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UTILIZATION OF MINERAL AND AMINO N SOURCES BY THE ERICOID MYCORRHIZAL ENDOPHYTE *HYMENOSCYPHUS ERICAE* AND BY MYCORRHIZAL AND NON-MYCORRHIZAL SEEDLINGS OF *VACCINIUM*

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The ability of the ericoid mycorrhizal endophyte *Hymenoscyphus ericae* and of mycorrhizal and non-mycorrhizal ericaceous plants to utilize mineral and simple organic nitrogen is analysed. Growth of the endophyte occurs readily on acidic, basic and neutral amino-acids and on the amino-sugar glucosamine, final yields on most of these sources being comparable with those obtained on ammonium. Mycorrhizal plants produce greater yields than non-mycorrhizal ones and have higher nitrogen contents on several of the amino-acids tested. The significance of these results is discussed in relation both to the physiology of the endophyte and to the availability of N sources in soils.

In most soils the bulk of the nitrogen is present in organic forms which, it is generally considered, must be mineralized before they can be utilized by either fungi or higher plants. Heathland soils have very high levels of organic N (Stribley & Read, 1974) and in the process of mineralization of these N sources it is inevitable that a number of products intermediate in size and complexity between humo-protein and ammonium must be released to the soil solution.

Working in sub-alpine areas, Labroue & Carles (1977) showed that low soil temperatures inhibited ammonification and that, as a consequence, amino-acids accumulate. More recently it has been shown (Read & Bajwa, 1985) that amino N occurs in concentrations at least as high as ammonium N in *Calluna* heathland soil. While amino-acids probably constitute the major portion of the simple organic N compounds in heathland soil, amino-sugars may also be an important source of nitrogen (Bremner & Shaw, 1954). It is therefore necessary to determine the ability of the ericoid mycorrhizal fungus to utilize these amino compounds and to examine the role of mycorrhizal infection in providing plants with access to these organic N sources. In an early study (Pearson & Read, 1975) it was shown that some amino-acids can be utilized by the endophyte as efficiently as ammonium. Stribley & Read (1980) later demonstrated that mycorrhizal infection produced a stimulus to growth of *Vaccinium* seedlings grown on a small number of amino-acids. These investigations have now been extended to cover a wider range of amino-acids and to provide a more detailed analysis of the growth responses of the endophyte in vitro and of the host plants in the mycorrhizal and non-mycorrhizal condition.

MATERIALS AND METHODS

Growth of endophyte on mineral, amine, amino-acid and amino-sugar nitrogen sources

Inoculum employed for the fungal growth studies was obtained from cultures of *Hymenoscyphus ericae* (Read) Korf & Kernan (≡ *Pezizella ericae* Read), which had recently been isolated from ericoid mycorrhizal roots of *Vaccinium* using methods described by Pearson & Read (1973).

The ability of the endophyte to utilize inorganic and organic N sources was compared. Ammonium and nitrate were employed as inorganic N sources, and a range of acidic, basic, neutral and sulphur amino-acids together with the amino-sugar glucosamine, all of which are known to occur in soil, were used as organic sources. Ammonium was supplied as $(NH_4)_2SO_4$ and nitrate as $Ca(NO_3)_2$. The following amino compounds, obtained as chromatographically pure compounds (Sigma Chemical Company Ltd), were used: glutamic acid, aspartic acid, arginine, glutamine, alanine, 2-amino-*n*-butyric acid, proline, α-phenylalanine, tyrosine, tryptophane, valine, threonine, serine, leucine, methionine, cystine, cysteine and glucosamine.

The nitrogen sources were added individually to the N-free basal medium of Rorison (see Hewitt, 1966), to give a final N concentration of 100 mg l^{-1}. After calculation of the carbon content of each N treatment, supplementary glucose was added to give a final C : N ratio of 20 : 1 in all flasks. The pH of the media were adjusted to 4·5 using 5% H_2SO_4

or 10% KOH and solutions were sterilized by autoclaving at 15 lb pressure for 15 min. Autoclaving had minor effects upon the pH of the media, though in some cases levels were raised or lowered by as much as half a unit. Analyses of subsamples of amino-acid-amended solutions using thin-layer chromatography showed that they were structurally intact after autoclaving.

