section 1.2.1) has led to highly contaminated mine spoil soils. C. vulgaris is often the dominant vegetation on these spoil soils (Porter & Peterson, 1975) and is present at these sites in association with the ericoid mycorrhizal fungus H. ericae (Chapter 3).
1.2. Ericoid mycorrhizas

Ericoid mycorrhizas represent a distinctive form of symbiosis between plants in the order Ericales and ascomycete fungi (Bonfante, 1980). The hyphae of the fungi form a loose network over hair roots and surface network branches emerge at right angles to penetrate the epidermal cells. Once inside the cell, the fungal hyphae proliferate extensively and often there is little or no vacuolar volume apparent. The fungal hyphae produce hyphal ‘coils’ within cells, the most characteristic feature of the ericoid mycorrhizal association (Smith & Read, 1997).

Early classification of ericoid mycorrhizal fungi was based purely on morphological characteristics (McNabb, 1961; Pearson & Read, 1973a; Singh, 1974; Reed, 1987). The first ericoid mycorrhizal fungal endophyte described was an ascomycete with a dark, slow growing sterile mycelium, later identified as *H. ericae* (= *Pezizella ericae*) (Read, 1974). Species of *Oidiodendron* have subsequently been described as symbionts of ericaceous plants in Canada (Couture *et al.*, 1983; Dalpé, 1986) and Europe (Douglas *et al.*, 1989; Perotto *et al.*, 1996). The development of molecular techniques, such as PCR-RFLP, RAPD and DNA sequencing, has enabled the identity and diversity of ericoid mycorrhizal fungi to be more thoroughly investigated (Perotto & Bonfante, 1998; Perotto *et al.*, 2000). The use of PCR techniques have revealed close taxonomic relationships between fungi, for example *Scytalidium vaccinii* is an anamorph of *H. ericae* (Egger & Siegler, 1993). Recently, these techniques have also been used to show that the root system of a single ericaceous plant harbours several taxa of both mycorrhizal and non-mycorrhizal fungi (Perotto *et al.*, 1996a; Monreal *et al.*, 1996, 1999). While the development of molecular techniques is significantly enhancing our understanding of genetic diversity within ericoid mycorrhizal fungi, further investigation of the functional significance of this diversity is required.
1.2.1. Role of ericoid mycorrhizas

Plants belonging to Ericales are widely distributed throughout the world and dominate heathland communities in both arctic and temperate climates (Perotto & Bonfante, 1998). While ericaceous plants are the dominant vegetation on heathlands, they also have the ability to associate with other endo- and ectomycorrhizal plants as understorey vegetation (Perotto & Bonfante, 1998). Soils colonized by the Ericaceae are usually acidic and nutrient deficient, due to the inhibition of decomposition and mineralisation processes, which leads to the production of nutrient deficient mor humus soils. Because of their putative saprotrophic capabilities, ericoid mycorrhizas play an important ecological role in the nutrition of the host in such environments (Read, 1991). The enzymatic abilities of ericoid mycorrhizal fungi suggest that they are able to degrade many of the complex organic compounds found in mor humus soils (Leake & Read, 1991; Cairney & Burke, 1998). Evidence also exists which suggests that selected ericoid mycorrhizal endophytes may increase the resistance of their hosts to toxic metal contamination (Bradley et al., 1981, 1982) (section 1.2.2).

While recent evidence suggests that several fungal taxa form ericoid mycorrhizas with ricaceous hosts (Hambleton et al., 1998), our limited knowledge of their functional importance has mainly resulted from the study of H. ericae. The ability of H. ericae to enhance N status of its host has been well documented (Bajwa et al., 1985; Bajwa & Read, 1986; Leake & Read, 1989a). Stribley and Read (1974b) grew Vaccinium macrocarpon Ait. both in mycorrhizal association with H. ericae and in its non-mycorrhizal state and examined the ability of V. macrocarpon to utilise NH₄⁺. They demonstrated a stimulation of growth and N concentration when V. macrocarpon was in mycorrhizal association. Given the low rates of mineralisation in soils colonized by the Ericaceae, the majority of available N in these environments is present in the form of organic molecules (Straker, 1996). Stribley and Read (1980) revealed that
mycorrhizal ericaceous plants could use amino acids as a N source, however, in the absence of the fungus, amino acids were not readily utilised. *H. ericae* can utilise both amino acids and protein as sole N sources (Stribley & Read, 1980; Bajwa & Read, 1986; Bajwa et al., 1985; Cairney et al., 2000). Most physiological studies involving *H. ericae* have been based upon a single isolate, therefore our understanding of physiological variation at the population level is relatively poor. Cairney et al., (2000) investigated the ability of two *H. ericae* populations to grow on inorganic and organic N sources. They concluded that while *H. ericae* could utilise both inorganic and organic N, the extent of utilisation varied within the populations (Cairney et al., 2000), as previously observed in ectomycorrhizal fungi (Laiho, 1970; Keller, 1996; Anderson et al., 1999). *H. ericae* has also been reported to degrade necromass of fungi and mobilise the N contained in this material (Kerley & Read, 1997). This ability not only broadens the sources of available N to *H. ericae* in the environment, but given the ability of the fungus to transfer N to the host plant (Kerley & Read, 1997), also increases the N supply available to the plant. The ability of *H. ericae* to release N from otherwise recalcitrant complexes demonstrates the essential role that ericoid mycorrhizal associations play in nutrient cycling (Read et al., 1989).

*H. ericae* can degrade and utilise P in the form of phosphodiesters such as DNA, via what are thought to be constitutively-produced extracellular and cell surface-bound phosphodiesterase enzymes (Leake & Miles, 1996; Myers & Leake, 1996). The production of phosphodiesterases may enable the fungus to utilise phosphodiesters, regardless of their rapid turnover in soil, under low levels of inorganic P availability. The ability to produce plant cell-wall degrading enzymes may enable *H. ericae* to utilise compounds such as nucleic acids in moribund plant tissue (Cairney & Burke, 1998). *H. ericae* is also capable of degrading lignin.

Heathland soils also contain high levels of toxic organic acids, which are produced by microbial conversion of both fatty acids and phenolic acid-rich residues (Jalal & Read, 1983a, b). While many of the phenolic compounds are potentially phyto- and fungi-toxic (Jalal & Read, 1983a), *H. ericae* can effectively detoxify (via metabolism) a number of phenolic acids and tannic substrates (Leake & Read, 1989b). *H. ericae* can also mobilise N from protein-polyphenol complexes (Bending & Read, 1996). Leake and Read (1989b) suggested that the ability of *H. ericae* to detoxify phenolic compounds may be one of the means by which ericaceous plant species can exclude competitors and become dominant on heathland soils.

These investigations provided evidence, which showed the ability of *H. ericae* to assimilate organic nutrients representative of the compounds found in heathland soil.

**1.2.2. Metal resistance in ericoid mycorrhizas**

Mycorrhizas have been shown to exhibit both constitutive and adaptive resistance to metals (Meharg & Cairney, 2000). If variation in metal resistance occurs within a fungal population from uncontaminated soils, it suggests that increased metal resistance is conferred by physiological mechanisms selected for in, at least some, uncontaminated habitats (Hartley *et al*., 1997a). Such resistance is referred to as constitutive resistance. Adaptation leading to metal resistance will occur only under strong selection pressures such as those observed on mine tailings (Bradley *et al*., 1981, 1982), soils polluted by metal processing (Hunter *et al*., 1987) or in soils with naturally elevated concentrations of metals (Baker & Walker, 1989). Soils
dominated by the Ericaceae are acidic, which facilitates the mobilisation of transition metals. Essential micronutrients such as Cu, Fe, Mn, Ni and Zn are freely available however, they can become toxic if present at high concentrations. Non-essential metals such as Al, Cd, Cr and Pb are also phytotoxic and are bioavailable in acidic soil (Meharg & Cairney, 2000). Ericaceae have also been shown to be the dominant vegetation on some metal contaminated sites, which has led to the investigation of their ability to tolerate toxic metals, along with the functional role of mycorrhizal associations in such habitats.

Bradley et al., (1981, 1982) investigated the ability of unidentified ericoid mycorrhizal endophytes to ameliorate Cu and Zn toxicity to their host plant (C. vulgaris). Plants growing on both a Cu mine and an uncontaminated site were obtained and inoculated with endophytes from their site of origin. Non-mycorrhizal C. vulgaris demonstrated the same response to Cu, regardless of site of origin, a finding previously reported by Marrs and Bannister (1978). However, both mine and uncontaminated site C. vulgaris in mycorrhizal association with endophytes from their site of origin, exhibited an increased Cu resistance (Bradley et al., 1982). Growth of non-mycorrhizal plants was almost completely inhibited at Cu concentrations of 0.15 mol m$^{-3}$, while mycorrhizal plants continued to grow at 1.13 mol m$^{-3}$ Cu (Bradley et al., 1982). The fungal endophytes of C. vulgaris in the absence of their host were shown to be resistant to Cu at concentrations up to 0.75 mol m$^{-3}$, however growth ceased at 1.5 mol m$^{-3}$ Cu (Bradley et al., 1982). The results from this study clearly indicated that Cu resistance in C. vulgaris was conferred by mycorrhizal association (Bradley et al., 1981, 1982). The mechanism for Cu resistance in mycorrhizal C. vulgaris is unknown, however Bradley et al., (1981, 1982) postulated that Cu is bound to external fungal hyphae, which therefore restricts the transfer of Cu to the host plant. In other organisms, Cu resistance has been shown to be due to either the complete exclusion of Cu from cells, as has been demonstrated for selected filamentous fungi
(Gadd & White, 1985), yeasts (Gadd et al., 1984) and algae (Foster, 1977; Twiss et al., 1993), or the sequestration of Cu within cells, which has been suggested for selected bacteria (Silver & Misra, 1988).

Ericoid mycorrhizal fungi have also been shown to ameliorate Zn toxicity in host plants (Bradley et al., 1981, 1982). Denny and Ridge (1995) suggest that the production of a mass of slime by *C. vulgaris* endophytes removes Zn from solution, and therefore ameliorates Zn toxicity in the plant. Bradley et al., (1981, 1982) demonstrated reduced Zn toxicity in mycorrhizal Ericaceae in comparison to non-mycorrhizal plants. Biomass production of *V. macrocarpon* and *Rhododendron ponticum* L. was inhibited by 50 % at Zn concentrations of 68 and 17 mg l⁻¹ respectively in the absence of mycorrhizal infection. In mycorrhizal association however, biomass production was inhibited by 50 % at Zn concentrations twice as high (98 and 33 mg l⁻¹ respectively) (reanalysed data, Meharg & Cairney, 2000). In the case of *C. vulgaris*, non-mycorrhizal plants from Cu mine spoil soils showed increased resistance to Zn in comparison to non-mycorrhizal plants from uncontaminated environments (Bradley et al., 1982). As was the case for Cu, Zn resistance was conferred to mycorrhizal *C. vulgaris*, regardless of their site of origin (Bradley et al., 1982). It has been suggested that the dominance of *C. vulgaris* on soils with high levels of transition metals, may indicate that selection pressures on these plants are quite different to other plant species. Mechanisms that have evolved for the plants survival in such habitats may undergo further selection in metalliferous environments, leading to evolution of co-resistances not observed for other higher and lower plants (Meharg & Cairney, 2000).

Isolates of *H. ericae* have been shown to be resistant to both Al and Fe (Burt et al., 1986; Leake et al., 1990; Shaw et al., 1990). Leake et al. (1990) demonstrated that plants in
mycorrhizal association with *H. ericae* showed normal root growth in the presence of 400 mg l\(^{-1}\) Al. This contrasted to non-mycorrhizal plants, which showed complete inhibition of root growth at this concentration (Leake et al., 1990). Growth of *H. ericae* in pure culture was not affected by the presence of 800 mg l\(^{-1}\) Al (Burt et al., 1986). *H. ericae* also demonstrates resistance to high concentrations of Fe. Shaw et al. (1990) demonstrated resistance of the fungus at Fe concentrations up to 144 mg l\(^{-1}\), the concentration which is typical of many heathland soils (Jones & Etherington, 1970). These authors further demonstrated the ability of *H. ericae* to regulate Fe uptake by *C. vulgaris* when in mycorrhizal association. The results suggested that mycorrhizal association not only enhanced the uptake of Fe to the host at low Fe concentrations, but reduced Fe transport to the host at toxic Fe concentrations (Shaw et al., 1990).

Given the ability of ericoid mycorrhizal fungi to increase metal resistance, and the dominance of the Ericaceae on soils with high levels of transition metals, it is likely that mycorrhizal association contributes to the success of these plants in such soils. While conclusive evidence exists which clearly demonstrates the ability of the mycorrhiza to confer Cu resistance, further investigations must be performed in order to clarify the role of mycorrhizal fungi in metal resistance.

### 1.3. Research aims

In SW England, large tracts of land are contaminated with AsO\(_4^{3-}\), both through naturally elevated As (associated with a granitic intrusion) and via the mining and processing of Cu and As ores. The spoil soils associated with the mining and processing of ores are extremely toxic and support the growth of only a limited number of plant species, of which *C. vulgaris* is the dominant vegetative cover (Porter & Peterson, 1975). Research to date has
concentrated on the resistance of a limited number of grass species to AsO$_4^{3-}$, while no attention has been given to the ability of either *C. vulgaris* or their fungal endophytes, to colonise As contaminated soil.

Two abandoned As/Cu mines in Devon, England, provided an opportunity to investigate the response of *C. vulgaris* and its ericoid mycorrhizal endophyte *H. ericae* to AsO$_4^{3-}$. Populations of *C. vulgaris* and *H. ericae* from an uncontaminated natural heathland were used as comparisons and the majority of research was carried out at the population level. In order to determine why *C. vulgaris* has the ability to colonise As contaminated sites, the research was conducted with the following aims:

i. To examine, in the preliminary investigation, the ability of two isolates of *H. ericae* from uncontaminated soils to grow in AsO$_4^{3-}$, and compare their response to an ectomycorrhizal fungus, *Hebeloma crustuliniforme*.

ii. To isolate and identify, using molecular techniques, populations of *H. ericae* from the roots of *C. vulgaris* at As contaminated sites and an uncontaminated heathland site.

iii. To investigate the diversity of fungal endophytes isolated from *C. vulgaris* and compare the diversity of endophytes at As contaminated sites and a natural heathland site.

iv. To investigate the physiological responses of *H. ericae* populations from As contaminated soils and uncontaminated heathland site to high AsO$_4^{3-}$ concentrations and determine if mine populations have an increased resistance to AsO$_4^{3-}$.

v. To determine the mechanism, if any, used by *H. ericae* to increase AsO$_4^{3-}$ resistance, with particular reference to the high affinity PO$_4^{3-}$ uptake system.

vi. To investigate the growth of *C. vulgaris* populations from As contaminated sites and an uncontaminated heathland site in the presence of AsO$_4^{3-}$.
vii. To examine the effect of mycorrhizal association on the uptake of $\text{AsO}_4^{3-}$ in *C. vulgaris*, in order to determine whether *H. ericae* confers $\text{AsO}_4^{3-}$ resistance to its host.
Chapter 2

Arsenate sensitivity in ericoid and ectomycorrhizal fungi

2.1. Introduction

Mycorrhizal fungi play an essential role in the acquisition and transfer of nutrients from soil to their host plants. Pollution of soil with potentially toxic metals due to mining, industrial or agricultural processes may have detrimental effects on mycorrhizal colonization and their hosts (Hartley et al., 1997a). Some mycorrhizal fungi have been shown to exhibit resistance to concentrations of metals that are normally toxic to plants (Tam, 1995). Differential metal sensitivity between and within mycorrhizal fungal species have also been shown to exist (Hartley et al., 1997b; Thompson & Medve, 1984; Colpaert & Van Assche, 1992; Egerton-Warburton & Griffin, 1995). Colpaert and Van Assche (1992) showed that Suillus bovinus and Suillus luteus from metal contaminated soils demonstrated reduced metal sensitivity in comparison to isolates from unpolluted soils. Such sensitivity may be either evolved or constitutive low sensitivity (Denny & Wilkins, 1987; Wilkinson & Dickinson, 1995).

Calluna vulgaris is often found colonising mine sites (Porter & Peterson, 1975), where the low pH characteristic of these soils results in a high availability of potentially toxic metals (Rorison, 1973). C. vulgaris has colonized Cu and As contaminated mine spoil soils of SW England (Porter & Peterson, 1975). Several plant species have demonstrated a reduced sensitivity towards AsO$_4^{3-}$ (Rocovich & West, 1975) the dominant form of soil As (Porter & Peterson, 1975, 1977). Arsenate is a PO$_4^{3-}$-analogue and is absorbed by the PO$_4^{3-}$ uptake system in angiosperms (Meharg & Macnair, 1990). Phosphate has been shown to ameliorate AsO$_4^{3-}$-toxicity in some plants in solution culture (Rumberg et al., 1960) and in soil (Hurd-Karrer, 1939). Arsenate tolerance in some plants is achieved through suppression of the high affinity
PO$_4^{3-}$ uptake system (Meharg & Macnair, 1992a). The objective of this preliminary investigation was to examine the sensitivity of *H. ericae* isolated from natural heathland sites to AsO$_4^{3-}$, and to determine whether PO$_4^{3-}$ has the ability to ameliorate AsO$_4^{3-}$ toxicity in this fungus. Arsenate transport kinetics was also investigated. Since some ectomycorrhizal fungi have also been shown to exhibit constitutive tolerance to potentially toxic metals (Hartley *et al.*, 1997b), the response of *H. ericae* to AsO$_4^{3-}$ was compared with an isolate of the ectomycorrhizal fungus *Hebeloma crustuliniforme*.

2.2. Materials and Methods

2.2.1. Fungal Culture

Two isolates of the ericoid mycorrhizal endophyte *H. ericae* (Read 100, Read 101) and one isolate of the ectomycorrhizal fungus *H. crustuliniforme* were obtained from uncontaminated sites in England and Sweden respectively. *H. crustuliniforme* was used to compare the growth response to AsO$_4^{3-}$ between an ectomycorrhizal fungus and an ericoid mycorrhizal fungus. Cultures were maintained on modified Melin-Norkrans agar medium (MMN) (Marx & Bryan, 1975) at 22$^\circ$C in the dark. The basal medium for all growth experiments was liquid MMN containing (mol m$^{-3}$): (NH$_4$)$_2$HPO$_4$, 3.79; MgSO$_4$.7H$_2$O, 0.57; CaCl$_2$.6H$_2$O, 0.23; NaCl, 0.43; glucose, 55.5; thiamine, 0.0003, adjusted to pH 5.5 with 0.1M NaOH. KH$_2$PO$_4$ was added at 2.21 mol m$^{-3}$ to all basal media unless otherwise specified. Chemicals were purchased from Sigma (Australia) unless otherwise specified.

2.2.2. Determination of growth curves

In order to determine the time period of the linear growth phase of *H. ericae* (Read 100) and *H. crustuliniforme*, two circular plugs of the fungi (6 mm diam.) were cut from the edges of actively growing colonies on MMN. Fungal plugs were inoculated into 9 cm diam. Petri dishes
containing basal liquid medium (25 ml) and incubated in the dark at 22°C. After incubation for periods of 2, 4, 8, 12, 16, 20, 25 and 34 d, mycelial mats were removed from medium, placed onto pre-weighted aluminium foil and oven dried (24 h, 80°C). All treatments were replicated three times for each isolate. The mean fungal d wt was then plotted as a growth curve for each isolate.

2.2.3. Effects of PO₄³⁻, AsO₄³⁻ and AsO₂⁻ on biomass production

Using the above method of inoculation, the three fungal isolates were grown in basal medium supplemented with either PO₄³⁻, AsO₄³⁻ or AsO₂⁻ (in the form of KH₂PO₄, Na₂HAsO₄ and NaAsO₂ respectively). The concentrations used were: PO₄³⁻ 0, 0.01, 0.02, 0.05, 1.0, 1.5, 2.0 mol m⁻³; AsO₄³⁻ 0, 0.13, 0.33, 0.67, 1.00, 1.33, 2.00, 2.67, 3.34, 4.00, 4.67, 5.34, 6.00, 6.67, 10.00, 13.34 mol m⁻³ and AsO₂⁻ 0, 0.01, 0.13, 0.67, 1.33 mol m⁻³. For all AsO₄³⁻ and AsO₂⁻ treatments, the PO₄³⁻ concentration in the media was adjusted to 0.01 mol m⁻³.

Each treatment was replicated three times for each isolate and cultures were grown at 22°C in the dark for 17 d, the time determined from the growth curves at which all isolates had achieved approximately 50% of maximum growth (Fig. 2.1). This ensured each isolate was harvested at a similar stage of growth and during the linear phase of the growth cycle. Mycelial mats were harvested as in section 2.2.2. and biomass determined gravimetrically. The effective concentration of AsO₄³⁻ inhibiting growth by 50% (EC₅₀) was calculated by fitting exponential decay curves to the data.

2.2.4. Effects of PO₄³⁻ on AsO₄³⁻ and AsO₂⁻ sensitivity

The effect of PO₄³⁻ on AsO₄³⁻ and AsO₂⁻ sensitivity of all isolates was investigated by inoculating fungi in basal liquid medium containing PO₄³⁻ at the concentrations outlined in
section 2.2.3. and both AsO₄³⁻ and AsO₂⁻ at the pre-determined EC₅₀ value for each isolate (Fig. 2.3 and Fig. 2.4). Isolates were inoculated and harvested as outlined above with each treatment replicated three times.

