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Utilisation of organic nitrogen and phosphorus sources by mycorrhizal endophytes of *Woollsia pungens* (Cav.) F. Muell. (Epacridaceae)

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Abstract The ability of four ericoid mycorrhizal endophytes isolated from roots of *Woollsia pungens* (Cav.) F. Muell. (Epacridaceae) to utilise organic forms of nitrogen and phosphorus during growth in axenic culture was assessed. All isolates were able to utilise glutamine, arginine and bovine serum albumin (BSA), along with NH_4^+ or NO_3^-, in most cases yielding at least as much biomass as the ericoid mycorrhizal endophyte *Hymenoscyphus ericae* (Read) Korf & Kernan. All isolated endophytes were able to utilise BSA, arginine and glutamine as sole sources of N and C. With the exception of a single isolate (C40), which showed little growth on glutamine, biomass yields on glutamine as the sole N and C source was significantly greater for all isolates than on either of the other two organic N sources. Two isolates from *W. pungens* (C40 and A43) utilised DNA and sodium inositol hexaphosphate as sole P sources, in each case yielding significantly more biomass than *H. ericae*. The results suggest that mycorrhizal endophytes from epacrid plant hosts and those from ericaceous hosts have similar abilities to utilise organic forms of N and P.

Key words Ericaceae · *Hymenoscyphus ericae* · Ericoid mycorrhiza · Plant nutrition · Ascomycetes

Introduction

The Epacridaceae take the place in the Australian flora of the Ericaceae in the northern hemisphere, being found largely on soils of poor nutrient status (McLennan 1935; Read 1996). As with Ericaceae, Epacridaceae is considered to be a mycorrhiza-forming family, with most plants exhibiting mycorrhizal infection with morphological similarity to ericoid mycorrhizas (Mclean and Lawrie 1996; Steinke et al. 1996). Epidermal cells of hair roots of epacrid plants are thus frequently occupied by mycelial coils of fungal endophytes which produce small amounts of extramatrical mycelium around the root surface (e.g. Allen et al. 1989; Mclean and Lawrie 1996; Steinke et al. 1996).

A number of demonstrated and putative endophytes have been isolated from roots of the Epacridaceae. Although not yet taxonomically defined, they are generally described as slow-growing, dark, septate, sterile mycelia, presumed to be ascomycetes (Hutton et al. 1994, 1996; Steinke et al. 1996; Liu et al. 1998). Such characteristics are reminiscent of those displayed by many endophytes isolated from the Ericaceae (e.g. Perotto et al. 1990, 1996), and there is convincing evidence that endophytes from the Ericaceae and Epacridaceae can infect hosts in both families (Reed 1989; Read 1996; Liu et al. 1998). Despite this, pectic isozyme analyses have been unable to identify sterile endophytes from Western Australian epacrids as known ericoid endophyte taxa (Hutton et al. 1994, 1996) and the taxonomic relatedness of endophytes from different families within the Ericales remains to be clarified.

Ericoid plant species occur largely on soils characterised by low rates of mineralisation and organic matter decomposition, leaving key mineral nutrients in largely organic forms (Read 1991). It is now clear that the major benefit conferred upon the host by ericoid mycorrhizal symbiosis is access to organic forms of (largely) N and (to a lesser extent) P that would otherwise be unavailable. Thus endophytes from the Ericaceae produce extracellular carboxyl proteinases (e.g. Bajwa et al. 1985; Leake and Read 1989), phosphomonoesterases (e.g. Straker and Mitchell 1986; Shaw and Read 1989) and phosphodiesterases (Leake and Miles 1996; Myers and Leake 1996) capable of mobilising N and P, respectively, from organic sources. Some endophytes of the Ericaceae can produce ligninolytic (Haselwandter et al. 1990), cellulolytic (Burke and Cairney

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1997), hemicellulolytic (Burke and Cairney 1997), pectinolytic (Perotto et al. 1997) and chitinolytic (Kerley and Read, 1997) activities that may effect access to mineral nutrients sequestered within moribund plant and fungal tissue. Furthermore, some endophytes can access organic mineral nutrient sources complexed with polyphenolic compounds via production of phenol-oxidising enzymes (Bending and Read 1996a, b).