Nine replicate flasks each with 20 ml of medium were prepared for each treatment. Inoculum was obtained from endophyte cultures growing in Hagem's nutrient solution. A portion of mycelium was washed and macerated in sterile distilled water and a standard loop of macerate was aseptically transferred to each experimental flask and incubated at room temperature.

Harvests were taken at three intervals of 10 days and endophyte yield was determined on pre-weighed filter papers after oven drying at 80 °C for 24 h. The final pH values were recorded at each harvest.

Growth of seedlings of Vaccinium macrocarpon

Aseptically germinated seedlings of *V. macrocarpon* were raised in the mycorrhizal (M) and non-mycorrhizal (NM) condition on nitrogen-free nutrient agar medium. Once the infection was established, seedlings were aseptically transferred to various nitrogen regimes in sand culture.

Sterile acid-washed sand was poured into sterile plant containers (Flow Labs, Irving, Argyll), to give a sand depth of approximately 10 mm. The sand was saturated with 35 ml of Rorison nutrient medium. Batches of M and NM seedlings were grown on mineral, amine or amino-acid nitrogen at an N concentration of 25 mg l^{-1}. The nitrogen sources employed were calcium nitrate, ammonium sulphate, glutamine, glutamic acid, aspartic acid, arginine, serine and leucine.

The pH of all the media was adjusted to 4·5, then they were filter-sterilized using pre-washed membrane filters and added to the sand. Seedlings were grown in a controlled environment room (16 h day, 20° day and 15° night, irradiance 37 W m^{-2}).

The initial harvest comprised 8 M and 8 NM seedlings, randomly selected at the time of transplantation to sand cultures. Thereafter, 6 M and 6 NM plants were harvested from each treatment at 30 d and 10 plants at 60 d after planting. Plant dry weights were determined by oven drying at 80° for 24 h. At each harvest replicate samples of sand from the chambers were transferred to malt agar to check for the presence of microbial contaminants. No organism other than the ericoid endophyte was recovered.

Analysis of total plant N

Total nitrogen content of the seedlings was determined at each harvest, by Kjeldahl digestion followed by Buchi 322 semi-micro distillation and automatic titration. Six replicate M and NM plants were analysed at the 30 d harvest, but due to losses in the course of analysis only four replicates were available for the 60 d harvest.

Analysis of data

Two-way analysis of variance was used to evaluate the overall significance of the mycorrhizal infection and nitrogen treatment effects and their interaction, both in terms of mean dry weight and N-content of the seedlings. The significance of differences between M and NM treatment means within individual nitrogen treatments was determined using *t* tests.

RESULTS

Endophyte yields on mineral, amine and amino-acid N sources

The nitrogen sources which produced the highest yield at the first harvest were ammonium, arginine and glutamine (Figs 1*a*, *c*, 2*a*). Some sources produced only a small yield at this stage, notable amongst these being nitrate, 2-amino-*n*-butyric acid, tyrosine, valine and proline (Figs 1*a*, *c*, 2*b*, *c*). However, in most of these cases growth by the second harvest was as good as, or better than, that on ammonium. By the third harvest most of the amino-acids were seen to support good growth of the endophyte, though yields in aspartic acid, leucine, threonine, serine and the sulphur amino-acids were somewhat less good (Figs 1*b*, 2*b*, *c*, 3).

The form of nitrogen source had a marked effect upon the pH of the growth medium in the course of the experiment. When added in anionic form either as nitrate or in the form of acidic amino-acids, the pH increased markedly with increasing yield (Fig. 1*a*, *b*) and utilization of nitrogen. Conversely, when N sources were of the cationic, neutral or basic type, pronounced downward shifts of pH were obtained (Figs 1*a*, *c*, 2*a*–*c*, 3), so that in some cases the pH of the growth medium at the final harvest was as low as 2·1.

Glucosamine supported good growth of the ericoid endophyte (Fig. 4) although, as in the case of nitrate and some of the amino-acid treatments, the yield was lower than that obtained on ammonium at the first harvest. Again, a pronounced downward shift of pH of the growth medium was observed with increased utilization of the substrate.