2.2.5. Kinetics of AsO₄³⁻ accumulation

To determine AsO₄³⁻ accumulation in *H. ericae* and *H. crustuliniforme*, two circular plugs of mycelium (6 mm diam.) were incubated in basal medium for 17 d and then transferred to PO₄³⁻ free basal medium for 48 h prior to analysis. Mycelial mats were then incubated in 25 ml of aerated test solution for 20 min. Test solutions contained 10 mol m⁻³ 2-[N-Morpholino]ethanesulphonic acid (MES), 0.5 mol m⁻³ Ca(NO₃)₂ and different concentrations of AsO₄³⁻ in the form of Na₂HAsO₄.7H₂O at pH 5. Arsenate concentrations were: 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.25, 0.5, 0.75, 1.0 mol m⁻³. Using the methodology of Meharg and Macnair (1994), mycelial mats were rinsed after incubation in 25 ml of an ice cold solution containing 1 mol m⁻³ K₂HPO₄, 10 mol m⁻³ MES and 0.5 mol m⁻³ Ca(NO₃)₂. They were then transferred to 25 ml of an aerated ice cold solution of the same composition for 10 min to ensure desorption of AsO₄³⁻ from the cell free space. Samples were then oven dried (24 h, 80°C) before analysis.

Arsenic was determined by digesting mycelia in 2 ml concentrated nitric acid (Analar grade). Samples were digested using a block digester for 1 h at 120°C followed by 1 h at 180°C to evaporate samples to dryness. The arsenic residue was re-dissolved in 20 ml of a solution containing 5% conc. HCl (Analar grade) and 20 mol m⁻³ KI. The amount of arsenic present in the digests was determined using hydride generation interfaced with an atomic absorption spectrophotometer (ThermoUnicam Solaar 929, Cambridge, UK). A known set of standards were run at the start and finish of each analytical run with the top standard analyzed every ten samples to account for any instrumental drift.
2.2.6. Statistical analysis

Data were analyzed by ANOVA using the computer package Minitab v. 11 (Minitab, State College, PA, USA). If the variance was not normally distributed as determined by the Anderson-Darling test for normality, a suitable transformation was performed. Where transformations were performed, the transformations are noted in the Figure legends. Second order exponential decay curves were fitted to toxicity data using the computer package Sigma Plot (Jandel Scientific, Erkrath, Germany). Single Michaelis-Menten functions were fitted to the kinetics data using hyperbola curves from Sigma Plot.

2.3. Results

2.3.1. Growth curves

The growth of *H. ericae* (Read 100) and *H. crustuliniforme* followed a sigmoid curve, typical of microorganisms grown in batch culture (Fig. 2.1). *H. ericae* produced a final biomass of 70.4 mg after 34 d in basal medium, approximately twice the final biomass produced by the *H. crustuliniforme* isolate (28.2 mg). After 17 d incubation, both *H. ericae* and *H. crustuliniforme* had achieved approximately 50% of their final biomass production (Fig. 2.1).

![Graph showing growth curves](image)

*Figure 2.1* Growth curves for *H. ericae* (Read 100) (○) and *H. crustuliniforme* (▲). Each point is the mean of three replicates ± SE. Curves were fitted using a sigmoid equation (Sigma-plot, Jandel Corporation, Germany).
2.3.2 Effect of PO₄³⁻, AsO₄³⁻ and AsO₂⁻ on biomass production

Initially, increasing the amount of PO₄³⁻ in the incubation medium caused a significant enhancement of biomass production for all isolates (Fig. 2.2). Phosphate increased the amount of biomass produced at concentrations up to 1.0 mol m⁻³. At PO₄³⁻ concentrations greater than 1.0 mol m⁻³, biomass production was not further enhanced for any isolate (Fig. 2.2). Analysis of variance (Table 2.1) of the data presented in Fig. 2.2 revealed a highly significant individual effect of both PO₄³⁻ and isolate with respect to biomass production. Both isolates of *H. ericae* produced significantly more biomass at all PO₄³⁻ concentrations than *H. crustuliniforme* (Fig. 2.2).

![Graph showing the effect of PO₄³⁻ concentration on biomass yield](image)

**Figure 2.2** Growth of *H. ericae* (Read 100) (●), *H. ericae* (Read 101) (○) and *H. crustuliniforme* (▼) over a range of PO₄³⁻ concentrations. Each point is the mean of three replicates ± SE.
Table 2.1 The effect of $\text{PO}_4^{3-}$ on fungal biomass production as determined by ANOVA both in the presence (+$Hc$) and absence (-$Hc$) of $H. crustuliniforme$. $P$ is the probability of the source term not being significant.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of freedom</th>
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</tr>
</thead>
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<tr>
<td></td>
<td>+$Hc$</td>
<td>-$Hc$</td>
<td></td>
<td></td>
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<tr>
<td>$\text{PO}_4^{3-}$</td>
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<td>6</td>
<td>2273.6</td>
<td>2008.5</td>
</tr>
<tr>
<td>Isolate</td>
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<td>1</td>
<td>8368.9</td>
<td>609.3</td>
</tr>
<tr>
<td>$\text{PO}_4^{3-}$ * isolate</td>
<td>12</td>
<td>6</td>
<td>103.9</td>
<td>452.7</td>
</tr>
<tr>
<td>Error</td>
<td>41</td>
<td>28</td>
<td>57.9</td>
<td>77.8</td>
</tr>
</tbody>
</table>

Increasing $\text{AsO}_4^{3-}$ concentrations resulted in a decreased biomass yield for all isolates (Fig. 2.3). Fungal growth in the presence of $\text{AsO}_4^{3-}$ followed an exponential decay curve. A second order exponential decay curve was fitted to the data (Fig. 2.3) in order to determine the $\text{AsO}_4^{3-}$ EC$_{50}$ value. $H. crustuliniforme$ demonstrated the greatest sensitivity to $\text{AsO}_4^{3-}$ with an EC$_{50}$ of 0.33 mol m$^{-3}$, while the two $H. ericae$ isolates showed lower sensitivity, both having an EC$_{50}$ of 1.33 mol m$^{-3}$. $H. ericae$ produced significant biomass (10 $\mu$g h$^{-1}$) in the presence of 13.33 mol m$^{-3}$ $\text{AsO}_4^{3-}$ (the highest concentration tested) in contrast to $H. crustuliniforme$ which did not grow at this concentration (Fig. 2.3). Increasing $\text{AsO}_4^{3-}$ concentrations resulted in a highly significant ($P < 0.001$) decrease in the mean biomass produced (Table 2.2). The isolate source term was also significant ($P = 0.003$) when all three isolates were analyzed together. When $H. crustuliniforme$ was removed from the ANOVA, no significant difference was demonstrated between the two $H. ericae$ isolates (Table 2.2). The slope of the dose response curve comparing $H. crustuliniforme$ and $H. ericae$ differed significantly when all three isolates were analysed ($\text{AsO}_4^{3-}$ * isolate $P < 0.001$) (Table 2.2).
Figure 2.3 Growth of *H. ericae* (Read 100) (●), *H. ericae* (Read 101) (○) and *H. crustuliniforme* (→) over a range of AsO₄³⁻ concentrations. Each point is the mean of three replicates ± SE. Second order exponential decay curves were fitted to the data (Sigma-plot, Jandel Corporation, Germany).

Table 2.2 The effect of AsO₄³⁻ on fungal biomass production as determined by analysis of variance in the presence (+Hc) and absence (-Hc) of *H. crustuliniforme*. AsO₄³⁻ was used as a covariate. Data were log₁₀ transformed before statistical analysis to normalize residuals. Normality was checked using the Anderson-Darling normality test on the residuals from the General Linear Model. P is the probability of the source term not being significant.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree freedom of Adjusted squares</th>
<th>mean</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td></td>
<td>+Hc</td>
<td>-Hc</td>
<td>+Hc</td>
<td>-Hc</td>
</tr>
<tr>
<td>As²⁺</td>
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<td>1</td>
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</tr>
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<td>Error</td>
<td>104</td>
<td>85</td>
<td>0.0275</td>
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</tr>
</tbody>
</table>
Biomass production for all isolates was not affected by the presence of AsO$_2^-$ at concentrations less than 0.67 mol m$^{-3}$ AsO$_2^-$ (Fig. 2.4). In the presence of 1.33 mol m$^{-3}$ AsO$_2^-$, both isolates of *H. ericae* produced a biomass yield below 10 μg h$^{-1}$, while *H. crustuliniforme* produced no growth (Fig. 2.4).

![Graph](image)

*Figure 2.4* Growth of *H. ericae* (Read 100) (●), *H. ericae* (Read 101) (◊) and *H. crustuliniforme* (▼) over a range of AsO$_2^-$ concentrations. Each point is the mean of three replicates ± SE.

**2.3.3. Effect of PO$_4^{3-}$ on AsO$_4^{3-}$ and AsO$_2^-$ sensitivity**

When the interactive effect of PO$_4^{3-}$ on AsO$_4^{3-}$ toxicity was investigated, high concentrations of PO$_4^{3-}$ ameliorated toxicity of both AsO$_4^{3-}$ and AsO$_2^-$ for all three isolates (Fig. 2.5). At 1.0 mol m$^{-3}$ PO$_4^{3-}$ in the absence of AsO$_4^{3-}$, biomass produced by *H. ericae* (105.1 μg h$^{-1}$) was not significantly different to the biomass yield at 1.0 mol m$^{-3}$ PO$_4^{3-}$ in the presence of 1.33 mol m$^{-3}$ AsO$_4^{3-}$ (96.0 μg h$^{-1}$) and 1.00 mol m$^{-3}$ AsO$_2^-$ (120 μg h$^{-1}$) (P<0.01). *H. crustuliniforme* demonstrated a similar trend, with growth at 1.0 mol m$^{-3}$ PO$_4^{3-}$ in the absence of AsO$_4^{3-}$ (43.5 μg h$^{-1}$) being similar to that at the same PO$_4^{3-}$ concentration in the presence of 0.33 mol m$^{-3}$ AsO$_4^{3-}$ (41.2 μg h$^{-1}$) and 1.00 mol m$^{-3}$ AsO$_2^-$ (55 μg h$^{-1}$). From the ratio of biomass produced in the absence and presence of AsO$_4^{3-}$ (Fig. 2.5b), it appears that the two isolates of *H.*
*ericae* had differing PO$_4^{3-}$/AsO$_4^{3-}$ interactions at low PO$_4^{3-}$ concentrations. Isolate 100 showed little effect of AsO$_4^{3-}$ on biomass production at PO$_4^{3-}$ concentrations of 0.01 and 0.02 mol m$^{-3}$, while isolate 101 demonstrated a much greater biomass production in the absence of AsO$_4^{3-}$ at the low PO$_4^{3-}$ concentrations (Fig. 2.5b). Statistical analysis (Table 2.3) indicated a highly significant individual effect of AsO$_4^{3-}$, PO$_4^{3-}$ and isolate with respect to biomass production for all three isolates. The main effects plots for AsO$_4^{3-}$, PO$_4^{3-}$ and isolate on biomass production are shown in Fig. 2.6. The presence of AsO$_4^{3-}$ had a negative effect on the total biomass produced (Fig. 2.6a) while the presence of increasing PO$_4^{3-}$ concentrations had a positive effect. The amount of biomass produced was dependent on the isolate, with *H. crustuliniforme* producing less overall biomass in comparison to the two isolates of *H. ericae*. When all three isolates were analyzed together there were also significant effects between AsO$_4^{3-}$ * PO$_4^{3-}$, AsO$_4^{3-}$ * isolate and PO$_4^{3-}$ * isolate with respect to biomass production (Table 2.3). The interaction plots (Fig. 2.7) demonstrated an overall positive regression for the response of PO$_4^{3-}$ on biomass increase in the presence and absence of AsO$_4^{3-}$ (Fig. 2.7a). A negative regression slope was obtained for both the AsO$_4^{3-}$ * isolate (Fig. 2.7b) and PO$_4^{3-}$ * isolate interaction (Fig. 2.7c) with respect to biomass production. When *H. crustuliniforme* was removed from the ANOVA there were no significant interactions between AsO$_4^{3-}$ * PO$_4^{3-}$, AsO$_4^{3-}$ * isolate or between all three source terms. The interaction of isolate * PO$_4^{3-}$ on biomass production remained significant between the two *H. ericae* isolates, however a different slope of regression was obtained (Fig. 2.7f). A significant positive regression was demonstrated at 1.0 mol m$^{-3}$ PO$_4^{3-}$ with the amount of biomass produced by *H. ericae* isolate 101 larger than *H. ericae* isolate 100. This contrasted to the negative regression obtained when *H. crustuliniforme* was included in the ANOVA.
Figure 2.5  a Growth of *H. ericae* (Read 100) (●), *H. ericae* (Read 101) (●) and *H. crustuliniforme* (●) in the presence of AsO₄³⁻ (open symbols) at the pre-determined EC₅₀ value (1.33 and 0.33 mol m⁻³ AsO₄³⁻ for *H. ericae* and *H. crustuliniforme* respectively) and in the absence of AsO₄³⁻ (closed symbols) at a range of PO₄³⁻ concentrations.  b. The ratio of growth in the absence and in the presence of 1.33 mol m⁻³ AsO₄³⁻ for *H. ericae* (Read 100) (●) and *H. ericae* (Read 101) (●) and 0.33 mol m⁻³ AsO₄³⁻ for *H. crustuliniforme* (●).  c. Growth of *H. ericae* (Read 100) (●), *H. ericae* (Read 101) (●) and *H. crustuliniforme* (●) in 1.0 mmol m⁻³ AsO₂ over a range of PO₄³⁻ concentrations. Each point is the mean of three replicates ± SE.
Table 2.3 The interactive effects of PO$_4$$^{3-}$ and AsO$_4$$^{3-}$ on fungal biomass production as determined by analysis of variance in the presence (+Hc) and absence (-Hc) of *H. crustuliniforme*. $P$ is the probability of the source term not being significant.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of freedom</th>
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<td>-Hc</td>
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<tr>
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<td>83.3</td>
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Figure 2.6 General Linear Modeling main effects plots for the effect of $\text{AsO}_4^{3-}$, $\text{PO}_4^{3-}$ and isolate on biomass production. (From data in Fig. 2.5a and Table 2.3). Main effects are $\text{AsO}_4^{3-}$ concentration (a, d), $\text{PO}_4^{3-}$ concentration (b, e) and isolate (c, f). A, b and c represent the presence of *H. crustuliniforme*, plots d, e and f the absence of *H. crustuliniforme*. Dashed line = overall mean.
Figure 2.7 General Linear Modelling interaction plots for the effects of $\text{AsO}_4^{3-}$, $\text{PO}_4^{3-}$ and isolate on biomass production. (From data in Figure 2.5a and Table 2.3). A and d = presence of $\text{AsO}_4^{3-}$ at the EC$_{50}$ concentration ($\bullet$) and absence of $\text{AsO}_4^{3-}$ ($\circ$) against $\text{PO}_4^{3-}$ concentration. B and e = presence of $\text{AsO}_4^{3-}$ at the EC$_{50}$ concentration ($\bullet$) and the absence of $\text{AsO}_4^{3-}$ ($\circ$) against isolate. C and f = $\text{PO}_4^{3-}$ concentration against isolate ($\bullet=0.01$; $\circ=0.02$; $\blacktriangle=0.05$; $\triangledown=0.5$; $\blacksquare=1.0$ mol m$^{-3}$ $\text{PO}_4^{3-}$). Plots a, b and c represent the presence of *H. crustuliniforme*, plots d, e and f the absence of *H. crustuliniforme*. 
2.3.4. Kinetics of high affinity AsO$_4^{3-}$ accumulation

Concentration dependent accumulation of AsO$_4^{3-}$ was analyzed by fitting Michaelis-Menten functions to the data (Fig. 2.8). Single Michaelis-Menten functions were fitted to the data representing the high affinity uptake system, which is utilized at low substrate concentrations.

The two isolates of *H. ericae* demonstrated similar patterns of AsO$_4^{3-}$ accumulation. The $V_{\text{max}}$ for AsO$_4^{3-}$ accumulation for *H. ericae* isolate 100 and 101 was 18 and 17 μmol g$^{-1}$ d. wt. h$^{-1}$ respectively, while the $V_{\text{max}}$ of *H. crustuliniforme* was approximately two fold higher (39 μmol g$^{-1}$ d. wt. h$^{-1}$) (Table 2.4). The two isolates of *H. ericae* had a $K_m$ of 0.071 and 0.068 mol m$^{-3}$ respectively, while *H. crustuliniforme* had a $K_m$ value five times higher (Table 2.4). The $K_m$ calculated for *H. crustuliniforme* may be slightly distorted as the AsO$_4^{3-}$ influx at the two highest concentrations (Fig. 2.8) may be the start of the low affinity uptake system for which a different carrier becomes dominant (Epstein, 1976). When the two highest concentrations were removed a $K_m$ of 0.14 mol m$^{-3}$ and $V_{\text{max}}$ of 7.75 μmol g$^{-1}$ d. wt. h$^{-1}$ were obtained.
Figure 2.8  AsO₄³⁻ accumulation for a. *H. ericae* (Read 100), b. *H. ericae* (Read 101) and c. *H. crustuliforme*. Each point is the mean of three replicates ± SE.
Table 2.4  Kinetic parameters for AsO₄³⁻ influx in *H. ericae* and *H. crustuliniforme*. Data represent kinetic parameters ± SE of the mean. P is the probability of the source term not being significant.

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<th>Isolate</th>
<th>Kᵣ (mol m⁻³)</th>
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<th>R² value</th>
<th>P</th>
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<td><em>H. ericae</em> (Read 100)</td>
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<td><em>H. ericae</em> (Read 101)</td>
<td>0.068 ± 0.029</td>
<td>17 ± 2.0</td>
<td>0.50</td>
<td>0.0001</td>
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<tr>
<td><em>H. crustuliniforme</em></td>
<td>0.35 ± 0.14</td>
<td>39 ± 6.9</td>
<td>0.77</td>
<td>&lt; 0.0001</td>
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</table>

2.4. DISCUSSION

The results presented in this chapter demonstrate the negative effect of increasing AsO₄³⁻ concentrations on the growth of two isolates of the ericoid mycorrhizal fungal endophyte *H. ericae* and a single isolate of the ectomycorrhizal fungus *H. crustuliniforme* in solution culture. The sensitivity of *H. ericae* to AsO₄³⁻ was significantly lower than *H. crustuliniforme*, however the sensitivity of both *H. ericae* and *H. crustuliniforme* to AsO₂⁻ was similar (Fig. 2.4). *H. ericae* had an EC₅₀ of 1.33 mol m⁻³ AsO₄³⁻ in comparison to 0.33 mol m⁻³ for *H. crustuliniforme* (Fig. 2.3). *H. ericae* also demonstrated the ability to grow at high AsO₄³⁻ concentrations in pure culture (<13.34 mol m⁻³) (Fig. 2.3). The two isolates of *H. ericae* used in these experiments were isolated from heathland soils that were not contaminated with As and the ability to grow in pure culture at high AsO₄³⁻ concentrations may suggest constitutive AsO₄³⁻ resistance in this fungus. However, the two *H. ericae* isolates showed different growth rates at low concentrations of both AsO₄³⁻ and AsO₂⁻ and this may reflect some intraspecific differences in growth rates in this species (Figs 2.3 & 2.4). It must be noted however, that the liquid MMN solution was not changed for the duration of the experiment and some changes in nutrient levels due to depletion by the fungus may have occurred.
While many studies have been performed on higher plants in relation to \( \text{AsO}_4^{3-} \) sensitivity, there have been relatively few studies on fungi. The range of \( \text{AsO}_4^{3-} \) sensitivities observed in the previous studies on plants and fungi however indicate that the two \( H. \ ericae \) isolates demonstrate particularly low sensitivity. For example, the deuteromycete fungus \( \text{Neurospora crassa} \) (at 0.01 mol m\(^{-3}\) \( \text{PO}_4^{3-} \)) suffers complete growth inhibition at 0.133 mol m\(^{-3}\) \( \text{AsO}_4^{3-} \) (Beever & Burns, 1980), while the marine yeast \( \text{Rhodotorula rubra} \) undergoes complete growth inhibition at 0.534 mmol m\(^{-3}\) \( \text{AsO}_4^{3-} \) at \( \text{PO}_4^{3-} \) concentrations of between 0.01 and 0.02 mmol m\(^{-3}\) (Beever & Burns, 1980). Arsenate EC\(_{50}\) values of plants from uncontaminated soils range between 5-67 mol m\(^{-3}\) (Pollard, 1980; Paliouris & Hutchinson, 1991; Meharg & Macnair, 1992b).