The functional significance of ericoid mycorrhizal endophytes of the Epacridaceae is far less clear. While epacrid species occur in a range of habitats, including wet mires and dry sand plains (Read 1996), they are typically associated with mineral-nutrient poor soils (Bell and Pate 1996). Nutrient enrichment studies indicate that the growth of some Epacridaceae from Western Australian heathland sites is limited primarily by N rather than P availability (Bell et al. 1994). Thus it has been suggested that mycorrhizal infection, as with the Ericaceae, may benefit the host primarily by mediating access to the soil organic N pool (Bell and Pate 1996; Read 1996). Indeed in some subtropical heathlands colonised by epacridaceous species, soluble proteins and amino acids constitute the major N fractions and δ^{15}N-enrichment studies support the utilisation of soil organic N sources by ericoid mycorrhizal associations in the Epacridaceae (Schmidt and Stewart 1997). To date, however, there have been no investigations published of interactions between endophytes from the Epacridaceae and organic nutrient sources. As a first step towards characterising the potential for accessing such material, we report here the results of an investigation of the ability of endophytes isolated from *Woollsia pungens* (Cav.) F. Muell. (Epacridaceae) to utilise organic sources of N and P during growth in axenic culture.

Materials and methods

The fungal endophytes used in this study were four sterile, unidentified isolates (D01, D02, C40 and A43) from *W. pungens* confirmed as mycorrhizal endophytes (Liu et al. 1998), along with a single isolate of *Hymenoscyphus ericae* (Read) Korf & Kernan (DJ Read 100). All isolates were maintained on 1/3-strength malt extract agar (MEA) medium (Oxoid). For growth experiments, two plugs of fungus (6 mm diameter) were cut from the leading edge of colonies growing on MEA and inoculated into 9-cm Petri dishes containing 25 ml liquid medium.

The influence of N source on fungal yield was determined by growing the fungi in a basal medium containing either $(NH_4)_2HPO_4$, $Ca(NO_3)_2$, glutamine, arginine or bovine serum albumin (BSA) to give a starting N concentration of 7.6 mM. The basal medium was as described by Sharples and Cairney (1997), containing (per litre): KH_2PO_4 300 mg; $MgSO_4.7H_2O$ 140 mg; $CaCl_2$ 50 mg; $ZnSO_4$ 3.0 mg; ferric EDTA 12.6 mg; thiamine 100 mg. Glucose was added to each treatment to give a standard C:N ratio of 39 : 1. The basal medium was adjusted to pH 5.5 prior to autoclaving, with organic N sources (pH 5.5) added by filter sterilisation into autoclaved media as described by Finlay et al. (1992). A treatment containing no N and the maximal amount of glucose added to the N treatments (10.34 g l^{-1}) was also included to determine the growth in the presence of the small amount of N in the inoculum plugs.

To determine the growth on different P sources, fungi were grown in a basal medium containing either $NaHPO_4.2H_2O$, sodium inositol hexaphosphate or salmon sperm DNA (Sigma) to give a starting P concentration of 5.0 mM. The basal medium was as described by Leake and Miles (1996), containing (per litre): $(NH_4)_2SO_4$ 1.15 g; KCl 228 mg; $MgSO_4$ 45 mg; ferric citrate 10 mg; $MnSO_4$ 5.0 mg; $ZnSO_4$ 4.0 mg; glucose 5.0 g. The basal medium was adjusted to pH 4.5 prior to autoclaving and after sodium inositol hexaphosphate was added by filter sterilisation to the autoclaved medium the pH of the medium increased to 7.8. DNA was added after autoclaving following the procedure outlined in Leake and Miles (1996), without significantly altering the pH of the basal medium. A treatment containing no P was also included as outlined above.