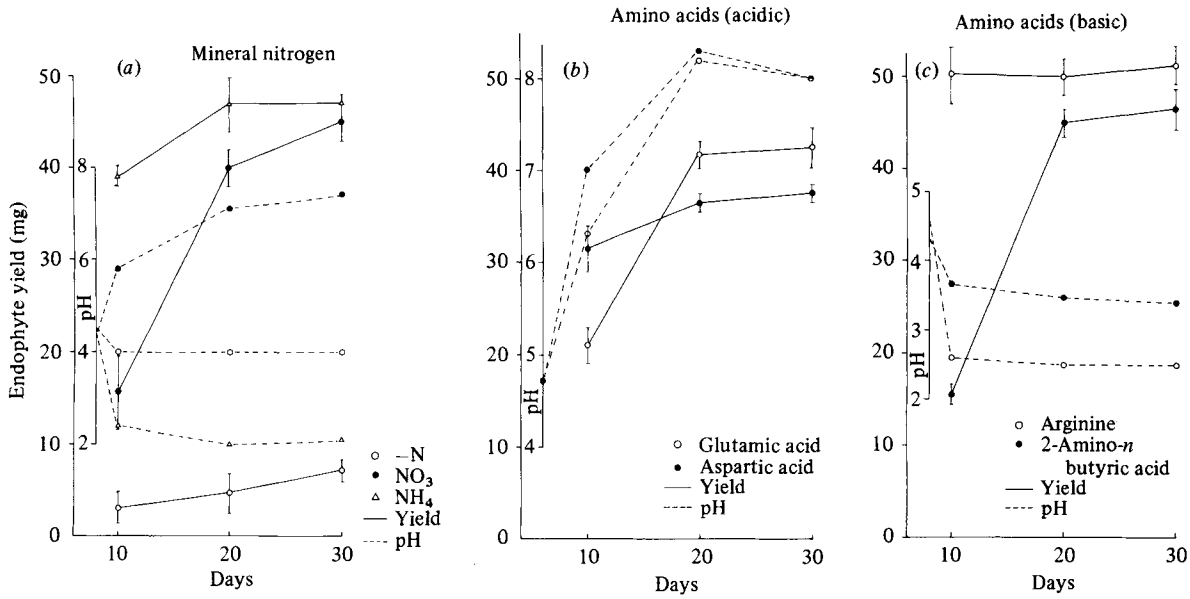

Fig. 1. Dry-weight yields of *H. ericae* and changes of pH of media after growth of fungi on mineral and on acidic and basic amino-N sources. All values are means of three replicates. Vertical bars represent 95% confidence limits. (*a*) Mineral nitrogen or minus nitrogen (−N) treatments; (*b*) acidic amino-acid treatments; (*c*) basic amino-acid treatments.