It has previously been reported that \( \text{PO}_4^{3-} \) may exhibit an ameliorating effect on \( \text{AsO}_4^{3-} \) sensitivity. \( \text{PO}_4^{3-} \) is rarely found in soil solution at concentrations above 0.01 mol m\(^{-3}\) (Bielski, 1973). This chapter demonstrated that increasing \( \text{PO}_4^{3-} \) concentrations up to 1.0 mol m\(^{-3}\) enhanced growth of all isolates, however at \( \text{PO}_4^{3-} \) concentrations above 1.0 mol m\(^{-3}\) no further biomass increase was demonstrated (Fig. 2.2). A similar trend has been shown in other fungi (Beever & Burns, 1980). However, as stated earlier, the nutrient solution was not changed for the duration of the experiment and therefore the depletion of \( \text{PO}_4^{3-} \) from the media by the fungus over time may explain the growth of the fungus at such high \( \text{PO}_4^{3-} \) concentrations. When the combined effects of \( \text{AsO}_4^{3-} / \text{PO}_4^{3-} \) and \( \text{AsO}_2^- / \text{PO}_4^{3-} \) were studied it was found that, at the highest \( \text{PO}_4^{3-} \) concentration (1.0 mol m\(^{-3}\)), growth in the presence of \( \text{AsO}_4^{3-} \) and \( \text{AsO}_2^- \) by all isolates was similar to the growth produced in the absence of \( \text{AsO}_4^{3-} \) (Fig. 2.5). Since \( \text{PO}_4^{3-} \) does not enhance growth at concentrations greater than 1.0 mol m\(^{-3}\), it can be suggested that the
observed increase in growth is due to amelioration of $\text{AsO}_4^{3-}$ and $\text{AsO}_2^-$ by $\text{PO}_4^{3-}$, as has earlier been suggested in higher plants (Meharg & Macnair, 1992a; Asher & Reay, 1979; Lee, 1982).

The ameliorative effect of $\text{PO}_4^{3-}$ on $\text{AsO}_4^{3-}$ toxicity in *H. ericae* may be due to competition for uptake into the fungal hyphae. Arsenate behaves as a $\text{PO}_4^{3-}$ analogue (Asher & Reay, 1979; Lee, 1982; Ullrich-Eberius *et al.*, 1989) and is absorbed by the $\text{PO}_4^{3-}$ transport system in a wide range of organisms (Meharg & Macnair, 1992b). Phosphate transport across membranes is carrier-mediated and described by Michaelis-Menten kinetics (Beever & Burns, 1980; Woolhouse, 1975). Meharg and Macnair demonstrated that adaptation of the $\text{PO}_4^{3-}$ uptake system is a mechanism of $\text{AsO}_4^{3-}$ tolerance in the grasses *Holcus lanatus* L. (Meharg & Macnair, 1990, 1991, 1992a, 1992b) *Deschampsia cespitosa* (L.) Beauvois and *Agrostis capillaris* L. (Meharg & Macnair, 1991). They further showed that $\text{AsO}_4^{3-}$ tolerance was due to the constitutive suppression of the high affinity uptake system (dominant at low $\text{PO}_4^{3-}$ concentrations) by carrier synthesis inhibition independent of plant P status (Meharg & Macnair, 1992a).

The $K_m$ values for the two isolates of *H. ericae* (0.071 and 0.068 mol m$^{-3}$ respectively) obtained in the experiments in the present chapter are similar to the $K_m$ values previously reported for $\text{AsO}_4^{3-}$ transport in tolerant plants of *H. lanatus* (0.074 mol m$^{-3}$) (Meharg & Macnair, 1992a), yet are high in comparison to other fungi. For example, *Saccharomyces cerevisiae* has a $K_m$ for $\text{AsO}_4^{3-}$ of 0.004 mol m$^{-3}$ (Jung & Rothstein, 1965) while *Candida tropicalis* has a $K_m$ value of 0.005 mol m$^{-3}$ (Beever & Burns, 1980). The $V_{\text{max}}$ values obtained for *H. ericae* (Table 2.4) are similar to those reported for *S. cerevisiae* (10.2 µmol g$^{-1}$ d wt h$^{-1}$) (Jung & Rothstein, 1965), but higher than those found in tolerant *H. lanatus* (Meharg & Macnair, 1992a). This increase in $V_{\text{max}}$ in comparison to tolerant *H. lanatus* was expected due
to the larger surface area to mass ratio of *H. ericae* in comparison to plant tissue. The $K_m$ value for *H. crustuliniforme* reported in this study was 5 fold higher than *H. ericae*, however the $V_{max}$ value is double that of *H. ericae*. *H. crustuliniforme* was much more sensitive to high $\text{AsO}_4^{3-}$ concentrations than *H. ericae* (Fig. 2.2) which may be related to the higher $V_{max}$.

In summary, results from this chapter indicate that isolates of *H. ericae* from non-contaminated environments demonstrate low $\text{AsO}_4^{3-}$ sensitivity in comparison to other fungi. This may indicate constitutive resistance to $\text{AsO}_4^{3-}$ in single isolates of *H. ericae*. In order to investigate further the resistance of *H. ericae* to $\text{AsO}_4^{2-}$, populations of *H. ericae* from $\text{AsO}_4^{3-}$ contaminated soils needed to be examined. The isolation of *H. ericae* populations from two As/Cu mine sites and an uncontaminated natural heathland in SW England (Chapter 3) provided this opportunity. Furthermore, the present chapter indicated that $\text{PO}_4^{3-}$ has the ability to decrease the negative effects of $\text{AsO}_4^{2-}$ toxicity in *H. ericae*. It was hypothesised that the low sensitivity toward $\text{AsO}_4^{3-}$ in *H. ericae* may be due to a high $K_m$ for $\text{AsO}_4^{3-}$, however it is possible other factors may have an effect. These include competition between $\text{PO}_4^{3-}$ and $\text{AsO}_4^{3-}$ for absorption into fungal hyphae, the possibility of an altered intracellular metabolism and the possibility of a potential efflux pump in operation must also be considered. These factors are investigated in detail in Chapter 4.
Chapter 3

Genetic diversity of ericoid mycorrhizal fungal endophytes from *Calluna vulgaris* at contrasting field sites.

3.1. Introduction

Plants of the Ericales are widespread in arctic, temperate and tropical climates, and dominate on nutrient-poor heathland soils in both northern and southern hemispheres (Read, 1996). The presence of ericoid mycorrhizal associations is regarded as a key factor in the success of the Ericaceae on extremely nutrient deficient soils and at sites contaminated with toxic metals (Read, 1991, 1996). Ericoid mycorrhizas typically produce little extramatrical mycelium and an intracellular fungus-root interface comprising hyphal coils of variable density within epidermal cells of hair roots (section 1.2). These mycorrhizal associations enhance nutrient (particularly N and P) availability to the host plant and may also alleviate metal toxicity in some circumstances (Bradley et al., 1981).

The ascomycete *Hymenoscyphus ericae* (Read) Korf and Kernan (along with its anamorph *Scytalidium vaccinii* Dalpè, Litten & Sigler (Pearson & Read, 1973; Egger & Sigler, 1993; Hambleton & Currah, 1997) and several *Oidodendron* spp. (Couture et al., 1983; Dalpè, 1986; Douglas et al., 1989; Perotto et al., 1995, 1996; Hambleton & Currah, 1997) are known to form mycorrhizas with several taxa of the Ericaceae, however the diversity of mycorrhizal endophytes of the Ericaceae appear to be much greater than this. Thus, a range of sterile mycelia has also been isolated from hair roots of Ericaceae species and confirmed as mycorrhizal endophytes in laboratory synthesis experiments (Duclos & Fortin, 1983; Perotto et al., 1990, 1995, 1996). While few of these isolates have been studied in detail, recent molecular
analyses suggest that most, at least in North America, probably have taxonomic affinities with the ascomycete order Leotiales (Hambleton et al., 1998).

In the southern hemisphere the order Ericales is largely represented by the Epacridaceae, which form ericoid mycorrhizas that are known to be structurally similar to those of northern hemisphere Ericaceae (Smith & Read, 1997). Further evidence is provided by the fact that endophytes isolated from Epacridaceae can form morphologically typical mycorrhizal associations with Ericaceae hosts (Reed, 1989; Read, 1996; Liu et al., 1998). Only an Oidiodendron species (probably O. maius Barron) has been identified as an endophyte of an Epacridaceae host in the field (Chambers et al., 2000) and molecular data suggest that, in common with those from Ericaceae, most endophytes probably have affinities with Leotiales (McLean et al., 1999; Chambers et al., 2000). Interestingly, although H. ericae has been identified as an endophyte of leafy liverwort rhizoids in the southern hemisphere (Chambers et al., 1999), it has yet to be found as an endophyte of any Epacridaceae host.

While the taxonomic status of ericoid mycorrhizal endophytes of Ericales hosts has received some attention, far less effort has to date been focused on determining the diversity of endophyte assemblages present within root systems of a host taxon at a single field site or of the same taxon at different field sites. Results of several studies indicate that a single stand of an Ericales host species may be colonised by several endophyte taxa, as can the root system of an individual plant within such a stand (Perotto et al., 1990, 1995; Xiao & Berch, 1996; Hambleton & Currah, 1997; Liu et al., 1998; Chambers et al., 2000). Although detailed functional studies have yet to be performed on endophytes other than H. ericae, it seems likely such diversity may be of considerable functional significance to the hosts (Smith & Read, 1997).
Restriction fragment length polymorphism analysis of the internal transcribed spacer region (ITS-RFLP) and ITS sequence comparison were used to identify populations of *H. ericae* from *C. vulgaris* at contrasting field sites in SW England. The diversity of fungal endophytes in root systems of the host was also investigated.

### 3.2. Materials and Methods

#### 3.2.1. Site description

Three field sites in SW England were used for this study (Fig. 3.1). Two sites were disused As/Cu mines, abandoned in the late 1800's: Devon Great Consols, Devon (SX426733) and Gawton United Mine, Devon (SX453688). The third field site used was Aylesbeare Common, Exeter (SX898060), a natural heathland which has not been contaminated by mining and other industrial processes. The two mine site spoil soils were highly contaminated with As (section 4.3.3) and Cu, due to the mining and processing of As and Cu ore. Levels of other elements, such as Zn, were also expected to be high due to geochemical association with As in the original ores. Mine spoil soils are usually low in organic matter (Colbourn *et al.*, 1975) and both mine site soils were composed of relatively coarse waste material. Vegetation colonising Devon Great Consols was extremely sparse and dominated by *C. vulgaris*. The grass species, *Holcus lanatus* and *Agrostis capillaris* were also present. In comparison, species richness of plants colonizing Gawton United Mine site was much greater, however *C. vulgaris* was the dominant vegetation. The heathland site, Aylesbeare Common, was dominated by a wide range of grass species and Ericaceae plants including *Erica tetralix* L. and *Erica cinerea* L. *C. vulgaris* was also present at this site.
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Figure 3.1 Map of the United Kingdom. Inset represents SW England and approximate locations of the three field sites are represented by a cross (x).
3.2.2. Collection of plant material and isolation of fungi

Six plants of *C. vulgaris* were collected randomly from each of the three field sites. Root systems of individual plants were washed under running H₂O and any remaining soil was removed with forceps. Hair roots were excised, transferred to a muslin bag and surface sterilised in a solution of 37.5% commercial bleach (1.5% available chlorine) for 3 min, then in 10 changes (5 min each) of sterile deionised H₂O.

Fungal endophytes were isolated using a modified method of Pearson & Read (1973). Sterilised root pieces (1 cm length) were placed in 9 cm diam. Petri dishes containing 2% malt agar, 0.5 g l⁻¹ streptomycin sulphate (added after autoclaving), adjusted to pH 5.5. Petri dishes were incubated in the dark at 25°C and observed daily for hyphal emergence. Root pieces from which fast-growing fungi emerged were discarded, while slow-growing fungal colonies were subcultured onto MMN agar medium (Marx & Bryan, 1975) adjusted to pH 5.5 (section 2.2.1). Isolated fungi were maintained on MMN at 25°C.

3.2.3. Mycorrhiza synthesis

In order to confirm the mycorrhizal status of the putative fungal endophytes, all isolates were inoculated onto *Vaccinium macrocarpon* under sterile conditions. Seeds of *V. macrocarpon* were extracted from frozen commercially available fruit and infected with the putative endophytes as described by Duclos and Fortin (1983). Plants were incubated for approx. 12 weeks at 20°C (16 h/8 h day/night cycle, approx 350 μmol m⁻² s⁻¹) after which root pieces were stained with 0.05% lactophenol blue for 15 min and observed with a Zeiss® Axiolab light microscope. The presence of hyphal coils in epidermal cells (section 1.2) suggested the isolates as putative mycorrhizal endophytes.
3.2.4. DNA extraction and ITS-RFLP analysis

Genomic DNA was extracted from axenic fungal mycelia using the modified cetyltrimethylammonium bromide (CTAB) method of Gardes and Bruns (1993). Amplifications of the internal transcribed spacer region (ITS) were performed with the primers ITS1 and ITS 4 using a method modified from White et al. (1990). Amplifications (in duplicate) were performed in a 50 μl reaction volume containing ca 100 ng genomic DNA, 25 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 200 mol each of dATP, dCTP, dGTP and dTTP, and 1.5 units of Taq polymerase (Promega). All amplifications were performed in a PTC-100 thermocycler (MJ Research) with 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min. A negative control, containing no fungal DNA, was included in each PCR reaction run to test for the presence of contaminants. Amplification products were subjected to electrophoresis in 1.5% (w/v) agarose gels, stained with ethidium bromide and viewed under UV light.

All ITS products were digested with the restriction endonucleases Hinf I, Rsa I or Hae III (Promega) by incubating ca 2 μg of PCR product with each enzyme at 37°C for 3 h. Restriction fragments were separated by electrophoresis in 3% (w/v) agarose gels and viewed as above.

3.2.5. Sequencing of amplified ITS regions

Endophytes were grouped based on ITS-RFLP analysis and the ITS region of at least one endophyte from each group was sequenced. PCR products were cloned with the pGEM-T easy vector system (Promega) and two or three clones for each isolate sequenced with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an automated
fluorescent DNA sequencer ABI model 373-A (Applied Biosystems). Sequencing reactions were performed with the primers T7 and SP6 (Promega).

3.2.6. Data Analysis

ITS sequences were analysed using the FASTA 3.0 program (Pearson & Lipman, 1988) and sequences aligned using the Pileup and Pretty programs [within the EGCG extensions to the Wisconsin Package, Version 8.1.0 (Rice, 1996)]. All sequences were screened for restriction sites for *Hinf I, Rsa I* and *Hae III* using the Map program (Rice, 1996) to confirm band sizes estimated from RFLP gels and to account for small (< ca 30 bp) fragments that were not observed on gels. In order to infer relationships between the taxa most similar to the endophytes as indicated by FASTA analysis, a neighbour-joining analysis of the patristic distance matrix (1000 bootstraps re-sampling replicates) was conducted using the Beta version of PAUP (4.01b). Details of isolates used in this analysis are given in Table 3.1. ITS sequences of *Tuber melanosporum* and *Morchella esculenta* were used for outgroup rooting. Parsimony analysis (with 500 replicates) was also conducted using the heuristic search option of PAUP.
Table 3.1 GenBank accession codes for ITS sequences of isolates included in the neighbour-joining analysis.

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<tr>
<td>salal root-associated fungus II</td>
<td>AF149073</td>
<td>Unknown</td>
</tr>
<tr>
<td>salal root-associated fungus III</td>
<td>AF149075</td>
<td>Unknown</td>
</tr>
<tr>
<td>salal root-associated fungus IV</td>
<td>AF149076</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trichopezetta niduluis</td>
<td>TNU57813</td>
<td>Hyaloscyphaceae</td>
</tr>
<tr>
<td>Tuber melanosporum</td>
<td>TMU89359</td>
<td>Tuberaeae</td>
</tr>
</tbody>
</table>
3.3. Results

A total of 107 slow-growing, sterile mycelial isolates were obtained from root systems of *C. vulgaris* at the three sites, comprising 26-44 isolates from each site (Table 3.2). Seventy percent of these isolates formed typical mycorrhizal structures (coils) in epidermal cells of *V. macrocarpon* hair roots during *in vitro* synthesis experiments, identifying them as putative mycorrhizal endophytes (Table 3.2). DNA was extracted from all isolates, regardless of the outcome of the *in vitro* synthesis experiments, and in each case ITS amplification produced a single band of approximately 550-600 bp in size (data not shown). Subsequent digestion with restriction endonucleases *Rsa* 1, *Hinf* 1 and *Hae* III resulted in RFLP patterns for 76 isolates that were consistent with a known isolate of *H. ericae* (Read 101) (Tables 2 and 3). All of these isolates (ITS type 1) formed segmented arthroconidial chains characteristic of *H. ericae* (Smith & Read, 1997) (data not shown). Three randomly chosen isolates from this group were selected for ITS sequencing and FASTA analysis indicated that they had 96.1-99.2% identity over 488-512 bp with known *H. ericae* sequences (Table 3.4), suggesting that this group of isolates are congeneric, or maybe conspecific, with *H. ericae*. The majority (62-92%) of isolates obtained from the three sites were thus *H. ericae* (Table 3.2). Interestingly, of the 76 *H. ericae* isolates obtained, only 58 formed typical ericoid mycorrhiza-like hyphal coils in epidermal cells of hair roots of *V. macrocarpon* (Table 3.2), with 12-43% of the *H. ericae* isolates from each site failing to form a mycorrhizal association under the experimental conditions adopted. Furthermore, given the % sequence divergence within this group, and the possibility that they may represent more than one species, it may be that different species are differentially mycorrhizal.
Table 3.2 Numbers of root endophyte isolates obtained from *C. vulgaris* roots at three field sites in SW England. The number of isolates that had RFLP patterns consistent with *H. ericae* is also shown, along with the number of isolates (in parentheses) that formed mycorrhizas with *V. macrocarpon* in dual culture experiments.

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolates obtained</th>
<th><em>H. ericae</em> isolates</th>
<th>Other endophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Devon Great Consols</td>
<td>26</td>
<td>24 (21)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>Gawton United</td>
<td>44</td>
<td>29 (24)</td>
<td>15 (9)</td>
</tr>
<tr>
<td>Aylesbeare Common</td>
<td>37</td>
<td>23 (13)</td>
<td>14 (10)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>107</td>
<td>76 (58)</td>
<td>31 (19)</td>
</tr>
</tbody>
</table>

ITS-RFLP patterns grouped the 31 remaining endophytes into 13 RFLP types, each type having a distinctive pattern with at least one restriction endonuclease (Table 3.3). Although in some cases, several isolates were grouped as the same RFLP type, in no case were isolates of the same RFLP type obtained from more than one site (Table 3.3). Nineteen of these isolates formed mycorrhizal associations with *V. macrocarpon* (Table 3.2). ITS regions of at least one representative isolate from each group were sequenced and sequences submitted to the GenBank nucleotide database (see Table 3.4 for accession codes). The full length ITS sequences were screened for closest matches to other fungal taxa for which ITS sequences are available. With the exception of RFLP types 7 and 13, all RFLP types showed >84% similarity over at least 462 bp with sequences of endophytes from roots of Ericaceae or Epacridaceae hosts; indeed in most cases the % similarity was considerably higher (Table 3.4). RFLP types 7 and 13 had greatest similarity (ca. 80% over 362-489 bp) with non-mycorrhizal Hyaloscyphaceae and Myxotrichaceae respectively. Types 2 and 3 showed highest identity (90-92% over 488-531 bp) identity to known *Hymenoscyphus* spp. (Table 3.4), however the RFLP patterns obtained were not consistent with *H. ericae* (Read 101) (Table 3.3).
Table 3.3 Grouping of endophytes isolated from *C. vulgaris* roots at three field sites in SW England as RFLP types according to the sizes of restriction fragments (bp) produced following digestion of the ITS region with three restriction endonucleases.

<table>
<thead>
<tr>
<th>RFLP type</th>
<th>Isolates</th>
<th><em>Hae</em> III</th>
<th><em>Hin</em> f I</th>
<th><em>Rsa</em> I</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. ericae</td>
<td>Read 101</td>
<td>292, 135, 85</td>
<td>259, 245, 8</td>
<td>355, 157</td>
</tr>
<tr>
<td>1</td>
<td>DGC1, DGC2, DGC3, DGC4, DGC5, DGC6, DGC7, DGC8, DGC9, DGC10, DGC11, DGC12, DGC13, DGC14, DGC15, DGC16, DGC17, DGC18, DGC19, DGC20, DGC21, DGC22, DGC23, DGC24, GU1, GU2, GU3, GU4, GU5, GU6, GU7, GU8, GU9, GU10, GU11, GU12, GU13, GU14, GU15, GU16, GU17, GU18, GU19, GU20, GU21, GU22, GU23, GU24, GU25, GU26, GU27, GU28, GU44, AC1, AC2, AC3, AC4, AC5, AC6, AC7, AC8, AC9, AC10, AC11, AC12, AC13, AC14, AC15, AC16, AC17, AC18, AC19, AC20, AC21, AC22, AC23</td>
<td>292, 135, 85</td>
<td>259, 245, 8</td>
<td>355, 157</td>
</tr>
<tr>
<td>2</td>
<td>GU30, GU31</td>
<td>283, 132, 97, 19</td>
<td>279, 234, 18</td>
<td>355, 126, 50</td>
</tr>
<tr>
<td>3</td>
<td>GU32, GU33, GU34</td>
<td>292, 137, 101</td>
<td>275, 167, 80, 8</td>
<td>no restriction sites</td>
</tr>
<tr>
<td>4</td>
<td>AC24, AC25</td>
<td>300, 111, 79, 28, 11</td>
<td>267, 252, 8</td>
<td>363, 164</td>
</tr>
<tr>
<td>5</td>
<td>GU35</td>
<td>280, 78, 60, 49, 28, 28</td>
<td>265, 250, 8</td>
<td>360, 163</td>
</tr>
<tr>
<td>6</td>
<td>GU36</td>
<td>430, 79</td>
<td>254, 167, 80, 8</td>
<td>357, 152</td>
</tr>
<tr>
<td>7</td>
<td>DGC25</td>
<td>436, 128, 48</td>
<td>354, 181, 69, 8</td>
<td>361, 223, 28</td>
</tr>
<tr>
<td>8</td>
<td>GU28</td>
<td>422, 79, 12</td>
<td>234, 169, 82, 20, 8</td>
<td>245, 152, 116</td>
</tr>
<tr>
<td>9</td>
<td>GU37, GU38, GU39, GU40, GU41, GU42, GU43</td>
<td>430, 79</td>
<td>254, 167, 80, 8</td>
<td>242, 115, 113, 39</td>
</tr>
<tr>
<td>10</td>
<td>AC26, AC27, AC28, AC29, AC30, AC31</td>
<td>298, 79, 75, 33, 28, 10</td>
<td>249, 161, 105, 8</td>
<td>no restriction sites</td>
</tr>
<tr>
<td>11</td>
<td>AC32, AC33, AC34, AC35</td>
<td>300, 108, 80, 28, 10</td>
<td>269, 249, 8</td>
<td>no restriction sites</td>
</tr>
<tr>
<td>12</td>
<td>AC36</td>
<td>298, 105, 99, 28, 10</td>
<td>249, 187, 105, 8</td>
<td>no restriction sites</td>
</tr>
<tr>
<td>13</td>
<td>DGC26</td>
<td>304, 86, 75, 33, 18</td>
<td>440, 109</td>
<td>no restriction sites</td>
</tr>
<tr>
<td>14</td>
<td>AC37</td>
<td>438, 80, 8</td>
<td>No restriction sites</td>
<td>no restriction sites</td>
</tr>
</tbody>
</table>

* letters denote the sites from which isolates were obtained (DGC = Devon Great Consols mine, GU = Gawton United mine, AC = Aylesbeare Common); isolates shown in bold formed ericoid structures in epidermal cells of *V. macrocarpon* in dual culture experiments.
Table 3.4 Closest matches from FASTA searches between ITS sequences from endophytes isolated from *C. vulgaris* roots and sequences from the GenBank nucleotide database.