For both N and P experiments, all treatments were replicated five times and cultures incubated at 20 °C in the dark. After 15 days, cultures were harvested by filtration, dried overnight at 80 °C and the biomass determined gravimetrically. Data from each experiment were analysed by one-way ANOVA and significant differences between yields determined by Fisher's PLSD test using Statview© software (Abacus Concepts).

Results and Discussion

All four endophytes from *W. pungens* and *H. ericae* utilised all inorganic and organic N sources for growth (Fig. 1). While the yield of *H. ericae* did not significantly differ on either N source, there were slight, in some cases significant, differences in the N preferences of the *W. pungens* endophytes, with isolates showing a slight preference for particular N sources (Fig. 1). Given that only single isolates were investigated and that their taxonomic status remains unclear (Liu et al. 1998), it seems unwise to over interpret such data at present. With the exception of isolate D01 on arginine, however, all *W. pungens* isolates utilised all N sources at least as well as *H. ericae*, indicating an ability to utilise simple organic N compounds on a par with the ericoid endophyte. Our data are thus in accord with those of Bajwa et al. (1985) and Bajwa and Read (1986), who previously showed, that *H. ericae* is able to utilise a range of amino compounds (including BSA, glutamine and arginine) and NH_4^+ equally well. In the case of BSA utilisation, *H. ericae* is known to produce extracellular proteinase activity which mediates protein hydrolysis, the fungus absorbing the resulting amino acids and effecting their transfer to the host (Read et al. 1989). *H. ericae* has further been shown to enhance host plant growth and N content in the presence of proteins and smaller amino compounds (Bajwa et al. 1985; Bajwa and Read 1986). Although further experiments are required for confirmation, data from the present study suggest that ericoid endophytes of the Epacridaceae have the potential to enhance host N status in a similar manner.

Isolates D01, D02 and A43 utilised the organic N compounds as sole N and C sources as well, or significantly better than, *H. ericae* (Fig. 2), suggesting that they are to some extent able to supplement the C supply from the host with C from organic N compounds. For each fungus, biomass yield on glutamine was significantly greater than on either BSA or arginine. Short-