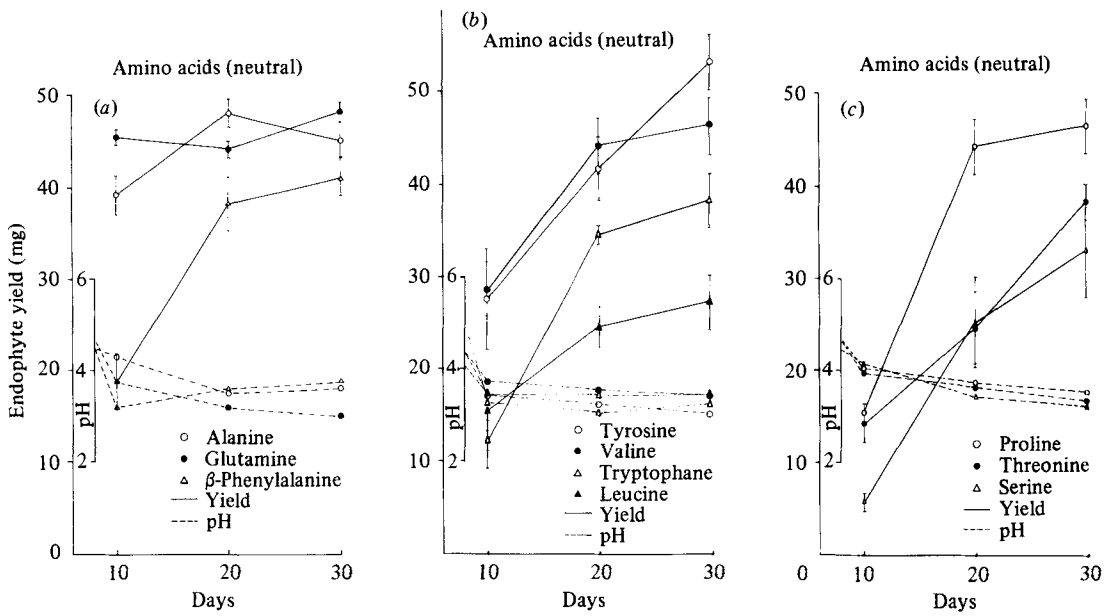

Fig. 2*a*, *b*, *c*. As in Fig. 1, but with neutral amino-acids as N sources.

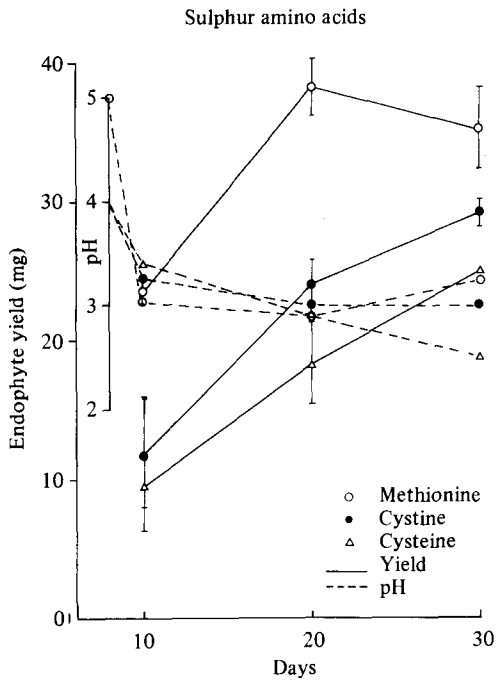

Fig. 3. As in Fig. 1, but with sulphur-containing amino-acids as nitrogen sources.

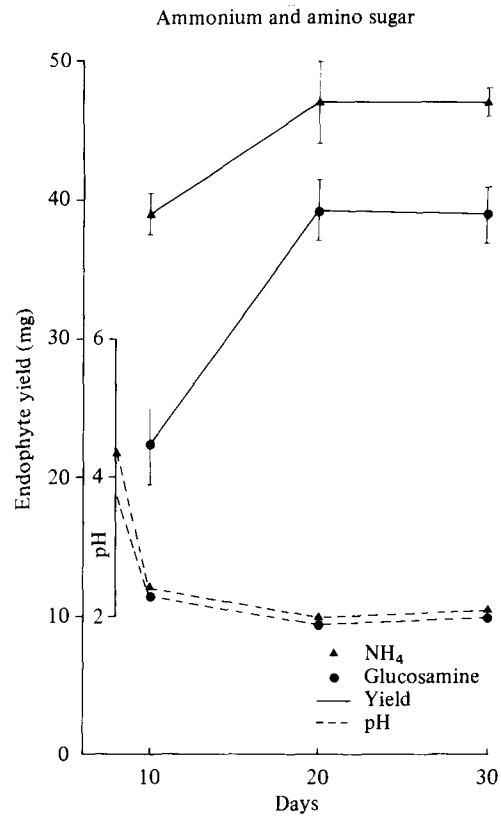

Fig. 4. As in Fig. 1, but with ammonium or glucosamine as nitrogen sources.