<table>
<thead>
<tr>
<th>RFLP type</th>
<th>Closest species match</th>
<th>Genbank Accession code</th>
<th>Sequence Similarity (%)</th>
<th>Nucleotide Overlap (bp)</th>
<th>Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (DG23)</td>
<td><em>Hymenoxypus ericae</em></td>
<td>AF069505</td>
<td>99.2</td>
<td>511</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AP252833)</td>
<td><em>Hymenoxypus sp.</em></td>
<td>AF069439</td>
<td>98.4</td>
<td>511</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AP252834)</td>
<td><em>Hymenoxypus sp.</em></td>
<td>AF069439</td>
<td>96.1</td>
<td>488</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AT21)</td>
<td><em>Hymenoxypus ericae</em></td>
<td>AF069505</td>
<td>96.1</td>
<td>488</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AP252831)</td>
<td><em>Hymenoxypus ericae</em></td>
<td>AF069505</td>
<td>99.0</td>
<td>511</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(GU27)</td>
<td><em>Hymenoxypus ericae</em></td>
<td>AF069439</td>
<td>98.4</td>
<td>511</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AP252835)</td>
<td><em>Hymenoxypus ericae</em></td>
<td>AF069505</td>
<td>98.6</td>
<td>512</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>2 (GU30)</td>
<td><em>Hymenoxypus sp.</em></td>
<td>AF069439</td>
<td>92.3</td>
<td>492</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AP252836)</td>
<td><em>Hymenoxypus sp.</em></td>
<td>AF149069</td>
<td>92.2</td>
<td>488</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>3 (GU32)</td>
<td><em>Hymenoxypus sp.</em></td>
<td>AF069439</td>
<td>90.0</td>
<td>531</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>(AP252837)</td>
<td><em>Hymenoxypus sp.</em></td>
<td>AF069505</td>
<td>89.8</td>
<td>531</td>
<td>Leotiaceae</td>
</tr>
<tr>
<td>4 (AC24)</td>
<td>Epacrid root endophyte</td>
<td>AF097312</td>
<td>94.6</td>
<td>465</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252838)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149077</td>
<td>95</td>
<td>520</td>
<td>Unknown</td>
</tr>
<tr>
<td>5 (GU35)</td>
<td>Salal root associated fungus</td>
<td>AF149073</td>
<td>95.3</td>
<td>514</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252839)</td>
<td>Salal root associated fungus</td>
<td>AF149075</td>
<td>95.1</td>
<td>514</td>
<td>Unknown</td>
</tr>
<tr>
<td>6 (GU36)</td>
<td><em>Dactylella lobata</em></td>
<td>DLU151958</td>
<td>86.9</td>
<td>513</td>
<td>Mitosporic ascomycete unknown</td>
</tr>
<tr>
<td>(AP252840)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149083</td>
<td>85.9</td>
<td>467</td>
<td>Hyaloscyphaceae</td>
</tr>
<tr>
<td>7 (DG25)</td>
<td><em>Perrotia flamma</em></td>
<td>PFU57988</td>
<td>80.2</td>
<td>489</td>
<td>Hyaloscyphaceae</td>
</tr>
<tr>
<td>(AP252841)</td>
<td><em>Perrotia distincta</em></td>
<td>PDU57989</td>
<td>77.5</td>
<td>494</td>
<td>Hyaloscyphaceae</td>
</tr>
<tr>
<td>8 (GU44)</td>
<td><em>Dactylella lobata</em></td>
<td>DLU151958</td>
<td>84.3</td>
<td>516</td>
<td>Mitosporic ascomycete unknown</td>
</tr>
<tr>
<td>(AP252842)</td>
<td>Salal root associated fungus</td>
<td>AF149072</td>
<td>84.2</td>
<td>475</td>
<td>Hyaloscyphaceae</td>
</tr>
<tr>
<td>9 (GU37)</td>
<td><em>Dactylella lobata</em></td>
<td>DLU151958</td>
<td>85.4</td>
<td>513</td>
<td>Mitosporic ascomycete unknown</td>
</tr>
<tr>
<td>(AP252843)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149083</td>
<td>84.5</td>
<td>465</td>
<td>Hyaloscyphaceae</td>
</tr>
<tr>
<td>10 (AC26)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149077</td>
<td>91.7</td>
<td>517</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252844)</td>
<td>Salal root associated fungus</td>
<td>AF149076</td>
<td>90.7</td>
<td>515</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AC31)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149077</td>
<td>92.7</td>
<td>517</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252845)</td>
<td>Salal root associated fungus</td>
<td>AF149076</td>
<td>91.7</td>
<td>515</td>
<td>Unknown</td>
</tr>
<tr>
<td>11 (AC22)</td>
<td>Salal root associated fungus</td>
<td>AF149073</td>
<td>89.4</td>
<td>519</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252846)</td>
<td>Salal root associated fungus</td>
<td>AF149075</td>
<td>89.2</td>
<td>519</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AC35)</td>
<td>Eriocid mycorrhizal fungus</td>
<td>AF072301</td>
<td>91.3</td>
<td>526</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252847)</td>
<td>Eriocid mycorrhizal fungus</td>
<td>AF072296</td>
<td>91.3</td>
<td>526</td>
<td>Unknown</td>
</tr>
<tr>
<td>12 (AC26)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149077</td>
<td>91.3</td>
<td>517</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252848)</td>
<td>Epacrid root endophyte</td>
<td>AF097312</td>
<td>92.2</td>
<td>462</td>
<td>Unknown</td>
</tr>
<tr>
<td>13 (DG26)</td>
<td><em>Pseudogymnascus roseus</em></td>
<td>AF062819</td>
<td>79.6</td>
<td>362</td>
<td>Myxotrichaceae</td>
</tr>
<tr>
<td>(AP252849)</td>
<td><em>Umbilicaria ruebliana</em></td>
<td>AF096219</td>
<td>71.6</td>
<td>469</td>
<td>Umbilicariaceae</td>
</tr>
<tr>
<td>14 (AC37)</td>
<td>Salal mycorrhizal fungus</td>
<td>AF149077</td>
<td>89.0</td>
<td>517</td>
<td>Unknown</td>
</tr>
<tr>
<td>(AP252850)</td>
<td>Eriocid mycorrhizal fungus</td>
<td>AF072301</td>
<td>88.5</td>
<td>523</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
The topologies of the neighbour-joining and parsimony trees were similar, hence only the neighbour joining tree is shown (Fig. 3.2). Two strongly supported major clades were observed, one of which (Clade I) comprised the *Hymenoscyphus* species along with RFLP types 1-3, three mycorrhizal endophytes from *Gaultheria shallon* Pursh (salal) and the mitosporic ascomycete *Dactylella lobata*. The other major clade (Clade II) contained all remaining isolates with the exception of DGC26 (RFLP type 13). All *Hymenoscyphus* isolates and RFLP type 1 (putative taxon 1) formed a strongly supported (100%) monophyletic group; isolates within this group having <9.0% sequence divergence. This group was nested within a larger group that comprised two mycorrhizal endophytes from *G. shallon* and RFLP types 2 and 3 (putative taxon 2). Within Clade I, RFLP types 6-9 formed a further strongly-supported monophyletic group with *D. lobata* and a mycorrhizal endophyte from *G. shallon* (Fig. 3.2). Within this group, types 6, 8 and 9 were grouped together (1.8-5.5% sequence divergence) and probably represent a single or closely-related taxon (putative taxon 3). RFLP type 7 (putative taxon 4) and a salal root associated fungus (AF149072), although grouped on a terminal branch, had only 68.5% sequence identity and probably represent two separate taxa.

Clade II comprised RFLP types 4, 5, 10, 11, 12 and 14, along with various endophytes from Ericales hosts, all Hyaloscyphaceae isolates, *Oidiodendron* spp. and the two Onygenales isolates (Fig. 3.2). Within this clade, all unidentified endophytes from Ericales hosts (including those from *C. vulgaris*) formed a strongly-supported monophyletic group, as did the *Oidiodendron* species, the Hyaloscyphaceae isolates and the two Onygenales taxa. Three strongly-supported sister groups were discerned within the unidentified endophyte group. Thus *C. vulgaris* endophytes AC26, AC31, AC36, AC32 and AC35 (RFLP types 10-12) formed a monophyletic group within which isolates had <11.1% sequence divergence, suggesting that they may be conspecific (putative taxon 5). Similarly, isolates AC24, AC25 and AC37 (RFLP
types 4 and 14) grouped strongly with several endophytes from North American Ericaceae and Australian Epacridaceae. Sequence dissimilarity in this grouping was up to 21%, although RFLP types 4 and 14, along with a mycorrhizal endophyte from salal (AF149077), differed by <13%. While the larger group may thus represent more than one taxon, RFLP types 4 and 14 may well be a single taxon (putative taxon 6). Isolate GU35 (RFLP type 5) formed a further monophyletic group with other endophytes from North American Ericaceae and Australian Epacridaceae (<11.0% sequence divergence) and may be conspecific with these isolates (putative taxon 7). Neighbour joining analysis placed isolate DGC26 (RFLP type 13) on a branch outwith the two major clades (Fig. 3.2). The low sequence identity (<60%) between this isolate and all other isolates included in the neighbour joining analysis clearly indicates that this is a further taxon (putative taxon 8, which has little affinity with Leotiaceae or Hyaloscyphaceae.
Figure 3.2 Neighbour-joining tree based on ITS sequence data from endophytes of the 14 RFLP types isolated from root systems of C. vulgaris, along with known ericoid mycorrhizal endophytes and selected ascomycetes. Numerical values above the branches indicate bootstrap percentiles from 1000 replicates. RFLP types shown in **bold** formed typical ericoid mycorrhizal structures in dual culture experiments with V. macrocarpon.
3.4. Discussion

The data presented in this chapter indicate that considerable genetic diversity exists within the assemblages of ericoid mycorrhizal endophytes and putative mycorrhizal endophytes isolated from roots of *C. vulgaris* from three field sites. This is consistent with several previous studies that have revealed diverse communities of mycorrhizal fungal endophytes in root systems of Ericales hosts at individual field sites (Perotto *et al*., 1996; Xiao & Berch, 1996; Chambers *et al*., 2000). While RFLP analysis separated the endophytes from the present study into 14 groups, the most frequently isolated endophytes, regardless of site of origin, displayed RFLP patterns (type 1) that were consistent with those for known isolates of *H. ericae*. ITS sequence analysis confirmed these isolates as a *Hymenoscyphus* species (possibly *H. ericae*), suggesting that this taxon may be the dominant mycorrhizal endophyte of *C. vulgaris* at heathland and mine sites in south-western England. RFLP types 2 and 3, which showed some similarities to the *H. ericae* RFLP pattern and formed a strongly-supported group with *H. ericae*, other *Hymenoscyphus* isolates and RFLP type 1 in Clade I may represent further *Hymenoscyphus* species, as may the mycorrhizal fungi from salal that were placed in the same group. These data are consistent with other recent reports that, far from being a single species, *H. ericae* comprises an aggregate of related genotypes for which the specific and/or generic boundaries remain to be defined (Monreal *et al*., 1999; Vrålstad *et al*., 2000).

*H. ericae* is thought to be important in survival of *C. vulgaris* on heathland sites by facilitating host access to otherwise unavailable forms of organic nitrogen and phosphorus (Smith & Read, 1997). Soil conditions at the mine sites were, however, markedly different to the heathland site. The mine sites were characterised by soils that contained little organic matter accumulation and extractable As concentrations 20-75 times greater than the Aylesbeare Common site (section 4.3.3.). We hypothesise that populations of *H. ericae* colonising the
mine sites may have an increased $\text{AsO}_4^{3-}$ resistance in comparison to populations from an uncontaminated natural heathland (Chapter 4).

Although *H. ericae* was the most common taxon at all three field sites, several other less common RFLP types (representing 1-2 putative taxa per site) were also present at each site. Perotto *et al.* (1996) found that mycorrhizal fungal endophyte assemblages from *C. vulgaris* at a heathland site in Italy were dominated by a single taxon (*O. maius*) and that several other RFLP types, although present, were isolated less frequently. A similar pattern of relative species abundance has been observed for ectomycorrhizal fungi in forest ecosystems (Gardes & Bruns, 1996; Gehring *et al.*, 1998) and is known to occur also in some animal and plant communities (eg. Terborgh *et al.*, 1990). A striking difference between the results presented in this chapter and that of Perotto *et al.* (1996) is the most frequently isolated taxon, *H. ericae* and *O. maius* respectively. Indeed, while there was no evidence for the presence of *Oidiodendron* spp. in roots of *C. vulgaris* from the three field sites in SW England, Perotto *et al.* (1996) isolated no *Hymenoscyphus* spp. at their field site. Both studies used the endophyte isolation technique of Pearson and Read (1973) modified to include the use of a sterillant at similar concentrations to each other, thus differences in the endophytes isolated cannot be attributed simply to the methods employed. It seems more likely that they reflect a real difference in the endophyte communities at the heathland sites at the two locations. Similarly, Xiao and Berch (1996) isolated no *H. ericae* from roots of *G. shallon* in Canadian forest clearcuts. *H. ericae* is often cited as a ubiquitous endophyte of Ericaceae (eg. Leake & Read, 1991). While, the data of Perotto *et al.* (1996) and Xiao and Berch (1996) do not appear to support this, my data emphasise the importance of this taxon at some contrasting heathland and mine spoil sites. It is also interesting to note that at the Devon Great Consols site only two endophytes apart from *H.*
ericace were isolated (Table 3.2), which appears to indicate a reduced diversity of endophytes at this site.

With the exception of RFLP type 13, which is clearly taxonomically distant from the other isolates considered here, all other types (putative taxa 5-7) were placed in Clade II in the neighbour joining tree. Aside from the C. vulgaris endophytes, this clade comprised Oidiodendron spp., several known Hyaloscyphaceae taxa and unidentified mycorrhizal/putative mycorrhizal endophytes from Ericaceae and Epacridaceae hosts. Putative taxa 5-7 thus appear to have strong affinity with Hyaloscyphaceae. RFLP type 13 notwithstanding, all mycorrhizal endophytes from C. vulgaris at my field sites in SW England appear to be aligned with either Leotiaceae (Clade I) or Hyaloscyphaceae (Clade II), supporting the suggestion of Hambleton et al. (1998) that formation of ericoid mycorrhizas may be limited to Leotiales.

It must be emphasised that, while at least some isolates from most RFLP types formed recognisable mycorrhizal structures in epidermal cells of V. macrocarpon during mycorrhiza synthesis experiments, this was not the case for RFLP types 6, 7, 8, 12 or 13. Since RFLP type 13 (putative taxon 8) is clearly genetically quite different to known mycorrhizal endophytes, its failure to form mycorrhizas with V. macrocarpon probably indicates that this as a non-mycorrhizal isolate. RFLP types 6 and 8, on the other hand, grouped strongly with the confirmed mycorrhizal RFLP type 9 (putative taxon 3) in the neighbour-joining analysis/sequence similarity analyses. Similarly, RFLP type 12 was grouped in putative taxon 5 with the mycorrhizal RFLP types 10 and 11. This suggests either that genetically similar endophytes may be quite different in terms of their mycorrhiza-forming abilities, that RFLP types 6, 8 and 12 are mycorrhizal endophytes that failed to infect V. macrocarpon under the experimental conditions used in this study, or that these are mycorrhizal endophytes, but show a
degree of host specificity (i.e., may form mycorrhizal with *C. vulgaris*, but not *V. macrocarpon*). In the light of the demonstrated inter-compatibilities between mycorrhizal endophytes from Ericaceae and Epacridaceae (Reed, 1989; Read, 1996; Liu et al., 1998; Mclean et al., 1998), the latter may appear unlikely. It must be noted, however, that the extent of mycorrhiza establishment by different ericoid mycorrhizal fungi has been shown to vary on different host taxa and under different conditions (Gianinazzi-Pearson & Bonfante-Fasolo, 1986). It is of further interest that almost 25% of the endophytes that were confirmed by RFLP and ITS sequence analyses to be *H. ericae* failed to form recognisable mycorrhizal structures in roots of *V. macrocarpon*. A recent study has identified marked intraspecific physiological variation (in terms of nitrogen source utilisation) in populations of the *H. ericae*-like endophytes from the three sites investigated in the present study (Cairney et al., 2000), and it is possible that similar variation exists in the ability of different isolates to form mycorrhizas. Some may thus be more weakly mycorrhizal than others. Although regarded as the model ericoid mycorrhizal fungus, *H. ericae* is also recognised as having considerable saprotrophic potential (Cairney & Burke, 1998), and it is certainly possible that some isolates may be more saprotrophic than others. However, the possibility that the experimental conditions adopted were not optimal for mycorrhiza formation by some of these isolates cannot be discounted.

The grouping of mycorrhizal endophytes from Ericaceae and Epacridaceae hosts variously within the Hyaloscyphaceae clade is significant. Previous studies (McLean et al., 1999; Chambers et al., 2000) have concluded from limited comparisons of sequences from endophytes isolated from the two families that endophytes from Epacridaceae hosts are probably Leotiales. Data presented in this chapter provide strong support for this suggestion. They further indicate that all mycorrhizal endophytes isolated from southern hemisphere Epacridaceae, for which ITS sequences have so far been obtained, have strong affinities with
the family Hyaloscyphaceae. While the phylogenetic analysis conducted by McLean et al. (1999) grouped two endophytes from an Australian epacrid host with *H. ericae*, the low degree of sequence identity and the limited range of taxa included in their analysis, suggest that these endophytes were, in fact, probably quite dissimilar to *H. ericae*. Similarly, although pectic enzyme analysis found some endophytes of Australian Epacridaceae to be more similar to *H. ericae* than others (Hutton et al., 1994), this provides no indication that they were, per se, related to *H. ericae*. These observations are of particular interest in the light of the recent discovery of *H. ericae* or, at least a *Hymenoscyphus* species, as a rhizoid endophyte of an Australian leafy liverwort (Chambers et al., 1999). This may simply reflect that relatively few endophytes from Epacridaceae have been investigated in detail so far, rather than indicate that *H. ericae*, or indeed other Leotiaceae, do not exist as mycorrhizal endophytes of southern hemisphere epacrids. Further studies are required to resolve this important question. Recent evidence suggests that *H. ericae* may also have a close association with ectomycorrhizal plants (Vrålstad et al., 2000) and it has been hypothesised that *H. ericae* is actually an aggregate of separate fungal taxa (Vrålstad et al., 2000). The positioning of RFLP type 1-3 within the *Hymenoscyphus* group (Clade 1) in the present study supports this hypothesis. Vrålstad et al. (2000) showed that the ectomycorrhizal fungus *Piceirhiza bicolorata* shared a 95% ITS1 sequence identity with *H. ericae* and could be successfully amplified with a taxon-selective primer designed exclusively for amplification of *H. ericae* like strains. Parsimony analysis strongly supported the single evolutionary lineage of both *P. bicolorata* and *H. ericae* (Vrålstad et al., 2000). These authors hypothesised that both ericoid and ectomycorrhizal plants share mycobionts of the *H. ericae* aggregate (Vrålstad et al., 2000).

The Ericaceae and Epacridaceae, while traditionally regarded as separate families of the order Ericales, are now considered to be more closely related, with epacrids perhaps
representing a sub-clade of the Ericaceae (Crayn et al., 1998). Cullings (1996) suggested that Ericales have a monophyletic origin and may have evolved from Gondwanan ancestors with subsequent spread to other parts of the globe. As a consequence of their monophyletic origin, he further suggested that their mycorrhizal endophytes may also be closely related. This suggestion finds support in the apparent inter-compatibility of host-mycorrhizal endophyte combinations from Ericaceae and Epacridaceae (Reed, 1989; Read, 1996; Liu et al., 1998; McLean et al., 1998). The grouping of endophytes from the two families together in the Hyaloscyphaceae clade in this chapter provides further support for this hypothesis.