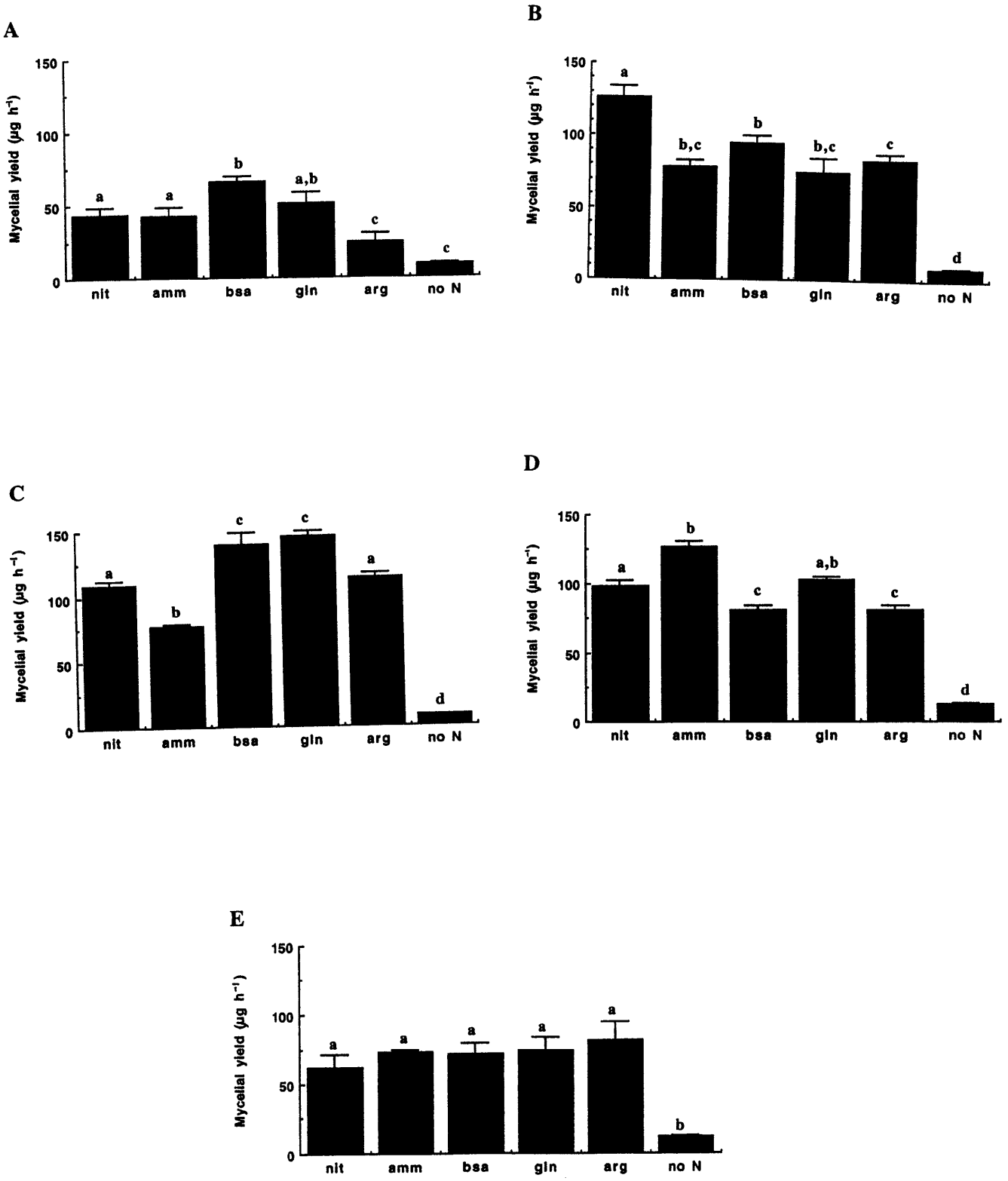

Fig. 1 Mean dry weight yields of endophytes isolated from *Woollsia pungens* (**A** = D01, **B** = DO2, **C** = C40, **D** = A43) and *Hymenoscyphus ericae* (**E**) on a range of nitrogen sources: nitrate (*nit*), ammonium (*amm*), bovine serum albumin (*bsa*), glutamine (*gln*), arginine (*arg*), no added nitrogen (*no N*). *Bars* represent standard errors and different *letters* above the error bars indicate significant differences ($P < 0.001$) between yields (as determined by Fisher PLSD)

chain amino acids, such as glutamine and arginine, are readily assimilated by *H. ericae* (Bajwa and Read 1986). The rapid assimilation of glutamine in the endophytes from *W. pungens* and *H. ericae* may result from direct absorption and incorporation into the glutamate synthase pathway, as was recently demonstrated for the ec-