Table 1. *Summary of analysis of variance of mean dry weight and total nitrogen content of mycorrhizal and non-mycorrhizal plants grown on a range of N sources for 30 or 60 d*

Variable	Treatment effect or interaction	D.F.	*F* ratio	Probability
Mean dry wt at 30 d	Mycorrhiza	(1, 80)	17·82	$P < 0{\cdot}001$
	Nitrogen source	(7, 80)	5·04	$P < 0{\cdot}001$
	Mycorrhiza × N source	(7, 80)	1·96	NS
Mean dry wt at 60 d	Mycorrhiza	(1· 144)	17·35	$P < 0{\cdot}001$
	Nitrogen source	(7, 144)	12·47	$P < 0{\cdot}001$
	Mycorrhiza × N source	(7, 144)	3·44	$P < 0{\cdot}001$
Total N content of plants at 30 d	Mycorrhiza	(1, 80)	45·43	$P < 0{\cdot}001$
	Nitrogen source	(7, 80)	34·41	$P < 0{\cdot}001$
	Mycorrhiza × N source	(7, 80)	3·11	$P < 0{\cdot}001$
Total N content of plants at 60 d	Mycorrhiza	(1, 48)	2·22	NS
	Nitrogen source	(7, 48)	78·72	$P < 0{\cdot}001$
	Mycorrhiza × N source	(7, 48)	24·51	$P < 0{\cdot}001$

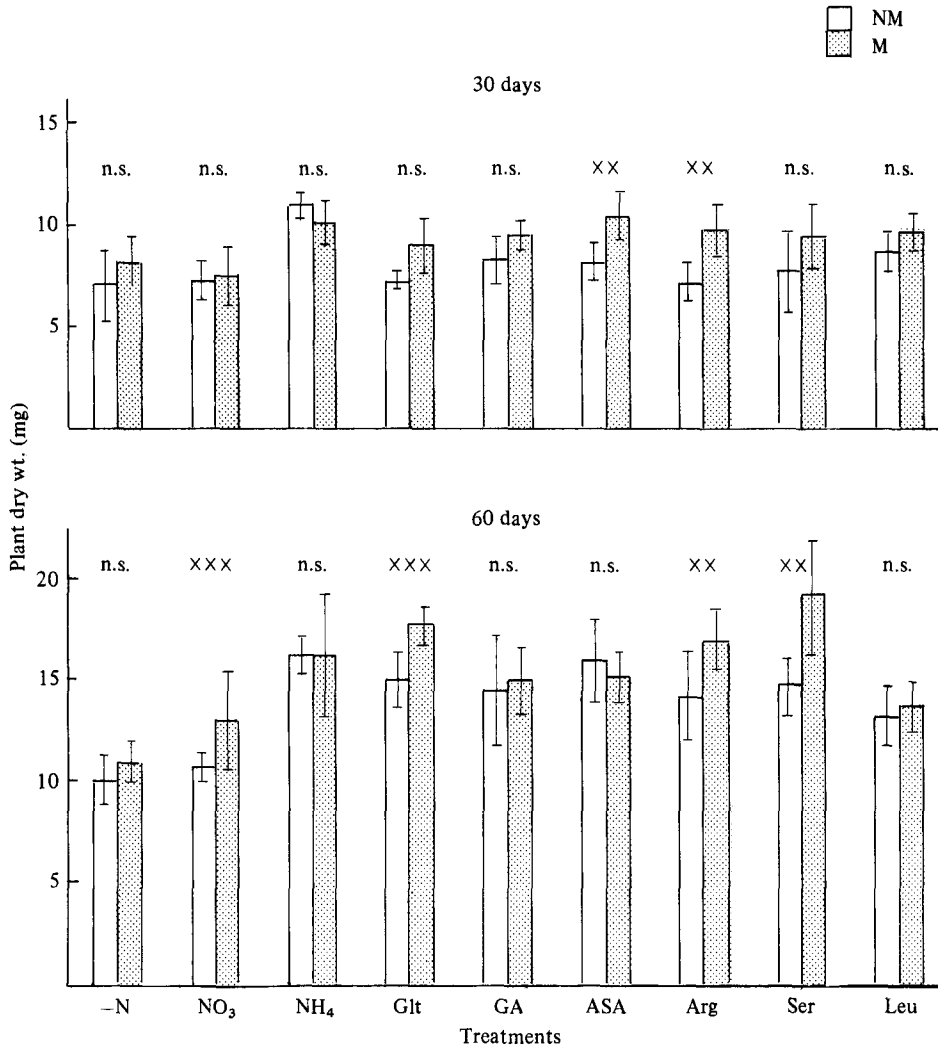

Fig. 5. Mean dry-weight yields of whole plants of mycorrhizal (M) and non-mycorrhizal (NM) plants of *Vaccinium macrocarpon* when grown on a range of mineral and amino-N sources (–N, no nitrogen; Glt, glutamine; GA, glutamic acid; ASA, aspartic acid; Arg, arginine; Ser, serine; Leu, leucine) at 25 mg l^{-1} N and harvested at 30 and 60 d. Vertical bars represent 95% confidence limits, $n = 6$ at 30 d, 10 at 60 d. Significance levels refer to results of t tests between M and NM treatment means within each N treatment. × = $P < 0.005$, × × = $P < 0.01$, × × × = $P < 0.001$. n.s., not significant.