Three populations of *H. ericae* were isolated from two AsO$_4^{3-}$ contaminated mine sites and an uncontaminated natural heathland site in SW England. In order to determine whether *H. ericae* populations from As mine spoil soils show constitutive resistance to AsO$_4^{3-}$ (section 2.4), or if they have evolved an alternative adaptive AsO$_4^{3-}$ resistance, the populations of *H. ericae* isolated in this chapter were screened for their resistance to AsO$_4^{2-}$ (Chapter 4).
Chapter 4

Arsenate resistance in the ericoid mycorrhizal fungus *Hymenoscyphus ericae*.

4.1. Introduction

*Calluna vulgaris* is often found colonising mine spoils in Europe (Meharg & Cairney, 2000). It forms ericoid mycorrhizal associations and it has been reported that this confers Cu and Zn resistance in the plant (Bradley *et al*., 1981, 1982). Bradley *et al*. (1981) showed that Cu resistance was constitutive in mycorrhizal endophytes of *C. vulgaris* from mine and uncontaminated sites, and that no evolution to the high metal concentrations had occurred at mine sites (Bradley *et al*., 1981, 1982) (section 1.2). Evidence of evolution of metal resistances in ectomycorrhizal fungi is conflicting (Meharg & Cairney, 2000), though there is limited evidence that Zn and Cd resistance evolves in arbuscular mycorrhizal fungi at metal contaminated sites (Meharg & Cairney, 2000).

In SW England, the mining and processing of As and Cu ores has led to highly contaminated mine spoil soils. Arsenic may be present in these spoil soils at 35 mmol kg$^{-1}$, with AsO$_4^{3-}$ being the dominant form of available soil As (Colbourn *et al*., 1975). A limited number of AsO$_4^{3-}$-tolerant angiosperms, including *Agrostis capillaris* and *Holcus lanatus* (Meharg & Macnair, 1990, 1991a, 1991b) colonise these extremely toxic mine soils, with *C. vulgaris* being the dominant vegetative cover (Porter & Peterson, 1977).

Given the importance of mycorrhizal infection in Cu and Zn resistance in *C. vulgaris* (Bradley *et al*., 1981, 1982), a role of the dominant *C. vulgaris* endophyte, *Hymenoscyphus ericae*, in AsO$_4^{3-}$ resistance was considered. Previous studies of metal tolerance in both
mycorrhizal fungi and plants have generally compared tolerance between limited numbers or, more often, single isolates of various taxa, isolated from either contaminated or uncontaminated areas (Hartley et al., 1997a). The results of such investigations may not be representative of populations and are therefore inconclusive. The experiments carried out in this chapter utilised 53 isolates of *H. ericae* from AsO$_4^{3-}$-contaminated mine spoil soils and 23 from an uncontaminated natural heathland. Sensitivity of *H. ericae* populations toward Cu was also investigated.

### 4.2. Materials and Methods

#### 4.2.1. Fungal culture

Seventy six *H. ericae* isolates were obtained from roots of *C. vulgaris* from three contrasting field sites (Chapter 3).

#### 4.2.2. Effect of AsO$_4^{3-}$ and Cu on biomass production

Two plugs (6 mm diam.) of each *H. ericae* isolate were cut from the edge of actively growing mycelia on MMN and inoculated into nine cm diam. Petri dishes containing 25 ml liquid MMN supplemented with either AsO$_4^{3-}$ or Cu. Arsenate was supplied in the form of Na$_2$HAsO$_4$ at concentrations of 0, 0.67, 1.33 and 4.67 mol m$^{-3}$ for experiments utilising all isolates of the population and at 0, 3.33, 6.66, 13.30, 33.30, and 66.66 mol m$^{-3}$ for six randomly selected isolates from the two mine site populations. Copper was supplemented in the form of CuSO$_4$ at concentrations of 0, 0.004, 0.015, 0.038, 0.15, 0.38, 1.13, 1.5, 7.5 and 15 mol m$^{-3}$ for six randomly selected isolates from each population. For all treatments, PO$_4^{3-}$ concentration in the media was adjusted to 0.01 mol m$^{-3}$. After 17 d incubation, mycelial mats were removed from the growth medium, oven dried overnight (80°C) and the biomass increase determined gravimetrically. This harvest time was chosen using growth curves for *H. ericae* in the same
medium (section 2.2.2.). All treatments were replicated three times. Data were analysed and graphically displayed using the computer package Sigma Plot (Jandel Scientific, Erkrath, Germany).

4.2.3. Analysis of soil material

Soil samples were obtained from around the root systems of all C. vulgaris plants collected at the field sites. Extractable AsO$_4^{3-}$ concentration was determined in soil samples using the mixed acid extractant, 0.05 M HCl and 0.025 M H$_2$SO$_4$ (Woolson et al., 1973) as a comparison to the values obtained by Meharg & Macnair, 1990. A total of six soil samples were analysed from each of the three sites. Soil solution extracts were digested in 2 ml concentrated nitric acid (Analar grade) using a block digester for 1 h at 120°C followed by 1 h at 180°C to evaporate samples to dryness. The As residue was redissolved in 20 ml of a solution containing 5% HCl (v/v) (Analar grade) and 20 mol m$^{-3}$ KI. The amount of As present in the digests was determined using hydride generation interfaced with an atomic absorption spectrophotometer (ThermoUnicam Solaar 969, UK).

4.2.4. Statistical Analysis

Arsenate toxicity data were ranked and a two way ANOVA performed using the computer package Minitab v. 11 (Minitab, state College, PA, USA).

4.3 Results

4.3.1. Effect of AsO$_4^{3-}$ on biomass production of the *H. ericae* populations

All *H. ericae* isolates, regardless of their site of origin, produced a biomass yield of 10-80 µg h$^{-1}$ in the absence of AsO$_4^{3-}$, with the mean yield for each population ranging from 49.4 to 53.1 µg h$^{-1}$ (Fig. 4.1a, e, i). Each of the *H. ericae* populations demonstrated reduced mean
yields with increasing extracellular AsO₄³⁻ concentration, however there were clear differences between the heathland and mine site populations in this regard. Increasing AsO₄³⁻ concentrations and the site of origin of the *H. ericae* isolates had a highly significant effect on the growth of the population (p < 0.001). The site * AsO₄³⁻ concentration source term was also highly significant (p < 0.001). The presence of 0.67 mol m⁻³ AsO₄³⁻ reduced the mean yield of the heathland population significantly, with >60% of isolates producing a biomass increase of <10 μg h⁻¹ at this concentration (Fig. 4.1b). A small proportion of the population (5% of isolates) yielded a biomass of >50 μg h⁻¹ in the presence of 0.67 mol m⁻³ AsO₄³⁻. At the highest AsO₄³⁻ concentration (4.67 mol m⁻³) 95% of the heathland *H. ericae* population produced little or no growth (Fig. 4.1d). In contrast to the heathland site, mean biomass yields of isolates in the two mine site populations were unaffected by the presence of 0.67 mol m⁻³ AsO₄³⁻, with no apparent significant effects on either the range of biomass yields or the population mean (Fig. 4.1f, j). In the presence of 4.67 mol m⁻³ AsO₄³⁻, the mean biomass yield of the two mine site populations was significantly inhibited by 42% (Fig. 4.1h, l), however, 72% and 82% of the Gawton United and Devon Great Consols populations respectively produced biomass yields of 10-70 μg h⁻¹. Indeed, 4% of the Devon Great Consols population and 12% of the Gawton United population produced biomass yields of >70 μg h⁻¹ in the presence of 4.67 mol m⁻³ AsO₄³⁻. There was no significant difference between the growth of the two mine populations.

Distribution of growth of the three *H. ericae* populations was normal in the absence of AsO₄³⁻, however, in the presence of AsO₄³⁻, the distribution of the heathland population became significantly one-tailed. The distribution of both mine site populations in the presence of AsO₄³⁻ were unchanged (Fig. 4.1).
The mean biomass yield of isolates from the heathland population was reduced by 50% (EC$_{50}$) at 0.22 mol m$^{-3}$ AsO$_4^{3-}$ while the growth of the Gawton United mine site population was not significantly affected at this concentration (Fig. 4.2a). Growth of isolates from the heathland site was completely inhibited at 6.7 mol m$^{-3}$ AsO$_4^{3-}$ (Fig. 4.2a), while the Gawton United and Devon Great Consols isolates were totally inhibited by 33.25 mol m$^{-3}$ and 66.5 mol m$^{-3}$ AsO$_4^{3-}$ respectively (Fig. 4.2b).
Figure 4.1  Distribution of biomass increases for heathland (a-d), Devon Great Consols (e-h) and Gawton United (i-l) populations of *H. ericae* over a range of AsO$_4^{3-}$ concentrations. The mean biomass increase of each population at every AsO$_4^{3-}$ concentration is indicated with an asterisk (*).
Figure 4.2. a. Growth of *H. ericae* isolates from heathland (●), Devon Great Consols (▼) and Gawton United (■) populations at AsO$_4^{3-}$ concentrations up to 4.67 mol m$^{-3}$. b Growth of six randomly selected isolates from Devon Great Consols (▼) and Gawton United (■) populations at extended AsO$_4^{3-}$ concentrations. Each point is the mean of three replicates ± SE. Second order exponential decay curves were fitted to the data (Sigma-plot, Jandel Corporation, Germany).
4.3.2. Effect of Cu on growth of H. ericae

The three H. ericae populations showed similar biomass yield decreases in response to increasing Cu concentrations (Fig. 4.3). The EC$_{50}$ value was similar for all populations (0.034 mol m$^{-3}$ Cu) and growth of all isolates was completely inhibited at 0.38 mol m$^{-3}$ Cu regardless of their site of origin (Fig. 4.3).

![Graph showing biomass increase as a function of Cu concentration.](image)

**Figure 4.3** Growth of six randomly selected H. ericae isolates from the heathland population (●), Devon Great Consols population (■) and Gawton United population (◆) over a range of Cu concentrations. Each point is the mean of three replicates ± SE.

4.3.3. Extractable soil As

Variable amounts of As were present in soil from the sites. The heathland soil contained 20 - 75 times less extractable As (0.01 ± 0.004 μmol g$^{-1}$ d wt) than Devon Great Consols and Gawton United mine sites respectively. Gawton United mine site demonstrated the highest concentration of extractable soil As (0.82 ± 0.32 μmol g$^{-1}$ d wt), four times greater than Devon Great Consols (0.19 ± 0.08 μmol g$^{-1}$ d wt).
4.4. Discussion

The results demonstrate AsO$_4^{3-}$ resistance in populations of *H. ericae* isolated from two abandoned As/Cu mine sites in SW England. The soils at the mine sites contain 20–75 times higher AsO$_4^{3-}$ concentrations than those found in natural heathland areas (including Aylesbeare Common) which are unaffected by mining or other industrial processes. Isolates of *H. ericae* from both mine sites were unaffected at concentrations of 0.67 mol m$^{-3}$ AsO$_4^{3-}$ and approximately 85% of isolates from these sites demonstrated growth at the highest concentration used in the first experiment (4.67 mol m$^{-3}$). There was no significant difference between the growth of the isolates from the two mine populations. The heathland population of *H. ericae* was significantly inhibited at the lower AsO$_4^{3-}$ concentrations and 95% of isolates in this population were effectively inhibited at 4.67 mol m$^{-3}$ AsO$_4^{3-}$.

Previous investigations have found that mycorrhizal endophytes from *C. vulgaris* demonstrate constitutive insensitivity towards Cu (Bradley *et al.*, 1981, 1982). In the present chapter, the three *H. ericae* populations showed similar growth responses to increasing Cu concentrations, with EC$_{50}$ values being similar to those reported for ectomycorrhizal fungi (Hartley *et al.*, 1997b). These findings confirm the earlier reports of constitutive resistance to Cu in mycorrhizal endophytes from *C. vulgaris* (Bradley *et al.*, 1981, 1982). In the case of AsO$_4^{3-}$, however, the results presented in this chapter indicate the evolution of resistance in mine site *H. ericae* populations.

The majority of investigations into metal insensitivity in ecto-, ericoid and arbuscular mycorrhizal fungi have relied upon single or limited number of isolates from areas of either metal contaminated or uncontaminated soils (Meharg & Cairney, 2000). Only a few investigations have considered the response of fungal populations to metals (Egerton-
Warburton & Griffin, 1995; Leski et al., 1995). Limited interpretations can be placed on studies that have not been conducted at the population level. Since the majority of studies that have been conducted to date have used a limited number of isolates, there has been uncertainty in the literature as to whether mycorrhizal fungi show constitutive resistance or have evolved resistance to metals in the environment. The investigations carried out in the present chapter, which utilised large numbers of isolates from these separate populations, conclusively demonstrate the evolution of \( \text{AsO}_4^{3-} \) resistance in populations of \( H. ericae \) from As/Cu mine sites.

Arsenate insensitivity has previously been reported in bacteria (Silver & Phung, 1996), algae (Takimura et al., 1996), yeast (Rothstein & Donovan, 1963) and higher plant species (Pollard, 1980; Paliouris & Hutchinson, 1991; Meharg et al., 1993). Genetic evidence also exists which confirms the evidence of the evolution of adaptive insensitivity to \( \text{AsO}_4^{3-} \) in higher and lower plants (Macnair, 1993; Silver & Phung, 1996). In stress conditions (such as pollution by toxic metals), all but the most resistant genotypes in the population are eliminated. The survivors interbreed and among their progeny are more resistant genotypes, which are further selected (Ashmore, 1997). Arsenic resistance in \( H. lanatus \) (Macnair et al., 1992) and \( A. capillaris \) (Watkins & Macnair, 1991) has been shown to be under major gene control, with the gene encoding for \( \text{AsO}_4^{3-} \) exclusion (Macnair et al., 1992). In the case of \( H. lanatus \), \( A. capillaris \) and \( Deschampsia cespitosa \), \( \text{AsO}_4^{3-} \) resistance is conferred by a suppression of the high affinity phosphate uptake system, since \( \text{AsO}_4^{3-} \) is a \( \text{PO}_4^{3-} \) analogue and taken up by the \( \text{PO}_4^{3-} \) uptake system (Meharg & Macnair, 1992a). A similar exclusion mechanism has been reported in algae (Takimura et al., 1996), yeasts (Rothstein & Donovan, 1963) and deutermycete fungi (Beever & Burns, 1980). In bacteria, \( \text{AsO}_4^{3-} \) resistance is coded for by the \textit{ars} genes and \( \text{AsO}_4^{3-} \) resistance has been shown to result from intracellular reduction of \( \text{AsO}_4^{3-} \).
to AsO$_4^{3-}$, followed by AsO$_2^{+}$ efflux (Silver & Phung, 1996). The mechanism used by *H. ericae* is investigated in Chapter 5.

Soils colonised naturally by Ericaceae are generally acidic and nutrient deficient (Marrs & Bannister, 1978; Bradley *et al.*, 1982). Low pH and anaerobic soil conditions facilitate mobilisation of metals such as Al, Fe and Mn, which are toxic at high concentrations (Meharg & Cairney, 2000). Ericoid mycorrhizal associations have been shown to confer constitutive Cu, Zn, Al, Ni and Fe resistance to their host plant (Bradley *et al.*, 1982; Freedman & Hutchinson, 1980; Marrs & Bannister, 1978). Results of the present chapter confirm the results of Bradley and Read (1982), demonstrating ericoid mycorrhizal endophytes of *C. vulgaris* display constitutive resistance to Cu. Meharg and Cairney (2000) suggest that mechanisms of toxicity and cellular uptake of Cu are similar to Al, Fe and Mn (the concentrations of which are likely to be increased in the natural habitat of *C. vulgaris*) resistance, and perhaps *H. ericae* has constitutive resistance to Cu, conferred by Al, Fe and Mn resistances. This contrasts with the AsO$_4^{3-}$ resistance in *H. ericae* reported here. AsO$_4^{3-}$ has no toxic analogues in the heathland environment of *C. vulgaris* and its ericoid mycorrhizal endophyte. Conversely, AsO$_4^{3-}$ is a PO$_4^{3-}$ analogue and is transported by the PO$_4^{3-}$ uptake system (Meharg & Macnair, 1990). To survive on AsO$_4^{3-}$ contaminated soils, therefore, the evolution of AsO$_4^{3-}$ resistance is essential. The *H. ericae* populations from the mine sites showed intraspecific differences in resistance, which did not correlate to the extent of As contamination on the mine site. The Gawton United site was the most contaminated, however, the population of *H. ericae* from the Devon Great Consols site demonstrated most AsO$_4^{3-}$ resistance (Fig. 4.2). It should be noted that the mixed acid extraction used to assess As contamination probably does not reflect As availability to the fungus in soil. Arsenic availability will depend on pH, soil organic matter content and the
presence of other minerals. Therefore the amount of available As in the soil from Devon Great Consols may be greater than the amount present at the Gawton United site.

These results presented in this chapter suggest the evolution of $\text{AsO}_4^{3-}$ resistance in populations of $H. \text{ ericae}$ from As/Cu mines, which contrasts to the constitutive resistance to Cu demonstrated by the same populations. While these mine site $H. \text{ ericae}$ populations can withstand high $\text{AsO}_4^{3-}$ concentrations, the physiological basis for this resistance is unknown. Possible mechanisms of $\text{AsO}_4^{3-}$ resistance in mine site $H. \text{ ericae}$ populations were therefore investigated in Chapter 5.
Chapter 5

Mechanism of arsenate resistance in the ericoid mycorrhizal fungus

*Hymenoscyphus ericae.*

5.1. Introduction

Arsenate resistance is exhibited by the ericoid mycorrhizal fungus, *Hymenoscyphus ericae* (Chapter 4) and some angiosperms, including the grasses *Holcus lanatus* (Meharg & Macnair, 1990), *Agrostis capillaris* and *Deschampsia cespitosa* (Meharg & Macnair, 1991b, 1992a), collected from As contaminated mine soils. Arsenate is a PO$_4^{3-}$ analogue and competes with PO$_4^{3-}$ as a substrate for the PO$_4^{3-}$ uptake system in angiosperms (Asher & Reay, 1979; Ullrich-Eberius et al., 1989), fungi (Rothstein & Donovan, 1963; Jung & Rothstein, 1965; Beever & Burns, 1980) mosses (Wells & Richardson, 1985), lichens (Nieboer et al., 1984), and bacteria (Silver & Misra, 1988). Resistance mechanisms to AsO$_4^{3-}$ in the bacteria, *Staphylococcus aureous* and *Eschericha coli*, involve reducing cellular concentrations of As and rapidly effluxing them via a plasmid-encoded arsenical pump (Rosen, 1986; Silver & Misra, 1988). Meharg and Macnair (1990) demonstrated that AsO$_4^{3-}$ resistance in the grass *H. lanatus*, is due to suppression of the high affinity PO$_4^{3-}$ uptake system in AsO$_4^{3-}$-tolerant plants, which leads to reduced uptake of both PO$_4^{3-}$ and AsO$_4^{3-}$.

Populations of AsO$_4^{3-}$-resistant *H. ericae* from roots of *Calluna vulgaris* on As contaminated mine spoil soils in SW England were isolated in Chapter 3. The objective of the present chapter was to investigate the mechanism of AsO$_4^{3-}$ resistance in these populations and to determine whether *H. ericae* shows a suppression of the high affinity PO$_4^{3-}$ uptake system,
which has previously been observed in AsO$_4^{3-}$-tolerant plant species (Meharg & Macnair, 1990, 1991b, 1992a).

5.2. Materials and Methods

5.2.1. Fungal material

The AsO$_4^{3-}$-resistant *H. ericae* genotype (GU27) was isolated from roots of *C. vulgaris* obtained from the Gawton United mine, Devon, while the non-resistant genotype (AC21) was obtained from *C. vulgaris* roots from Aylesbeare Common, Devon, SW England (Chapter 3). Fungi were maintained as previously outlined in Chapter 2. In AsO$_4^{3-}$ and AsO$_2^{-}$ uptake experiments, mycelia were grown in liquid MMN for 17 d and transferred to PO$_4^{3-}$-free MMN for 48 h prior to uptake analysis (see section 5.3.3.). In order to determine the effects of PO$_4^{3-}$ on AsO$_4^{3-}$ uptake, mycelia were grown in liquid MMN containing 5 mmol m$^{-3}$ PO$_4^{3-}$ for 17 d before AsO$_4^{3-}$ uptake. Mycelia for PO$_4^{3-}$ uptake studies were grown on liquid MMN containing 0.01 mol m$^{-3}$ PO$_4^{3-}$ for 17 d at 25°C.