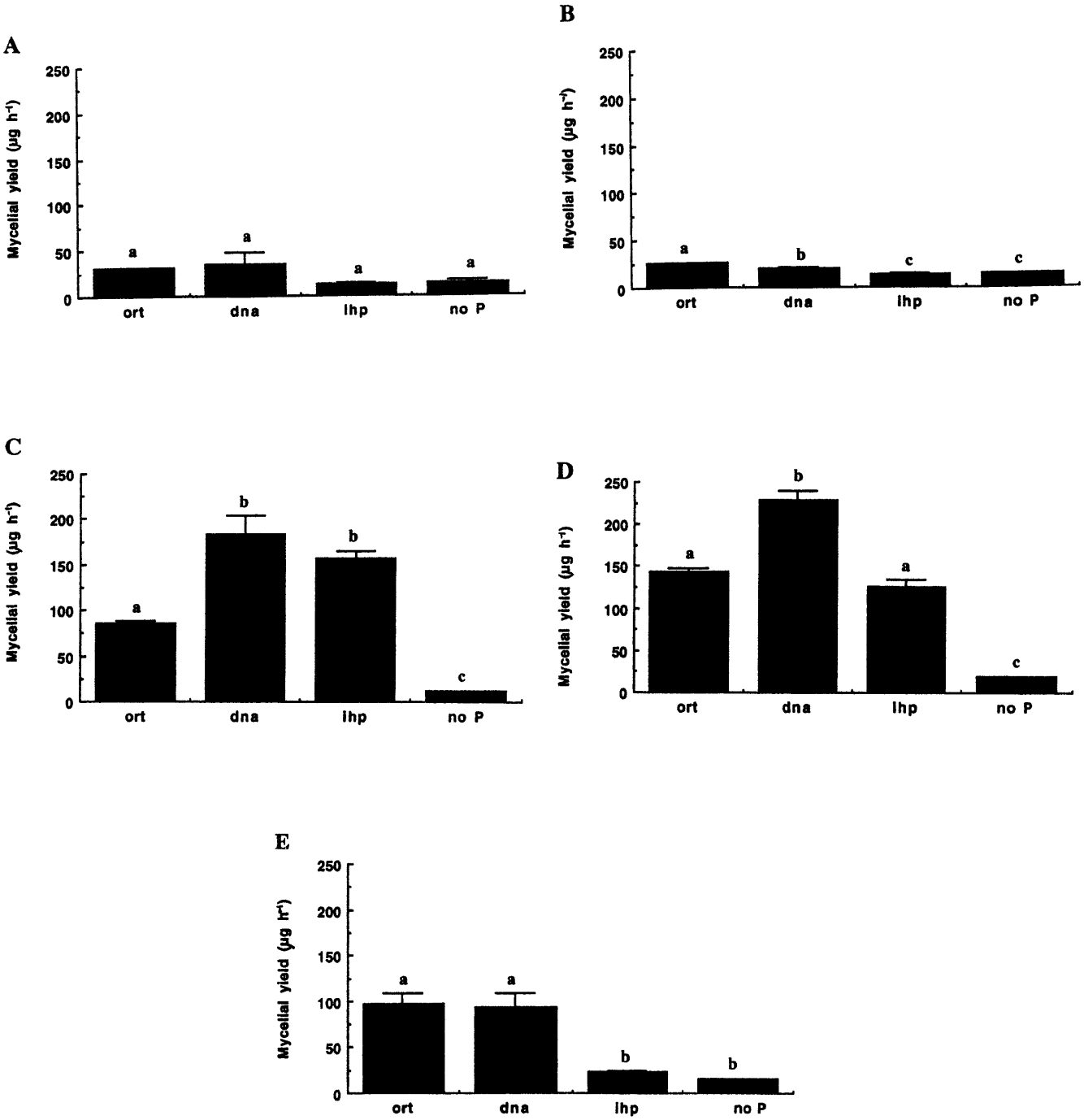

Fig. 2 Mean dry weight yields of endophytes isolated from *W. pungens* (**A** = D01, **B** = DO2, **C** = C40, **D** = A43) and *H. ericae* (**E**) on a range of organic nitrogen sources as sole sources of carbon and nitrogen: bovine serum albumin (*bsa*), glutamine (*gln*), arginine (*arg*). *Bars* represent standard errors and different *letters* above the error bars indicate significant differences ($P < 0.001$) between yields (as determined by Fisher PLSD)

tomycorrhizal basidiomycete *Paxillus involutus* (Chalot et al. 1994). The pattern of utilisation for C40 was somewhat different, with BSA producing the greatest yield and only very slight growth observed in the presence of glutamine (Fig. 2). Isolate C40 utilised glutamine-N effectively in the presence of exogenous glucose (Fig. 1), suggesting a limited ability to utilise the glutamine carbon skeleton rather than N relative to the other isolates. It is interesting to note in this context the findings of Bell and Pate (1996) that glutamine is the principal compound transported in xylem of mycorrhizal Epacridaceae plants. They suggested that transport of nitrogen from the endophyte to the host is accomplished via NH_4^+, and that glutamine in the host is formed by its own metabolism.