Growth of M and NM plants of V. macrocarpon *on different nitrogen sources*

Two-way analysis of variance of the mean dry weight for the first harvest (Table 1) shows that there was a significant effect both of nitrogen treatment and of mycorrhizal infection on yield. Interaction between treatments and mycorrhiza was not significant.

At the second harvest (Table 1) highly significant effects of N treatments and mycorrhizal infection were again found. There was also a significant interaction between treatments and mycorrhizal infection at this harvest.

The evaluation of data using t tests (Fig. 5) showed no significant difference between M and NM plants in the minus N or NH_4 treatments. Mycorrhizal infection provided a significant increase of yield in the NO_3 treatment but only at the second harvest (60 d). Yields of M plants on

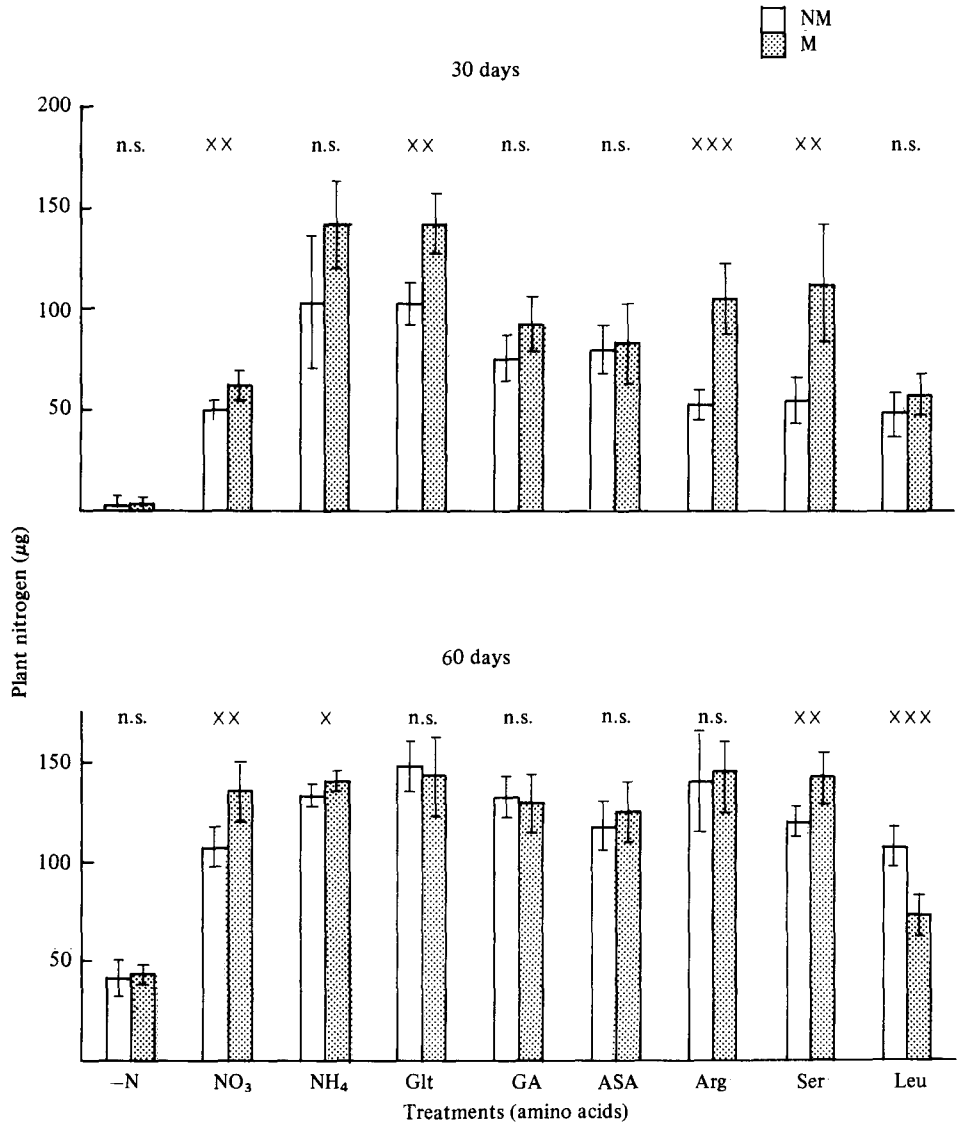

Fig. 6. Nitrogen contents of M and NM plants of *V. macrocarpon*. $n = 6$ at 30 d and 4 at 60 d. Other details as in Fig. 5.

glutamine were higher at both harvests but only significantly so at the second harvest. No significant mycorrhizal effect was observed in glutamic acid or leucine treatments but yields of M plants on arginine were significantly higher at both harvests. Aspartic acid provided a significantly higher yield of M plants at the first harvest and serine at the second. Yields of mycorrhizal plants in serine were higher than those in any other N treatment by the second harvest, and the yield of M plants in both arginine and glutamine was higher than that in ammonium.

Total N content of V. macrocarpon *seedlings*

Analysis of variance of total N content at the first harvest (Table 1) shows that it was significantly influenced by N treatments and by mycorrhizal infection. There was also a significant interaction between treatments and mycorrhizal infection. At

the second harvest, while total N content of plants was still significantly influenced by the N treatments, the effects of mycorrhizal infection had been lost. There was, however, still a significant interaction between treatments and mycorrhizal infection ($P < 0.001$).

The t tests show (Fig. 6) that at the first harvest M plants had significantly higher N content than their NM counterparts in NO_3, glutamine, arginine and serine treatments. The differences were associated with increased yield in all cases, though the yield increases were statistically significant only in the arginine treatment. The nitrogen contents of M seedlings in arginine and serine treatments were almost double those of the NM plants.