5.2.2. Effect of AsO$_4^{3-}$ and AsO$_2^{-}$ on biomass production

Two plugs (6 mm diam.) of each *H. ericae* isolate were cut from the edge of actively growing mycelia on MMN and inoculated into 9 cm diam. Petri dishes containing 25 ml liquid MMN. After 11 d incubation at 25°C, fungal plugs were transferred to 25 ml liquid MMN supplemented with either AsO$_4^{3-}$ or AsO$_2^{-}$, supplied as Na$_2$HAsO$_4$ and NaAsO$_2$ respectively, at concentrations of 0, 0.13, 0.33, 1.33, 3.33 and 13.34 mol m$^{-3}$. For all treatments, PO$_4^{3-}$ concentration in the medium was adjusted to 0.01 mol m$^{-3}$. After 7 d incubation, mycelial mats were removed from basal medium, dried overnight (80°C) and the biomass increase determined gravimetrically. All treatments were replicated three times.
5.2.3. Kinetics of $\text{AsO}_4^{3-}$ and $\text{PO}_4^{3-}$ uptake

To determine $\text{AsO}_4^{3-}$, $\text{AsO}_2^-$ and $\text{PO}_4^{3-}$ uptake, three replicate mycelial mats of each isolate were incubated in 25 ml of aerated test solution for 20 min (except when uptake was determined with respect to time). Test solutions contained 10 mol m$^{-3}$ 2-(N-morpholino)ethanesulfonic acid (MES), 0.5 mol m$^{-3}$ Ca(NO$_3$)$_2$, and different concentrations of either $\text{AsO}_4^{3-}$, $\text{AsO}_2^-$ or $\text{PO}_4^{3-}$ in the form of Na$_2$HAsO$_4$.7H$_2$O, NaAsO$_2$ and Na$_2$HPO$_4$ respectively at pH 5-5.5. In the experiments to determine the rate of $\text{PO}_4^{3-}$ uptake, [$^{32}\text{P}$] (as NaH$_3$PO$_4$, supplied by Amersham) was added to give an activity of 37 kBq ml$^{-1}$. Uptake was terminated as outlined in section 2.2.5. Mycelia were dried (80°C, 24 h) and biomass determined gravimetrically before analysis.

5.2.4. Repression of $\text{PO}_4^{3-}$ and $\text{AsO}_4^{3-}$ uptake

In order to investigate the effects of $\text{PO}_4^{3-}$ on $\text{AsO}_4^{3-}$ uptake, three replicate mycelial mats of each isolate were pre-incubated in liquid MMN containing 5 mmol m$^{-3}$ $\text{PO}_4^{3-}$ for 0, 20, 60, 120, 240 or 1440 min prior to $\text{AsO}_4^{3-}$ uptake. The effects of $\text{AsO}_4^{3-}$ on $\text{PO}_4^{3-}$ uptake were determined by pre-incubating three replicate mycelia in 0.75 mmol m$^{-3}$ $\text{AsO}_4^{3-}$ for 0, 20, 60, 120, 240 or 1440 min before exposure to [$^{32}\text{P}$] uptake solution.

5.2.5. Methylation and efflux of As by fungi

Methylation of $\text{AsO}_4^{2-}$ was investigated using a modified method of Jenkins et al. (1999). Conical flasks were inoculated with 20 ml of a solution containing 10 mol m$^{-3}$ MES, 0.5 mol m$^{-3}$ Ca(NO$_3$)$_2$ and 0.67 mol m$^{-3}$ $\text{AsO}_4^{3-}$ for mine site $\text{H. ericae}$ mycelia and 0.27 mol m$^{-3}$ $\text{AsO}_4^{3-}$ for heathland $\text{H. ericae}$ at pH 5-5.5. These $\text{AsO}_4^{3-}$ concentrations were the approximate $\text{AsO}_4^{3-}$ EC$_{50}$ values of the mine and heathland populations. Polyurethane plugs were soaked in
0.1 M HgCl and oven dried at 50°C for 12 h, before being placed inside a glass condensor fitted to the conical flasks. Three conical flasks were set up for each isolate. The presence of As was indicated by brown discoloration of the HgCl plugs and after 24 h of incubation, HgCl plugs were removed and analysed for As by atomic absorption spectrometry.

To investigate the AsO$_4^{3-}$ efflux, mycelia of each isolate ($n = 3$) were exposed to AsO$_4^{3-}$ uptake solution for 10 min, 20 min, 1 h, 4 h or 24 h. After termination of uptake, mycelia were transferred to 25 ml of liquid MMN containing no PO$_4^{3-}$ for 0, 30 min, 90 min, 5 h or 24 h. Mycelia were then dried (80°C, 24 h) and biomass determined gravimetrically before As analysis.

5.2.6. Speciation of As

Three replicate mycelia of each isolate were incubated in 0.75 mol m$^{-3}$ AsO$_4^{3-}$ uptake solution for 1 h. Uptake was terminated and mycelia transferred to a 2 ml test tube containing 1 ml of PO$_4^{3-}$-free liquid MMN. Fresh liquid MMN was continually pumped into and out of the test tube at a flow rate of 0.7 ml min$^{-1}$ and removed from the tube at the same rate for 5 h. After 5 h, fungal material was dried, digested and analysed for As. Two ml of MMN pumped from the test tube was also analysed for AsO$_4^{3-}$ and AsO$_2^{3-}$ using atomic absorption spectrometry (Glaubig & Goldberg, 1988).

5.2.7. Analysis

To determine [$^{32}$P]PO$_4^{3-}$ uptake, dried fungal mycelia were placed in 20 ml glass scintillation vials, to which 10 ml of deionised water was added. [$^{32}$P] activity was determined by Cerenkov counting using a Packard Tri Carb 2100TR liquid scintillation counter, with data
corrected for quenching. Arsenic was determined by digesting and analysing mycelia as outlined in section 2.2.5.

5.2.8. Statistical Analysis

Data were analysed by ANOVA using the computer package Minitab v. 11 (Minitab, State College, PA, USA). Curve fitting was carried out using the fitting regimes within the computer package Sigma Plot (Jandel Scientific, Erkrath, Germany), which utilises the Marquardt non linear curve fitting algorithm (Marquardt, 1963).

5.3. Results

5.3.1. Effect of $\text{AsO}_4^{3-}$ and $\text{AsO}_2^-$ on biomass production

The heathland $H. \text{ericae}$ isolate demonstrated significantly greater sensitivity to $\text{AsO}_4^{3-}$ and $\text{AsO}_2^-$ than the mine site isolate (Fig. 5.1). Growth of the heathland isolate was almost completely inhibited at 1.33 mol m$^{-3}$ $\text{AsO}_4^{3-}$ and above, while growth of the mine site isolate was inhibited by only 40% at the highest concentration tested (13.34 mol m$^{-3}$) (Fig. 5.1a). Biomass yield of the heathland isolate was 15 $\mu$g h$^{-1}$ in the presence of 13.34 mol m$^{-3}$ $\text{AsO}_2^-$, while the mine isolate produced a mean biomass yield of approximately 45 $\mu$g h$^{-1}$ at the same $\text{AsO}_2^-$ concentration (Fig. 5.1b).

5.3.2. Kinetics of high affinity $\text{AsO}_4^{3-}$ and $\text{PO}_4^{3-}$ uptake

In $\text{PO}_4^{3-}$ deficient tissue the rate of $\text{AsO}_4^{3-}$ and $\text{PO}_4^{3-}$ uptake was dependent on concentration, and the uptake of both ions displayed saturation kinetics (Figs 5.2 and 5.3). Single Michaelis-Menten functions were fitted to the data, representing the high affinity uptake
Figure 5.1 Growth of mine site (▼) and heathland (●) *H. ericae* over a range of a. AsO₄³⁻ concentrations and b. AsO₃⁻ concentrations. Each point is the mean of three replicates ± SE.

carrier, which would predominate at low substrate concentrations used here (Meharg & Macnair, 1990). Kinetics of both AsO₄³⁻ and PO₄³⁻ uptake were similar for the two *H. ericae* isolates (Table 5.1). There was no significant difference in the uptake of AsO₄³⁻ between the heathland and mine site *H. ericae* isolates, as determined by analysis of variance (data not shown). Growth in the presence of 5 mmol m⁻³ PO₄³⁻ prior to uptake suppressed AsO₄³⁻ uptake
in both the mine site and heathland isolates (Fig. 5.2). At 0.75 mol m\(^{-3}\) AsO\(_4^{3-}\) in PO\(_4^{3-}\)-
sufficient tissue, the rate of AsO\(_4^{3-}\) uptake was approximately two-three times lower than the
rate of uptake in the absence of PO\(_4^{3-}\) (Fig. 5.2). Michaelis-Menten kinetic parameters could
not be determined for isolates pre-cultured on high concentration PO\(_4^{3-}\) media, and a linear
regression was fitted to the data (Fig. 5.2).

![Graph](image-url)

**Figure 5.2** AsO\(_4^{3-}\) influx in the both the absence of PO\(_4^{3-}\) for mine site (▼) and heathland (●) *H. ericae* and in the
presence of 5 mmol m\(^{-3}\) PO\(_4^{3-}\) for mine site *H. ericae* (V) and heathland *H. ericae* (O). Each point is the mean of
three replicates ± SE.

Increasing PO\(_4^{3-}\) concentrations up to 0.1 mol m\(^{-3}\) resulted in an increase in the rate of
PO\(_4^{3-}\) uptake in both isolates (Fig. 5.3). At concentrations above 0.1 mol m\(^{-3}\), PO\(_4^{3-}\) uptake was
not further enhanced. Kinetic parameters for both isolates were similar (Table 5.1). There was
no significant difference between the rates of PO\(_4^{3-}\) uptake in the heathland or mine site isolate
as determined by ANOVA (Minitab, Jandel Scientific).
In comparison to the rate of AsO$_4^{3-}$ uptake, PO$_4^{3-}$ uptake was much greater in both *H. ericae* isolates at all concentrations. Both isolates demonstrated a much higher $V_{\text{max}}$ and a lower $K_m$ value for PO$_4^{3-}$ in comparison to AsO$_4^{3-}$ (Table 5.1), indicating a much higher affinity for PO$_4^{3-}$ uptake.

![Graph showing PO$_4^{3-}$ influx vs. concentration](image)

**Figure 5.3** PO$_4^{3-}$ influx for mine (▼) and heathland (●) *H. ericae*. Each point is the mean of three replicates ± SE.
Table 5.1 Kinetic parameters for AsO$_4^{3-}$, AsO$_2^-$ and PO$_4^{3-}$ influx in mine site and heathland *H. ericae*. Data represent kinetic parameters ± SE. P is the probability of the source term not being significant.

<table>
<thead>
<tr>
<th><em>H. ericae</em> Isolate</th>
<th>As</th>
<th>Regression</th>
<th>Y0</th>
<th>A</th>
<th>K$_m$ (mol m$^{-3}$)</th>
<th>V$_{max}$ (μmol g$^{-1}$d wt. h$^{-1}$)</th>
<th>R$^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mine <em>As</em>$_{3}^{3+}$</td>
<td>Linear</td>
<td>0.0068 ± 0.0068</td>
<td>0.19 ± 0.014</td>
<td>---</td>
<td>---</td>
<td>0.9729</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Mine <em>As</em>$_{5}^{3+}$</td>
<td>Hyperbola</td>
<td>---</td>
<td>---</td>
<td>3.5 ± 4.6</td>
<td>0.86 ± 0.91</td>
<td>0.9736</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Mine <em>As</em>$_{5}^{5+}$</td>
<td>Hyperbola</td>
<td>---</td>
<td>---</td>
<td>0.14 ± 0.06</td>
<td>3.1 ± 0.5</td>
<td>0.9432</td>
<td>0.0058</td>
<td></td>
</tr>
<tr>
<td>Mine PO$_4^{3-}$</td>
<td>Hyperbola</td>
<td>---</td>
<td>---</td>
<td>0.055 ± 0.048</td>
<td>64.9 ± 13.9</td>
<td>0.7734</td>
<td>0.0209</td>
<td></td>
</tr>
<tr>
<td>Heathland <em>As</em>$_{3}^{3+}$</td>
<td>Linear</td>
<td>0.024 ± 0.016</td>
<td>0.22 ± 0.033</td>
<td>---</td>
<td>---</td>
<td>0.9014</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>Heathland <em>As</em>$_{5}^{3+}$</td>
<td>Hyperbola</td>
<td>---</td>
<td>---</td>
<td>0.72 ± 0.60</td>
<td>0.40 ± 0.17</td>
<td>0.9297</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>Heathland <em>As</em>$_{5}^{5+}$</td>
<td>Hyperbola</td>
<td>---</td>
<td>---</td>
<td>0.28 ± 0.07</td>
<td>3.67 ± 0.37</td>
<td>0.9887</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>Heathland PO$_4^{3-}$</td>
<td>Hyperbola</td>
<td>---</td>
<td>---</td>
<td>0.051 ± 0.017</td>
<td>79.12 ± 6.51</td>
<td>0.9571</td>
<td>0.0007</td>
<td></td>
</tr>
</tbody>
</table>
5.3.3. Repression of AsO$_4^{3-}$ and PO$_4^{3-}$ uptake

Repression of AsO$_4^{3-}$ uptake by PO$_4^{3-}$, and PO$_4^{3-}$ uptake by AsO$_4^{3-}$, was investigated at fixed concentrations. Uptake of 0.75 mol m$^{-3}$ AsO$_4^{3-}$ from solution in heathland and mine site *H. ericae* isolates was reduced by pre-exposure of fungal mycelium to PO$_4^{3-}$ (5.0 mmol m$^{-3}$ PO$_4^{3-}$). The initial rate of AsO$_4^{3-}$ uptake in the absence of PO$_4^{3-}$ in mine site *H. ericae* was 0.22 μmol g$^{-1}$ d wt h$^{-1}$, which was lower than the initial rate of uptake in the heathland isolate (Fig. 5.4a). After 20 min exposure to PO$_4^{3-}$, a rapid decrease in AsO$_4^{3-}$ uptake was observed for both isolates, with mine site and heathland isolates demonstrating uptake rates of 0.14 and 0.29 μmol g$^{-1}$ d wt h$^{-1}$ respectively. After 2 h exposure to PO$_4^{3-}$, AsO$_4^{3-}$ uptake by both isolates was almost completely suppressed (Fig. 5.4a).

Uptake of 0.1 mol m$^{-3}$ PO$_4^{3-}$ was repressed by pre-exposure to 0.75 mol m$^{-3}$ AsO$_4^{3-}$ (Fig. 5.4b). Initially, the rate of PO$_4^{3-}$ uptake in the heathland isolate was greater than the mine site isolate, however on exposure to AsO$_4^{3-}$, rates of PO$_4^{3-}$ uptake decreased in both isolates (Fig. 5.4b). After 20 min exposure to AsO$_4^{3-}$, the rate of PO$_4^{3-}$ uptake was suppressed considerably, however, after 24 h exposure to AsO$_4^{3-}$, PO$_4^{3-}$ uptake in both isolates was completely inhibited (Fig. 5.4b). There was no difference between the effects of AsO$_4^{3-}$ on PO$_4^{3-}$ uptake and PO$_4^{3-}$ on AsO$_4^{3-}$ uptake between the heathland and mine isolate.

5.3.4. Efflux of As from fungal cells

In the methylation experiment, As was not present in the HgCl traps for either mine site or heathland isolates. Since HgCl complexes all volatile methylated arsines as well as AsH$_3$, this indicates that neither isolate methylated AsO$_4^{3-}$ (data not shown).
Figure 5.4 a Uptake of 0.75 mol m\(^{-3}\) AsO\(_4^{3-}\) after periods of exposure to 5.0 mmol m\(^{-3}\) PO\(_4^{3-}\) and b uptake of 0.1 mol m\(^{-3}\) PO\(_4^{3-}\) after periods of exposure to 0.75 mol m\(^{-3}\) AsO\(_4^{3-}\) in mine site (▼) and heathland (●) H. ericae. Each point is the mean of three replicates ± SE. Second order decay curves were fitted to the data (Sigma-plot, Jandel Corporation, Germany).
Efflux of As from mine site *H. ericae* mycelia was more rapid than for the heathland isolate (Fig. 5.5). After 1 h incubation in 0.75 mol m$^{-3}$ AsO$_4^{3-}$ (to load cells with As) and transfer to AsO$_4^{3-}$-free media for time periods of up to 24 h, As concentration in the mine site isolate tissue decreased significantly ($P < 0.001$) (Fig. 5.5). After 5 h in AsO$_4^{3-}$-free media, the mine site isolate lost 83% of its initial As concentration, in comparison to the heathland isolate which lost 13%, showing enhanced As cell efflux in the mine site *H. ericae* isolate. Similar trends were found after loading for 10 min, 20 min and 4 h exposure to 0.75 mol m$^{-3}$ AsO$_4^{3-}$ (data not shown). Of the total As effluxed from cells, 71.6% was AsO$_2^-$ with the remainder of As being lost as AsO$_4^{3-}$. Similarly, the majority of As lost from the heathland isolate was in the form of AsO$_2^-$ (71.3%). These results indicate an enhanced AsO$_2^-$ efflux mechanism in the mine site *H. ericae* isolate.

![Graph showing arsenic content over time](image)

**Figure 5.5** Arsenic content of mine site (▼) and heathland (●) *H. ericae* tissue over time as a percentage of total As content after incubation in 0.75 mol m$^{-3}$ AsO$_4^{3-}$. Each point is the mean of three replicates ± SE.
5.3.5. Accumulation of AsO$_4^{3-}$ over time

The long-term uptake of AsO$_4^{3-}$ was much greater in the heathland \textit{H. ericae} isolate than the mine site isolate (Fig. 5.6). Accumulation of AsO$_4^{3-}$ by the heathland isolate did not differ significantly from linearity with respect to time ($r^2 = 0.973$) over 2 h, however, after 2 h exposure, decreased accumulation was observed (Fig. 5.6). This decrease in As accumulation after 2 h seems likely to reflect a response to AsO$_4^{3-}$ toxicity. In contrast, while the mine site isolate demonstrated linear uptake over 20 min there was no further increase in the rate of uptake 2 h and this was sustained for up to 24 h.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure5_6.png}
\caption{Long-term accumulation of 0.75 mol m$^{-3}$ AsO$_4^{3-}$ in mine site (▼) and heathland (●) \textit{H. ericae}. Each point is the mean of three replicates ± SE.}
\end{figure}
5.3.6. Uptake of \( \text{AsO}_2^- \)

The rates of \( \text{AsO}_2^- \) uptake by heathland and mine site \( H. \text{ericae} \) isolates were similar, with uptake in both isolates increasing linearly in response to increasing \( \text{AsO}_2^- \) concentrations (Fig. 5.7). Although Michaelis-Menten functions could be fitted to the data, linear models demonstrated the best fits (Table 5.1). Uptake of \( \text{AsO}_2^- \) in mine site and heathland \( H. \text{ericae} \) isolates at low \( \text{AsO}_2^- \) concentrations (0.01 mol m\(^{-3}\) \( \text{AsO}_2^- \)) was three-four fold less than the rate of \( \text{AsO}_4^{3-} \) uptake at the same concentration (Figs 5.2 and 5.7). At 0.75 mol m\(^{-3}\) \( \text{AsO}_2^- \), the rate of \( \text{AsO}_2^- \) uptake was 15 times less than the rate of \( \text{AsO}_4^{3-} \) uptake, with both isolates have a lower affinity for \( \text{AsO}_2^- \) than \( \text{AsO}_4^{3-} \).

![Graph showing the relationship between \( \text{AsO}_2^- \) concentration and uptake rate.](image)

**Figure 5.7** \( \text{AsO}_2^- \) influx for mine site (▼) and heathland (●) \( H. \text{ericae} \). Each point is the mean of three replicates ± SE. Linear regressions were fitted to the data (Sigma-plot, Jandel Corporation, Germany).
5.4. Discussion

Populations of AsO$_4^{3-}$-resistant *H. ericae* have been isolated from As/Cu mine spoil soils (Chapter 4), however the present chapter also demonstrates increased AsO$_2^-$ resistance in an isolate of *H. ericae* obtained from an As/Cu mine site, in comparison to an isolate from an uncontaminated heathland site. Arsenite is usually considered to be the most toxic form of As available to plants and fungi, however both isolates of *H. ericae* demonstrated greater sensitivity to AsO$_4^{3-}$ (Fig. 5.1), although rates of growth of both isolates were similar in the absence of As. This is contrasts to the results presented in Figs 2.3 and 2.4, which indicate that *H. ericae* are more sensitive to AsO$_2^-$. This effect may indicate intraspecific differences within the *H. ericae* species with respect to their ability to grow in the presence of As.

Arsenate behaves as a PO$_4^{3-}$ analogue (Meharg & Macnair, 1990) and is accumulated by the PO$_4^{3-}$ transport system in a wide range of organisms (Meharg & Macnair, 1992a). Phosphate transport across membranes is carrier-mediated and described by Michaelis-Menten kinetics (Beever & Burns, 1980). Adaptation of the PO$_4^{3-}$ uptake system is a mechanism of AsO$_4^{2-}$ resistance in the grasses *H. lanatus*, *D. cespitosa* and *A. capillaris* (Meharg & Macnair, 1990, 1991b, 1992a, 1994). In *H. lanatus*, AsO$_4^{2-}$ resistance is achieved by constitutive suppression of the high affinity uptake system by carrier synthesis inhibition and is independent of plant P status (Meharg & Macnair, 1992a). The present chapter demonstrates similar $K_m$ and $V_{\text{max}}$ parameters for AsO$_4^{3-}$ transport and PO$_4^{3-}$ transport in AsO$_4^{3-}$-resistant (mine *H. ericae*) and non-resistant (heathland) *H. ericae* (Table 5.1). Mine site and heathland *H. ericae* $K_m$ values (0.14 ± 0.06 mol m$^{-3}$ and 0.28 ± 0.07 mol m$^{-3}$ respectively) were higher than values previously reported for AsO$_4^{3-}$-resistant *H. lanatus* (0.074 mol m$^{-3}$) (Meharg & Macnair, 1992b) and other fungi (Chapter 2). For example, *Saccharomyces cerevisiae* has a $K_m$ for AsO$_4^{3-}$ of 0.004 mol m$^{-3}$.
(Jung & Rothstein, 1965), and Candida tropicalis has a $K_m$ value of 0.005 mol m$^{-3}$ (Beever & Burns, 1980). The $V_{\text{max}}$ value for $\text{AsO}_4^{3-}$ uptake obtained for $H. ericae$ (Table 5.1) was similar to those reported for $S. cerevisiae$ (10.2 μmol g$^{-1}$ d wt h$^{-1}$), lower than the ectomycorrhizal fungus Hebeloma crustuliniforme (Chapter 2), yet higher than those found in $\text{AsO}_4^{3-}$-resistant $H. lanatus$ (Meharg & Macnair, 1990).