Isolates D01 and D02 showed no significant growth on either of the phosphorus sources (Fig. 3). Both iso-

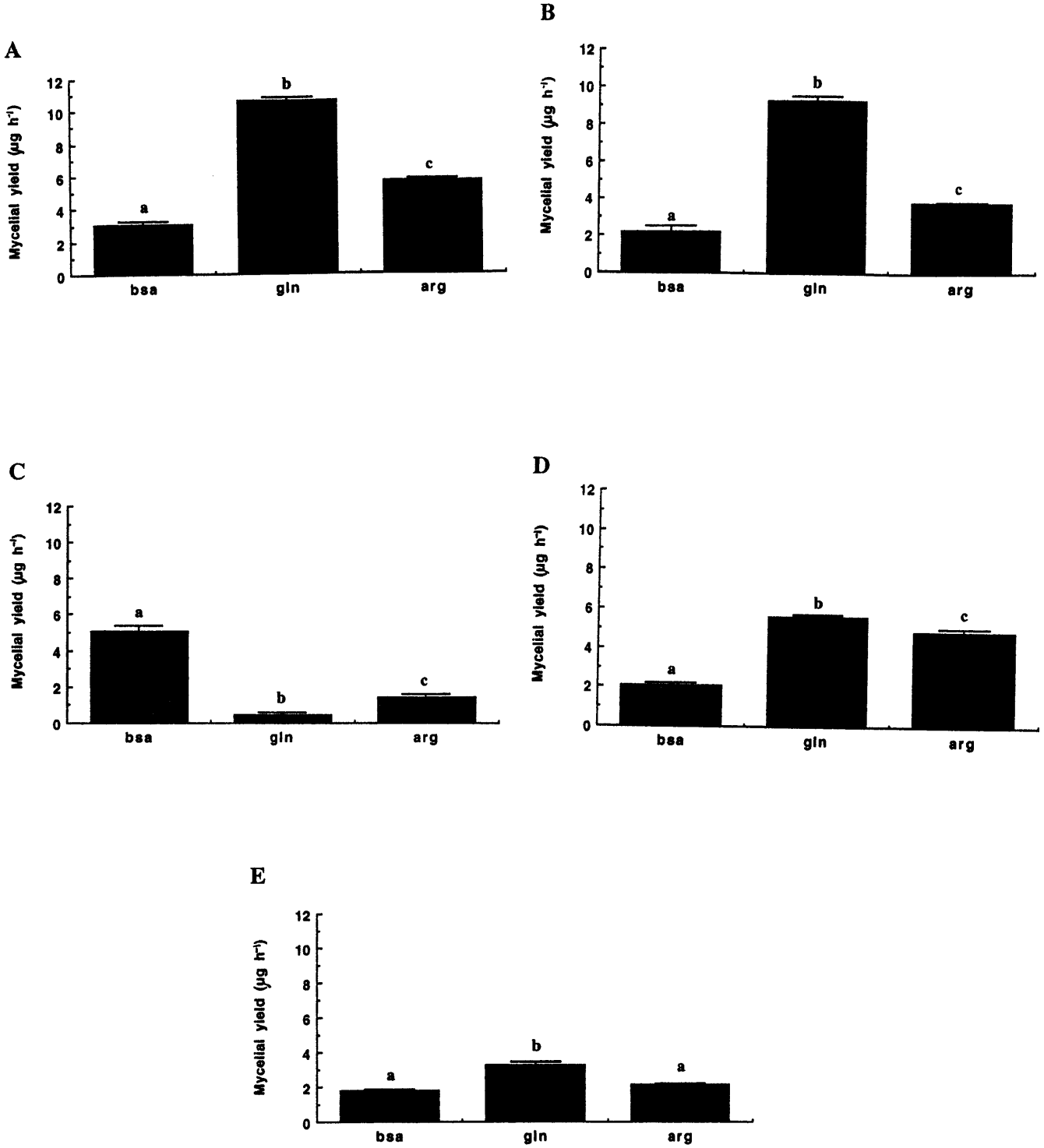

Fig. 3 Mean dry weight yields of endophytes isolated from *W. pungens* (**A** = D01, **B** = DO2, **C** = C40, **D** = A43) and *H. ericae* (**E**) on a range of phosphorus sources: orthophosphate (*ort*), DNA (*dna*), sodium inositol hexaphosphate (*ihp*), no added phosphorus (*no P*). *Bars* represent standard errors and different *letters* above the error bars indicate significant differences ($P < 0.001$) between yields (as determined by Fisher PLSD)

lates were, however, able to utilise orthophosphate in the N-utilisation experiment. The relative lack of growth on the phosphorus sources may thus reflect differences in the composition of the basal media used in the experiments. While there were differences in both the N and C content of the media, the most obvious difference was the inclusion of thiamine in the basal medium for the N-utilisation experiment, but not in the P-utilisation experiment. Given that the relative abili-

ties of different fungal taxa to synthesise vitamins differ (see Griffin 1994), the data may reflect an inability of the two isolates to synthesise thiamine, rather than an inability to utilise the organic P sources. Isolates C40 and A43 utilised all three phosphorus sources (Fig. 3). C40 grew significantly better on DNA or inositol hexaphosphate than on orthophosphate, while A43 grew significantly better on DNA than inositol hexaphosphate or orthophosphate. *H. ericae* grew equally well on orthophosphate and DNA, but showed no significant growth on inositol hexaphosphate (Fig. 3). Isolates C40 and A43 from *W. pungens* grew significantly better on either organic P substrate than *H. ericae* (Fig. 3).