By the second harvest, significant differences between nitrogen contents of M and NM plants were found only in the NO_3 and serine treatments. This was attributable to the fact that the N content of NM plants had increased relative to that of M plants in most of the treatments.

Calculations of the nitrogen budgets for the experiment provide a possible explanation for these observations. The total quantity of N provided in each treatment together with the starting N content of the seedlings is equivalent to 157 μg in the NM and 159 μg in the M cultures. In the case of NH_4, glutamine, arginine and serine more than 100 μg of this had been absorbed by 30 d and practically all of the remainder had probably been taken up within a short period following the first harvest. The M plants in many of the treatments would probably therefore have been N-limited for much of the period between the first and second harvest.

DISCUSSION

While there have been a number of detailed studies of amino-acid uptake and transfer in saprotrophic fungi (see review by Wolfinbarger, 1980), relatively little attention has been paid to the utilization of these N sources by mycorrhizal fungi. This situation reflects a general neglect of the possible importance of organic N sources in soils and as potential plant nutrients. The present study demonstrates not only that the ericoid endophyte utilizes a broad spectrum of amino compounds but also that the nitrogen so acquired by the heterotroph can be transferred to its autotrophic partner. While the present study does not attempt to reveal the mechanism of uptake and transport of amino-acids by the mycorrhizal fungi, it is evident that a very broad spectrum of amino-acids can be utilized by the endophyte.

Most of the acidic, basic and neutral amino-acids were used as readily as, or in some cases even more readily than, mineral sources of nitrogen. Some comparison can be drawn between the pattern of amino-acid utilization in the ericoid endophyte and that reported by Laiho (1970) for the facultative ectomycorrhizal fungus *Paxillus involutus*. Arginine and glutamic acid are used by both fungi as preferred N sources but, whereas the ericoid endophyte also readily used a wide range of neutral amino-acids like tyrosine, proline and valine, they were found to be very poor sources of amino-acid N in all the strains of *Paxillus*. While growth of the endophyte on aspartic acid was only slightly lower than that on mineral sources, *Paxillus involutus* showed negligible utilization of this compound.