Both $H. ericae$ isolates demonstrated similar $\text{AsO}_4^{2-}$ transport kinetics, however influx was reduced markedly when compared with the rate of $\text{AsO}_4^{3-}$ uptake (Figs 5.7 and 5.2). This decrease was expected due to the different chemical properties of $\text{AsO}_4^{2-}$ in comparison to $\text{AsO}_4^{3-}$. While the mechanisms by which $\text{AsO}_4^{2-}$ enters hyphae are unknown, $H. ericae$ is much less efficient at absorbing $\text{AsO}_4^{2-}$ compared with $\text{AsO}_4^{3-}$.

Pre-growth of $H. ericae$ in the presence of 5 mmol m$^{-3}$ $\text{PO}_4^{3-}$ significantly suppressed the uptake of $\text{AsO}_4^{3-}$ for both mine site and heathland isolates (Fig. 5.2). Suppression of $\text{AsO}_4^{3-}$ uptake by long-term exposure to $\text{PO}_4^{3-}$ has also been demonstrated in the plant Hordeum vulgare L. and Silene vulgaris (Lee, 1982; Paliouris & Hutchinson, 1991). In the case of $H. vulgare$, plants grown in the presence and absence of 0.5 mol m$^{-3}$ $\text{PO}_4^{3-}$, demonstrated $\text{AsO}_4^{3-}$ uptake rates of 27.7 and 81.6 nmol g$^{-1}$ f wt h$^{-1}$ respectively (Lee, 1982). Meharg and Macnair (1991a) suggested that $\text{AsO}_4^{2-}$ resistance in $H. lanatus$ was due to a decrease in the concentration of protein carriers in the plasma membrane rather than a change in the carrying capacity of the protein.

$\text{AsO}_4^{3-}$ uptake was rapidly repressed on exposure to $\text{PO}_4^{3-}$ in both mine site and heathland $H. ericae$ (Fig. 5.4). Similarly, the presence of $\text{AsO}_4^{3-}$ rapidly repressed $\text{PO}_4^{3-}$ uptake (Fig. 5.4). Rapid repression of the high affinity $\text{PO}_4^{3-}$ uptake system in plants under high plant
PO₄³⁻ status has long been reported (Meharg & Macnair, 1992b). The nature of this repression differs for different species. Repression may occur by a decrease in $V_{\text{max}}$ with little or no change in the $K_m$, which occurs in algae (McPharlin & Bieleski, 1987), bacteria (Lowendorf et al., 1975; Beever & Burns, 1980), and selected plants (Anghinoni & Barber, 1980; Lee, 1982; Cogliatti & Santa Maria, 1990; Jungk et al., 1990). Repression of the high affinity uptake system in the VAM fungus *Gigaspora margarita* is achieved by increase in the $K_m$ with little change in the $V_{\text{max}}$ (Thompson et al., 1990), while repression in the plant, *Solanum tuberosum* L., is achieved by both an increase in $K_m$ and a decrease in $V_{\text{max}}$ (Cogliatti & Clarkson, 1983). Changes in PO₄³⁻ uptake with changing PO₄³⁻ status may be under allosteric control (Lefebvre & Glass, 1982; Schjoring & Jensen, 1984) and by the synthesis and breakdown of transport sites (Jeanjean, 1973; Drew et al., 1984). Since the speed of repression is too rapid to be explained by protein turnover, under short exposure times it is likely that for *H. ericae* both PO₄³⁻ and AsO₄³⁻ act allosterically.

Short-term uptake of AsO₄³⁻ was similar between isolates, however in the longer term, accumulation of As by the heathland isolate decreased significantly during a 24 h incubation (Fig. 5.6). Such a decrease in the rate of As accumulation in the heathland isolate may reflect cell death in response to AsO₄³⁻ toxicity. Arsenate causes toxicity in fungi and plants by interfering with aerobic phosphorylation, following intracellular reduction of AsO₄³⁻ to AsO₂⁻ which then breaks down protein sulphhydryl groups (Ullrich-Eberius et al., 1989).

The present chapter demonstrates the ability of an isolate of *H. ericae* from a mine site to efflux AsO₂⁻ from its hyphae (Fig. 5.5), which may provide AsO₄³⁻ resistance to this isolate. Arsenite efflux has been reported as a mechanism of AsO₄³⁻ resistance in the bacterium *Staphylococcus aureous* (Broer et al., 1993) and the yeast *S. cerevisiae* (Wysocki et al., 1997),
and contrasts to the mechanism of resistance reported in higher plants (Meharg & Macnair, 1990). Arsenate resistance in *S. cerevisiae* is mediated by an AsO$_4^{3-}$ transporter (Wysocki *et al.*, 1997), and in the case of *S. aureous*, intracellular AsO$_4^{3-}$ is reduced to AsO$_2^{-}$ before being actively exported from the cells (Broer *et al.*, 1993). The results in the present chapter suggest a similar mechanism of As resistance in *H. ericae* at As contaminated mine sites. The steady state of As accumulation observed in the mine site isolate after 20 min is probably not due to a suppression of the high affinity uptake system, but rapid internal reduction of AsO$_4^{3-}$ to AsO$_2^{-}$, which then initiates efflux of AsO$_2^{-}$ from the hyphae. The ability of the mine site *H. ericae* isolate to efflux AsO$_2^{-}$ from cells into the surrounding soil indicates a need for enhanced resistance to AsO$_2^{-}$. Arsenate was much more toxic to *H. ericae* than AsO$_2^{-}$ (Fig. 5.1), which supports efflux of AsO$_2^{-}$ as the mechanism of AsO$_4^{3-}$ resistance in *H. ericae*.

The mechanism of AsO$_4^{3-}$ resistance we have described in *H. ericae* is likely to be of ecological importance for its host plant (*C. vulgaris*) on contaminated mine sites. Arsenite efflux may enable the fungus to retain its ability to transport PO$_4^{3-}$ from the soil (which in turn is accessed by the host plant), while effluxing absorbed AsO$_4^{3-}$. Hence, the ability of the fungus to supply PO$_4^{3-}$ to its host plant is not compromised. Efflux of AsO$_2^{-}$ from the fungal cells into the soil also may ensure that reabsorption of As from the soil is limited. However, in order to clarify the ecological role of *H. ericae* in the colonisation of *C. vulgaris* on As contaminated mine spoil soils, the ability of the host plant to grow in high AsO$_4^{3-}$ concentrations and the effect of mycorrhizal association on AsO$_4^{3-}$ uptake was investigated in Chapter 6.
Chapter 6

Arsenate resistance in *Calluna vulgaris* from As contaminated and uncontaminated sites.

### 6.1. Introduction

The high metal content of mine spoils can act as a toxic factor hindering the growth of vegetation (Paliouris & Hutchinson, 1991). Metal toxicity inhibits root elongation as well as affecting shoot growth, chlorophyll synthesis and enzyme activities (Cox & Hutchinson, 1980). However, selected plant species have the ability to colonise metalliferous soils, indicating that these plant populations may have evolved resistance to the effects of such metals (Paliouris & Hutchinson, 1991). Metal tolerant species and metal tolerant ecotypes have been found from a wide range of plant families including *Caryophyllaceae, Cruciferae, Composite, Leguminose* and *Gramineae* (Antonovics, 1975).

Metal tolerance in plant populations is usually specific to those metals present in soils in excess amounts (Jowett, 1964). For example, *Agrostis gigantea* Roth colonising a Cu-Ni mine demonstrated resistance to Cu and Ni (Hogan & Rauser, 1979), while *Agrostis capillaris* and *Holcus lanatus* colonising an abandoned As mine have shown resistance to As (Porter & Peterson, 1977; Meharg & Macnair, 1990, 1991b). Tolerance to Zn has also been reported for *Silene uniflora* L. (Baker, 1978; Chater & Walters, 1990) and *Silene vulgaris* (Harmens et al., 1989) collected from Zn contaminated sites, and exclusion of the metal has been demonstrated as a mechanism tolerance in these species (Baker, 1978). Exclusion has also been suggested as the mechanism for observed Co and Cu tolerance in *Silene cobalticola* L. (Baker et al., 1983).
AsO$_4^{3-}$-tolerant grasses, including *A. capillaris* and *H. lanatus*, have colonised As mine spoil soils in SW England (Porter & Peterson, 1975, 1977; Meharg & Macnair, 1990, 1991b, 1992a). Meharg and Macnair (1990, 1991b, 1992a) showed that the mechanism of AsO$_4^{3-}$-tolerance in *A. capillaris*, *H. lanatus* and *Deschampsia cespitosa* was, at least partly, due to a suppression of the high affinity PO$_4^{3-}$-uptake system. Arsenate competes with PO$_4^{3-}$ for uptake (Meharg & Macnair, 1990) and Meharg and Macnair (1990) demonstrated a reduced influx of both AsO$_4^{3-}$ and PO$_4^{3-}$ in AsO$_4^{3-}$-tolerant *H. lanatus* in comparison to non-tolerant genotypes. This reduced influx was also reflected in long-term accumulation of AsO$_4^{3-}$ from solution culture (Meharg & Macnair, 1991a). Suppression of the PO$_4^{3-}$-uptake system in plants, while acting as a mechanism for AsO$_4^{3-}$-tolerance, may however cause a PO$_4^{3-}$ deficiency.

*Calluna vulgaris* is the dominant vegetation colonising both natural heathland and mine sites in SW England, and it is found in mycorrhizal association with the ericoid ascomycete fungus *Hymenoscyphus ericae* (Chapter 3). Mycorrhizal fungi have long been shown to enhance acquisition and transfer of nutrients, such as PO$_4^{3-}$, from soil to their host plant. It has also been suggested that, in some cases, mycorrhizal fungi may alleviate metal toxicity to its host (Bradley et al., 1981, 1982). Isolates of *H. ericae* from Gawton United and Devon Great Consols As mine sites have an enhanced efflux of AsO$_2^-$ from their hyphae, in comparison to isolates from uncontaminated natural heathland (Chapter 5), which provides AsO$_4^{3-}$ resistance to these populations. The purpose of this chapter was to investigate the sensitivity of the host plant, *C. vulgaris* from both the heathland and Gawton United mine site to AsO$_4^{3-}$. The ability of *C. vulgaris* from these two sites to absorb PO$_4^{3-}$ was also investigated, along with the effects of mycorrhizal association with *H. ericae* on their ability to accumulate AsO$_4^{3-}$. 
6.2. Materials and Methods

6.2.1. Plant Material

*C. vulgaris* seeds were collected from Gawton United mine, Devon and Aylesbeare Common, Devon, SW England (section 3.2.1). All seeds were surface sterilised in a 50% bleach solution (4% available Cl) for 5 min. Following sterilisation, seeds were rinsed three times in sterile deionised H$_2$O and transferred to 9 cm diam. Petri dishes containing 25 ml 1.2% H$_2$O agar. Petri dishes were incubated under white light (16 h/8 h day/night cycle, approx 350 mol m$^{-2}$ s$^{-1}$) at 20°C for approximately 21 d or until germination had occurred.

6.2.2. Effect of AsO$_4^{3-}$ on root length

After germination, mine site and heathland seedlings were transferred to Petri dishes containing 1.2% H$_2$O agar supplemented with AsO$_4^{2-}$ at concentrations of 0, 0.01, 0.07, 0.13, 0.67 and 1.33 mol m$^{-3}$. Petri dishes contained five seedlings at each AsO$_4^{2-}$ concentration (n=3). After 21 d incubation at 20°C in the light (16 h/8 h day/night cycle, approx 350 (mol m$^{-2}$ s$^{-1}$), the length of the longest root of each seedling was measured. Mean maximum root lengths of the two populations were compared in order to determine if mine site *C. vulgaris* showed an enhanced AsO$_4^{3-}$ resistance.

6.2.3. AsO$_4^{3-}$ uptake

After germination, seedlings were transferred to 10% Rorison's solution containing (mol m$^{-3}$): Ca, 0.2; N, 1.4; Mg, 0.1; K, 0.2; P, 0.01; Fe, 0.005; Mn, 0.009; B, 0.046; Mo, 0.001; Zn, 0.002; Cu, 0.002 and agar, 12 g l$^{-1}$. Seedlings were incubated for approx. 12 weeks as above.
Both mine site and heathland seedlings were inoculated with an \( \text{AsO}_4^{3-} \) resistant (DGC23) and non-resistant \( H. \text{ericae} \) isolate (AC21) (the \( \text{AsO}_4^{3-} \) resistant and non-resistant genotype was isolated from Gawton United mine site and Aylesbeare Common respectively (Chapter 3)), using the method described by Duclos and Fortin (1983) (section 3.2.3). After 12 weeks incubation at 20\(^\circ\)C, randomly selected root pieces were stained with 0.05% lactophenol blue for 15 min and observed with a Zeiss® Axiolab light microscope. The presence of fungal hyphae coiled within cortical cells (section 1.2) confirmed the plants’ status as mycorrhizal. All plants showed a similar percentage of mycorrhizal colonisation.

To determine \( \text{AsO}_4^{3-} \) accumulation in both mine site and heathland mycorrhizal and non-mycorrhizal \( C. \text{vulgaris} \) populations, the roots of three replicate intact plants from each site were incubated in 100 cm\(^3\) of aerated \( \text{AsO}_4^{3-} \) test solution for 24 h. Test solution contained 10 mol m\(^{-3}\) 2-(N-morpholino)ethanesulfonic acid (MES), 0.5 mol m\(^{-3}\) Ca(NO\(_3\))\(_2\), and 0.75 mol m\(^{-3}\) \( \text{AsO}_4^{3-} \) in the form of Na\(_2\)HAsO\(_4\)·7H\(_2\)O. Using the methodology of Meharg and Macnair (1990), uptake was terminated by rinsing plant roots in 25 ml of an ice-cold solution containing 1 mol m\(^{-3}\) Na\(_2\)HPO\(_4\), 10 mol m\(^{-3}\) MES, and 0.5 mol m\(^{-3}\) Ca(NO\(_3\))\(_2\) before being transferred to 25 ml of an aerated ice-cold solution of the same composition for 10 min. In order to express \( \text{AsO}_4^{3-} \) accumulation on a root surface area basis, plant roots were excised and root length and diameter were measured for six randomly selected plants from each population. Root surface area was calculated and plant tissue was then analysed for As as previously described in section 2.2.5.
6.2.4. PO$_4^{3-}$ uptake

After germination, seedlings were incubated for approx. 12 weeks in 10% Rorisons solution containing agar (12 g l$^{-1}$) (section 6.2.3). After 12 weeks, plants were transferred to Petri dishes containing 1.2% H$_2$O agar for a further 2 weeks, prior to incubation in PO$_4^{3-}$ test solution. Plants were incubated for 20 min and 24 h (except when PO$_4^{3-}$ accumulation over time was to be determined) in PO$_4^{3-}$ test solution containing 10 mol m$^{-3}$ 2-(N-morpholino)ethanesulfonic acid (MES), 0.5 mol m$^{-3}$ Ca(NO$_3$)$_2$, and 0.01 mol m$^{-3}$ PO$_4^{3-}$ in the form Na$_2$HPO$_4$. $^{32}$P (as NaH$_2$PO$_4$, supplied by Amersham) was added to give an activity of 37 kBq ml$^{-1}$ at pH5-5.5. Uptake was terminated as described in section 2.2.5. Roots were excised from the plants exposed to PO$_4^{3-}$ test solution for 20 min, oven dried (24 h, 80°C) and biomass determined gravimetrically. Plants exposed to test solution for 24 h were oven dried as above. $^{32}$P accumulation was determined as outlined in section 5.2.7. Total PO$_4^{3-}$ accumulation was expressed in μmol g. dwt h$^{-1}$.

6.2.5. Statistical Analysis

Data were analysed by ANOVA using the computer package Minitab v. 11 (Minitab, State College, PA, USA). Curve fitting was carried out using the fitting regimes within the computer package Sigma Plot (Jandel Scientific, Erkraft, Germany).

6.3. Results

6.3.1. Effect of AsO$_4^{3-}$ on root length

Regardless of site of origin, root length of *C. vulgaris* decreased in the presence of increasing AsO$_4^{3-}$ (Fig. 6.1). *C. vulgaris* from the mine site was less affected by AsO$_4^{3-}$ than heathland plants, with root length showing no significant decrease at 0.013 mol m$^{-3}$ AsO$_4^{3-}$. In
contrast, root length of *C. vulgaris* from the heathland site was significantly reduced in the presence of the lowest AsO$_4^{3-}$ concentration tested (0.013 mol m$^{-3}$) (P < 0.01) (Fig. 6.1). In the presence of 1.33 mol m$^{-3}$ AsO$_4^{3-}$, *C. vulgaris* from the mine site showed no obvious root growth, in comparison to heathland plants, which demonstrated no obvious root growth at concentrations of AsO$_4^{3-}$ ten fold less (0.13 mol m$^{-3}$), emphasising enhanced AsO$_4^{3-}$ resistance in mine site *C. vulgaris* (Fig. 6.1).

![Figure 6.1](image)

**Figure 6.1** Root length of heathland (●) and mine site (▼) *C. vulgaris* over a range of AsO$_4^{3-}$ concentrations. Each point is the mean of three replicates ± SE.

**6.3.2. AsO$_4^{3-}$ uptake**

Mean root surface areas of *C. vulgaris* seedlings that were both non-mycorrhizal and in mycorrhizal association with *H. ericae* are shown in Fig. 6.2a. Root surface area of mine site *C. vulgaris*, regardless of mycorrhizal association, was greater than the root surface area of heathland *C. vulgaris*, however this difference was not significant (Fig 6.2a).
All *C. vulgaris* plants (regardless of site of origin or mycorrhizal status) absorbed AsO$_4^{3-}$ (Fig. 6.2b). Mine site *C. vulgaris* (regardless of mycorrhizal status) and non-mycorrhizal heathland plants absorbed a similar amount of AsO$_4^{3-}$ (Fig. 6.2b). Heathland *C. vulgaris* inoculated with non-resistant *H. ericae* showed an increase in AsO$_4^{3-}$ accumulation two fold greater (P < 0.01) than the non-mycorrhizal heathland plants and all mine plants (Fig 6.2b).

Arsenate accumulation was reduced in heathland plants inoculated with AsO$_4^{3-}$ resistant *H. ericae* (Fig. 6.2b).

![Figure 6.2](image.png)  
*Figure 6.2* AsO$_4^{3-}$ accumulation in mine (■) and heathland (■) *C. vulgaris*. a represents surface area and b represents AsO$_4^{3-}$ accumulation of *C. vulgaris* in non-mycorrhizal association (-), association with AsO$_4^{3-}$ resistant *H. ericae* (-) and non AsO$_4^{3-}$ resistant *H. ericae* (-). Bars represent SE of the mean (n = 6).
6.3.3. PO$_4^{3-}$ uptake

Phosphate uptake by both mine site and heathland *C. vulgaris* plants was linear over 20 min (Fig. 6.3). *C. vulgaris* from both the mine site and the heathland site absorbed similar amounts of PO$_4^{3-}$ (Table 6.1). After 20 min incubation in 0.01 mol m$^{-3}$ PO$_4^{3-}$, PO$_4^{3-}$ accumulation in root tissue was greater than the accumulation in shoot tissue of all plants (regardless of site of origin) (Table 6.1). After 24 h incubation, total PO$_4^{3-}$ uptake of all plants was similar (Table 6.1). There was no significant difference in PO$_4^{3-}$ absorption in *C. vulgaris*, regardless of site of origin, after either short or long term PO$_4^{3-}$ exposure (Table 6.1).

![Graph showing phosphate accumulation over time](image)

**Figure 6.3** Phosphate accumulation in heathland (○) and mine site (▼) *C. vulgaris* over time. Each point is the mean of three replicates ± SE.
Table 6.1 Phosphate accumulation in *C. vulgaris* after exposure to 0.01 mol m\(^{-3}\) PO\(_4^{3-}\). Data represent total PO\(_4^{3-}\) uptake ± SE of the mean.

<table>
<thead>
<tr>
<th>Time</th>
<th>Plant tissue</th>
<th><em>C. vulgaris</em> origin</th>
<th>Total PO(_4^{3-}) uptake (μmol g d wt h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>Root tissue</td>
<td>Gawton United</td>
<td>15.2 ± 4.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heathland</td>
<td>11.74 ± 1.55</td>
</tr>
<tr>
<td>20 min</td>
<td>Shoot tissue</td>
<td>Gawton United</td>
<td>3.65 ± 1.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heathland</td>
<td>5.74 ± 1.91</td>
</tr>
<tr>
<td>24 h</td>
<td>Total plant</td>
<td>Gawton United</td>
<td>7.29 ± 4.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heathland</td>
<td>8.51 ± 3.25</td>
</tr>
</tbody>
</table>

**6.4. Discussion**

The present chapter demonstrated the negative effect of increasing AsO\(_4^{3-}\) concentrations on growth of *C. vulgaris* from As contaminated mine site spoil soils and natural heathland. By comparing the maximum root length of *C. vulgaris* from each site in the presence of increasing AsO\(_4^{3-}\) concentrations, it was apparent that the Gawton United mine site population showed an increased AsO\(_4^{3-}\) resistance. Root length of *C. vulgaris* from the Gawton United mine site was decreased by the presence of 0.13 mol m\(^{-3}\) AsO\(_4^{3-}\), however there was no obvious root growth by heathland populations at this concentration (Fig. 6.1).