Utilisation of DNA by fungi requires production of phosphodiesterase (and perhaps phosphomonoesterase) activity, while phosphomonoesters such as inositol hexaphosphate are hydrolysed simply by the latter (Leake and Miles 1996). Our data are thus consistent with production of both enzyme activities by isolates C40 and A43 from *W. pungens*. Although *H. ericae* produced some growth on inositol hexaphosphate, this was not significantly greater than in the absence of phosphate ($P < 0.001$) (Fig. 3). *H. ericae* produces both cell surface-bound and extracellular phosphomonoesterase activities (Straker and Mitchell 1986; Shaw and Read 1989; Leake and Miles 1996), however the pH optimum for activity demonstrates intraspecific variability, ranging from 4.5 to 6.0 for different isolates (Lemoine 1992; Leake and Miles 1996). The starting pH for the P-utilisation experiments was 7.8 and this may have limited the growth of *H. ericae* on inositol hexaphosphate in our experiment. It also appears that isolates C40 and A43 from *W. pungens* produced phosphomonoesterase activities with more alkaline pH optima.

The data presented indicate that at least some mycorrhizal endophytes isolated from the root system of the epacridaceous plant species *W. pungens* can utilise simple forms of organic N and P as sole sources of these nutrients during growth in axenic culture. Provision of benefits to the host via organic nutrient utilisation remains to be demonstrated in the Epacridaceae; however, from the body of information relating to endophyte host associations in the Ericaceae and the data presented here, it seems likely that a similar relationship will prevail in the Epacridaceae as in the Ericaceae. This conclusion is supported by strong evidence for organic N utilisation by mycorrhizal epacrid plants in recent field studies (Schmidt and Stewart 1997).

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