Soil colonised by *C. vulgaris* at the Gawton United mine site contains approximately 75 times the level of As found in natural heathland (including Aylesbeare Common) (section 4.3.3), and previous studies have isolated AsO\(_4^{3-}\) resistant grasses from this site (Porter & Peterson, 1975, 1977; Meharg & Macnair, 1990, 1991a, 1992a). In the presence of 0.13 mol m\(^{-3}\), AsO\(_4^{3-}\) resistant *H. lanatus* demonstrated a root growth approximately six fold higher than non-resistant grass (Meharg *et al.*, 1992b). Arsenate tolerance in *H. lanatus* was shown to be due, at least partly, to suppression of the PO\(_4^{3-}\) uptake system (Meharg & Macnair, 1990, 1991b, 1992a, 1994) (Chapter 5). Non-tolerant *H. lanatus* accumulated twice the amount of AsO\(_4^{3-}\) in
comparison to tolerant genotypes after exposure to an AsO$_4^{3-}$ solution (Meharg & Macnair, 1991a). Uptake of PO$_4^{3-}$ was also suppressed in AsO$_4^{3-}$-tolerant genotypes (Meharg & Macnair, 1992a). The PO$_4^{3-}$ uptake system in *H. lanatus* accumulates PO$_4^{3-}$ much more efficiently than AsO$_4^{3-}$, which may explain how tolerant plants can survive high levels of AsO$_4^{3-}$ in soil solution and also how they adapt to growth on contaminated sites high in AsO$_4^{3-}$ and low in PO$_4^{3-}$ (Meharg *et al*., 1994).

The present chapter demonstrated similar rates of AsO$_4^{3-}$ uptake in the two *C. vulgaris* populations. The rate of AsO$_4^{3-}$ uptake in intact non-mycorrhizal *C. vulgaris* from the mine site and heathland site was similar (Fig. 6.2b). The rate of PO$_4^{3-}$ uptake in the two populations was also similar. There was no significant difference in PO$_4^{3-}$ accumulation in root or shoot tissue of either mine or heathland plants after short term (20 min) exposure to PO$_4^{3-}$. Total PO$_4^{3-}$ uptake after 24 h was similar in all *C. vulgaris* seedlings, regardless of site of origin (Table 6.1). These results contrast to the previous studies involving *H. lanatus* (Meharg & Macnair, 1991a) which clearly demonstrate reduced AsO$_4^{3-}$ and PO$_4^{3-}$ uptake in AsO$_4^{3-}$-resistant genotypes. The results of the present study indicated that AsO$_4^{3-}$ resistance in *C. vulgaris* from the mine site is not achieved by a suppression of the PO$_4^{3-}$ uptake system, and an alternative mechanism of AsO$_4^{3-}$ resistance may be utilised.

At the Gawton United mine site, *C. vulgaris* is found in mycorrhizal association with the ericoid ascomycete fungus *H. ericae* (Chapter 3), which is also resistant to high AsO$_4^{3-}$ concentrations (Chapter 4). However, *C. vulgaris* from the mine site was not as resistant to AsO$_4^{3-}$ as its fungal endophyte. At concentrations of 1.33 mol m$^{-3}$ AsO$_4^{3-}$, mine site *C. vulgaris* showed no obvious sign of root growth, while growth of *H. ericae* from the same mine site sustained growth at AsO$_4^{3-}$ concentrations ten fold higher (Fig. 5.1). Arsenate resistance in mine
site *H. ericae* is due to an enhanced AsO$_4^{3-}$ efflux mechanism, with no evidence of a suppressed PO$_4^{3-}$ uptake system (Chapter 5). Bradley *et al.* (1981, 1982) demonstrated the ability of mycorrhizal endophytes of *C. vulgaris* to decrease the sensitivity of the host plant to both Cu and Zn and the results of the AsO$_4^{3-}$ uptake experiment in the present chapter (Fig. 6.2), may indicate the ability of *H. ericae* to minimise AsO$_4^{3-}$ accumulation by *C. vulgaris*.

In this chapter, both mine site and heathland plants inoculated with non-resistant *H. ericae* showed an increase in AsO$_4^{3-}$ accumulation in comparison to non-mycorrhizal *C. vulgaris* (Fig. 6.2b). This increase was expected as mycorrhizal association increases the contact area with AsO$_4^{3-}$ due to the presence of fungal hyphae, which increase the plants surface area to mass ratio. However, in comparison to ectomycorrhizal associations, the affect of ericoid associations on plant surface area is minimal (Read, 1996). Heathland *C. vulgaris* in association with AsO$_4^{3-}$-resistant *H. ericae* showed half the AsO$_4^{3-}$ accumulation as non-mycorrhizal heathland *C. vulgaris* (Fig. 6.2b). This clearly indicates the ability of AsO$_4^{3-}$-resistant mycorrhizal endophytes to increase AsO$_4^{3-}$ resistance to the host plant. This trend however, was not observed in mine plants in association with AsO$_4^{3-}$-resistant *H. ericae*, which may provide further evidence that mine site plants have also evolved AsO$_4^{3-}$ resistance.

The isolation of an AsO$_4^{3-}$-resistant *H. ericae* population from the mine site (Chapter 4), and the data presented in this chapter, which shows that *C. vulgaris* from the mine site also exhibits AsO$_4^{3-}$ resistance, indicate that both host and symbiont have adapted to AsO$_4^{3-}$ contamination in parallel. We postulate that selection pressures in the mine site environment (ie the presence of high levels of As), have caused both plant and fungi to evolve mechanisms of AsO$_4^{3-}$ resistance, which has therefore resulted in their dominance on contaminated mine soils. However, further studies at the genetic level are required to investigate this hypothesis.
Chapter 7

Discussion

7.1. General Discussion

Certain higher plant taxa have adapted to polluted soils via micro-evolutionary processes (Macnair, 1993) and are generally symbiotic with mycorrhizal fungi on contaminated sites (Meharg & Cairney, 2000). Whether these fungi benefit their host plants on contaminated sites by simply fulfilling their normal ecological functions (Smith & Read, 1997) or confer enhanced resistance upon the plant, has not been resolved. Adaptation to AsO$_4^{3-}$ contaminated soils poses a particular challenge to mycorrhizal associations. Mycorrhizal fungi enhance plant PO$_4^{3-}$ uptake (Straker & Mitchell, 1987) but as AsO$_4^{3-}$ is a PO$_4^{3-}$ analogue for plant and fungal PO$_4^{3-}$ transporters (Lee, 1982) they will also enhance AsO$_4^{3-}$ uptake. The ericoid mycorrhizal fungus *Hymenoscyphus ericae* utilises organic forms of PO$_4^{3-}$ in soils by converting them to inorganic orthophosphates, which are then absorbed by the host plant (Straker & Mitchell, 1987). The mycorrhizal associations must therefore evolve strategies to obtain PO$_4^{3-}$ at the exclusion of AsO$_4^{3-}$.

The present study isolated populations of *H. ericae* from roots of *Calluna vulgaris* growing on two As/Cu mine spoils and an uncontaminated heathland site (Chapter 3). Extractable As levels at the mine sites were 20-75 times greater than the heathland site, which was uncontaminated by mining and other industrial or agricultural processes (section 4.3.3). Growth of mine and heathland populations of *C. vulgaris* and *H. ericae* in media supplemented with AsO$_4^{3-}$ (the dominant As species in soil solution) revealed that plant (section 6.3.1) and fungal populations (section 4.3.1) from the mine had considerably increased resistance to
AsO$_4^{3-}$ compared to the heathland populations. Mine site *H. ericae* demonstrated an AsO$_4^{3-}$ EC$_{50}$ value approximately 40 times higher than heathland *H. ericae* (section 4.3.1) and six-26 times higher than the values reported for individual *H. ericae* and *Hebeloma crustuliniforme* isolates utilised in the preliminary investigation (section 2.3.2). The Devon Great Consols population was more resistant to AsO$_4^{3-}$ than the Gawton United population, however, this did not correlate to the level of extractable soil As present at these sites. Soil from Gawton United mine site showed the highest amount of extractable AsO$_4^{3-}$; however, As availability in soil is dependent upon soil pH, organic matter content and the presence of other minerals, therefore the mixed acid extraction used in this study may not reflect the amount of AsO$_4^{3-}$ available at these sites. The majority of investigations into metal sensitivity in fungi have relied upon single or limited number of isolates from areas of either metal contaminated or uncontaminated soils (Meharg & Cairney, 2000). Limited interpretations can be placed on studies that have not been conducted at the population level, as the response of single isolates may not be indicative of the population response to metal contamination. The present study, which utilised seventy six *H. ericae* isolates (Chapter 4) conclusively demonstrated enhanced AsO$_4^{3-}$ resistance in mine site populations of *H. ericae*.

*C. vulgaris* from Gawton United mine site showed an increased resistance to AsO$_4^{3-}$ in comparison to heathland plants (section 6.3.1). However, plants were much more affected by the presence of AsO$_4^{3-}$ than their fungal endophytes. For example, *C. vulgaris* from the Gawton United mine site was unable to maintain growth at AsO$_4^{3-}$ concentrations of 1.33 mol m$^{-3}$ (Fig. 6.1) in comparison to *H. ericae* from the same site, which showed growth at AsO$_4^{3-}$ concentrations 25 fold higher (33.25 mol m$^{-3}$) (Fig. 4.2b). Both partners on the mine spoil have thus evolved resistance to AsO$_4^{3-}$. 
It has previously been reported that \( \text{PO}_4^{3-} \) may exhibit an ameliorating effect on \( \text{AsO}_4^{3-} \) sensitivity. In the preliminary investigation (Chapter 2), increasing \( \text{PO}_4^{3-} \) concentrations up to 1.0 mol m\(^{-3}\) enhanced growth of two \( H. \text{ ericae} \) isolates and one isolate of \( H. \text{ crustuliniforme} \), however at \( \text{PO}_4^{3-} \) concentrations above 1.0 mol m\(^{-3}\) no further biomass increase was demonstrated (Fig. 2.2). A similar trend has been shown in other fungi (Beever & Burns, 1980). When the combined effects of \( \text{AsO}_4^{3-}/\text{PO}_4^{3-} \) and \( \text{AsO}_2^{3-}/\text{PO}_4^{3-} \) were studied it was found that, at the highest \( \text{PO}_4^{3-} \) concentration (1.0 mol m\(^{-3}\)), growth in the presence of \( \text{AsO}_4^{3-} \) and \( \text{AsO}_2^{3-} \) by all isolates was similar to the growth produced in the absence of \( \text{AsO}_4^{3-} \) (Fig. 2.5). Since \( \text{PO}_4^{3-} \) stops increasing growth at \( \text{PO}_4^{3-} \) concentrations of 1.0 mol m\(^{-3}\) (Fig. 2.2), it was suggested that the observed increase in growth may be due to amelioration of \( \text{AsO}_4^{3-} \) and \( \text{AsO}_2^{3-} \) by \( \text{PO}_4^{3-} \), as has earlier been suggested in higher plants (Meharg & Macnair, 1992b; Asher & Reay, 1979; Lee, 1982). The ameliorative effects of \( \text{PO}_4^{3-} \) on \( \text{AsO}_4^{3-} \) toxicity in \( H. \text{ ericae} \) may be due to competition for uptake into the fungal hyphae. This amelioration effect may have important nutritional and ecological implications for the successful growth of plants and fungi on soils contaminated with \( \text{AsO}_4^{3-} \).

Short term \( \text{AsO}_4^{3-} \) and \( \text{PO}_4^{3-} \) uptake kinetics were identical for mine and heathland \( H. \text{ ericae} \) (section 5.3.2). Mine site and heathland \( K_m \) values were higher than those reported in single isolates of \( H. \text{ ericae} \) and \( H. \text{ crustuliniforme} \), while \( V_{\text{max}} \) values were lower (section 2.3.4). Arsenate uptake was rapidly repressed on exposure to \( \text{PO}_4^{3-} \) and similarly, \( \text{PO}_4^{3-} \) uptake was rapidly repressed by exposure to \( \text{AsO}_4^{3-} \). Changes in the rate of uptake may be under allosteric control (Lefebvre & Glass, 1982; Schorring & Jensen, 1984) or by the synthesis and breakdown of transport sites (Jeanjean, 1973; Drew et al., 1984). Since uptake of both \( \text{AsO}_4^{3-} \) and \( \text{PO}_4^{3-} \) was rapidly repressed in the presence of \( \text{PO}_4^{3-} \) and \( \text{AsO}_4^{3-} \) respectively, it is likely that for \( H. \text{ ericae} \), these changes are under allosteric control. Efflux kinetics of As species revealed
enhanced AsO$_2^-$ efflux as the mechanism of AsO$_4^{3-}$ resistance in mine site _H. ericae_ (section 5.3.4). Arsenite efflux has been reported as a mechanism of AsO$_4^{3-}$ resistance in the bacterium _Staphylococcus aureus_, whereby AsO$_4^{3-}$ is reduced to AsO$_2^-$ intracellularly, before being exported from the cells (Broer _et al._, 1993). _H. ericae_ has independently evolved an identical strategy. This strategy enables the fungus to retain its ability to transport PO$_4^{3-}$ from the soil, while effluxing absorbed AsO$_4^{3-}$. Hence the ability to supply PO$_4^{3-}$ to _C. vulgaris_ is not compromised. Mine site _H. ericae_ demonstrate much greater toxicity to AsO$_4^{3-}$ than AsO$_2^-$ (section 5.3.1) and are much less efficient at absorbing AsO$_2^-$ (section 5.3.6). This ensures that reabsorption of AsO$_4^{3-}$ from the soil is limited.

Arsenate resistance in _C. vulgaris_ is not achieved by exclusion. Non-mycorrhizal mine site and heathland plants demonstrated similar rates of both AsO$_4^{3-}$ uptake and PO$_4^{3-}$ uptake (section 6.3.2 and 6.3.3). Down-regulation of AsO$_4^{3-}$/PO$_4^{3-}$ transporters has been identified as a mechanism of AsO$_4^{3-}$ resistance in higher plants (Meharg & Macnair, 1992a). Meharg & Macnair (1992a) demonstrated that the high affinity PO$_4^{3-}$ uptake system in AsO$_4^{3-}$ resistant _H. lanatus_ shows a much higher affinity for PO$_4^{3-}$ than AsO$_4^{3-}$, which explains the ability of tolerant plants to adapt to growth on contaminated sites, high in AsO$_4^{3-}$ and low in PO$_4^{3-}$. In contrast, _C. vulgaris_ from the mine site showed no down regulation/suppression of the high affinity PO$_4^{3-}$ uptake system. Instead, the fungal endophyte dominates AsO$_4^{3-}$/PO$_4^{2-}$ accumulation and may act as a filter to maintain low plant AsO$_4^{3-}$ levels through AsO$_2^-$ efflux while enhancing plant PO$_4^{3-}$ status. Mine site _H. ericae_ maintained low levels of plant AsO$_4^{2-}$ by effluxing AsO$_2^-$ from its hyphae. When exposed to AsO$_4^{3-}$ for 24h, heathland _C. vulgaris_ inoculated with non-resistant heathland _H. ericae_ accumulated 100% more AsO$_4^{3-}$ than non mycorrhizal heathland plants. In contrast, AsO$_4^{3-}$ accumulation in heathland plants inoculated with resistant mine site _H. ericae_ was significantly reduced (section 6.3.2). In addition,
infection of mine site *C. vulgaris* with AsO$_4^{3-}$-resistant *H. ericae* did not place the host under additional AsO$_4^{3-}$ stress, further emphasising the evolution of AsO$_4^{3-}$ resistance in mine site populations of *H. ericae*. In stress conditions (such as pollution by AsO$_4^{3-}$), all but the most resistant genotypes in the population are eliminated. The survivors interbreed and among their progeny are more resistant genotypes, which are then further selected for (Ashmore, 1997). It is possible therefore, that selection has occurred amongst the fungal population and has resulted in the evolution of AsO$_4^{3-}$-resistant *H. ericae* populations.

In contrast to the evolution of AsO$_4^{3-}$ resistance, both mine site and heathland *H. ericae* demonstrated constitutive resistance to Cu. Regardless of the expected Cu contamination in mine spoil soils at both Gawton United and Devon Great Consols mine sites, *H. ericae* from these sites showed no increased resistance to Cu in comparison to the heathland population (section 4.3.2). Bradley *et al.* (1981, 1982) also reported constitutive Cu resistance in unidentified fungal endophytes of *C. vulgaris* and clearly demonstrated the ability of these endophytes to confer increased resistance to their host. Growth of non-mycorrhizal *C. vulgaris* was almost completely inhibited at Cu concentrations of 0.15 mol m$^{-3}$, however, in mycorrhizal association, plants continued to grow at concentrations of 1.13 mol m$^{-3}$ (Bradley *et al.*, 1982). Ericoid mycorrhizal associations have also been shown to confer constitutive Zn, Al, Ni and Fe resistance to their host plant (Bradley *et al.*, 1982; Freedman & Hutchinson, 1980; Marrs & Bannister, 1978).

The evolution of AsO$_4^{3-}$ resistance in mine site populations of *H. ericae*, may suggest that in these environments, the presence of AsO$_4^{3-}$ in the soil limits the number of fungal taxa colonising these environments. While *H. ericae* is the dominant endophyte on roots of *C. vulgaris* at the two mine sites and heathland site, there is also evidence of other fungal taxa
(section 3.3). The diversity of endophytes from the Devon Great Consols mine site was reduced in comparison to diversity from Gawton United mine site and the heathland population (Table 3.2) and no identical endophytes, with the exception of \textit{H. ericae}, were isolated from all three sites (section 3.3). Given that the Devon Great Consols \textit{H. ericae} population exhibited the greatest resistance to \(\text{AsO}_4^{3-}\), these results seem to indicate that soil As levels is the strongest selection pressure at this site. While the levels of extractable soil As do not appear to support this hypothesis (section 4.3.3), it is interesting to note that the vegetative species richness of Devon Great Consols mine site was reduced in comparison to the Gawton United mine site (section 3.2.1). \textit{H. ericae} is often cited as a ubiquitous endophyte of Ericaceae (eg. Leake & Read, 1991), however recent studies do not support this statement (Perotto \textit{et al}., 1996; Xiao & Berch, 1996). There was no evidence of \textit{H. ericae} on root systems of \textit{C. vulgaris} from Italian heathlands or root systems of \textit{Gaultheria shallon} in Canadian forests (Perotto \textit{et al}., 1996; Xiao & Berch, 1996). The present study demonstrates the important role of \textit{H. ericae} in \(\text{AsO}_4^{3-}\) resistance on the mine sites, however, the functional significance of the other \textit{C. vulgaris} endophytes is currently unknown.

In conclusion, the results presented in this study clearly demonstrate that \textit{H. ericae} and its host plant, \textit{C. vulgaris}, have evolved in parallel to \(\text{AsO}_4^{3-}\) contamination on the mine sites. The fungus has the ability to efflux absorbed As and confer enhanced \(\text{AsO}_4^{3-}\) resistance to \textit{C. vulgaris}. The data further demonstrates that evolution of host and symbiont is fundamental to colonisation of polluted soils by key plant taxa.
7.2. Further Investigations

The present investigation provided evidence of the evolution of AsO$_4^{3-}$ resistance in mine site populations of *H. ericae* based on physiological experiments. While physiological experiments are necessary and important in determining population responses to metals, an investigation at the genomic level would broaden our current knowledge and understanding of this area. Genetic evidence exists which confirms the physiological investigations demonstrating the evolution of AsO$_4^{3-}$ resistance in higher and lower plants (Macnair *et al.*, 1992). Arsenate resistance in *Holcus lanatus* and *Agrostis capillaris* has been shown to be under major gene control, with the gene encoding for AsO$_4^{3-}$ exclusion (Macnair *et al.*, 1992; Watkins & Macnair, 1991). In bacteria, AsO$_4^{3-}$ resistance is coded for by the *ars* genes (Silver & Phung, 1996). The gene/gene coding for AsO$_4^{3-}$ resistance in both mine site *H. ericae* populations and *C. vulgaris* is currently unknown and investigations at the genomic level are therefore desired. The identification of these genes may therefore lead to further experiments investigating the co-evolution of AsO$_4^{3-}$ resistance in this host/symbiont relationship.

The diversity of fungal endophytes colonising the roots of *C. vulgaris* at all three field sites in the present study may also provide the basis for further investigations. While the role of *H. ericae* in AsO$_4^{3-}$ resistance on mine sites has been well established, the functional significance of other fungal taxa present on the root systems of *C. vulgaris* is unknown. Is the function of these endophytes to increase nutrient availability to their host and if so, why is a diverse fungal taxon necessary? Or is it more likely that each of the fungal taxon provide a unique and specific function to their host? Further investigations are required to answer these
important questions and increase our knowledge of the ecological significance of specific host/symbiont associations.
7.3. Publications

Publications in refereed academic journals


Publications in preparation


Conference presentations


**Oral presentations**


Chapter 8

References