The relatively slow utilization of sulphur-containing amino-acids revealed in the present study is also of interest. Transport assays in *Neurospora* (Pall, 1971), *Aspergillus* (Poitrowska *et al.*, 1976) and *Penicillium* (Benko, Wood & Segel, 1967) have shown the presence of a methionine-specific transport system which is not activated when the fungi are grown on sulphur-sufficient medium. Similarly, uptake of other sulphur amino-acids has been shown to occur only in conditions of sulphur starvation. Thus the lower yields found on sulphur amino-acids in the present study may be attributed to the fact that a sulphur-sufficient growth medium was used in the assay.

Comparison of the results presented here with those of Laiho (1970) and of Lundeberg (1970) suggests that ectomycorrhizal fungi are more selective in their utilization of amino-acids than is the ericoid endophyte. Early studies of ectomycorrhizal systems (Melin & Nilsson, 1953) revealed that the mycorrhizal mycelium could absorb glutamine which was subsequently transferred through the hyphae to the host plant. Using detached beech mycorrhizas, Carrodus (1966) showed that the amides glutamine and asparagine were more readily absorbed from solution than were their amines. Alexander (1983) grew seedlings of *Picea sitchensis* in the mycorrhizal and non-mycorrhizal condition with NH_4 as sole N source and on NH_4 to which aspartic acid or serine was added. The presence of serine depressed the growth of both mycorrhizal and non-mycorrhizal plants despite the fact that the mycorrhizal fungus employed in the experiments had been shown to use serine as a sole N source. Again, therefore, it seems as if the responses of ectomycorrhizal plants to amino-acids are somewhat more variable than those found in the ericoid system, which in those studies carried out to date are generally positive.

The effects of mycorrhizal infection upon yields of *V. macrocarpon* grown on amino-acids are broadly comparable with those observed by Stribley & Read (1980), and confirm that infection

can lead to a stimulation of growth in the presence of amino compounds. However, some differences between the results of the two studies are evident. Stribley & Read used 20 p.p.m. N in their study, and the only nitrogen sources common to both studies were glutamine, glutamic acid and aspartic acid. The aspartic acid and glutamine results are comparable with those obtained by Stribley & Read, though the differences between the M and NM yields were not as dramatic as reported in their study. This is largely because the NM plants in the present experiment showed a much greater capacity to utilize amino-acids than did those of Stribley & Read. These workers found little utilization of some of the amino-acids, for example glutamic and aspartic acid, in the NM condition whereas in the present study, by the second harvest, there was no significant difference in yields of M and NM plants on these sources. Total yields of M plants over the experimental period were comparable in both studies, but those of NM plants reported by Stribley & Read are generally much lower. Of the amino-acids not examined by these workers, arginine gave consistently better yields in mycorrhizal plants, while serine gave yields significantly higher at the second harvest, and no difference was observed in leucine.

Bremner & Shaw (1954) in their studies with a number of different soils reported that 5–10% of the total N was in the form of amino sugars. They also compared the rates of decomposition of chitin, glucosamine, casein and yeast nucleic acid when incubated with soil under conditions found to produce rapid nitrification of ammonium sulphate. It was observed that glucosamine and chitin were readily decomposed by soil micro-organisms, although their breakdown was not as rapid as that of casein or yeast nucleic acid. Clearly, as the ericoid endophyte utilized glucosamine more readily than some of the amino-acid sources, it would be expected to have access to this compound in soils. It is likely that the endophyte will have the capacity to degrade and absorb both of the major forms of amino-sugars present in the soil.

Analysis of the pattern of utilization of the two mineral N sources indicates that, as in the case of the amino-acids, the ericoid endophyte may be more versatile than many ectomycorrhizal fungi. While some of the latter, such as *Paxillus involutus*, grow almost as well on nitrate as on ammonium, others, such as *Lactarius rufus*, are unable to utilize nitrate at all (Alexander, 1983). Of the ectomycorrhizal fungi tested by Lundeberg (1970) only 60% could utilize nitrate N. It appears that nitrate reductase activity is normally low or absent in many ectofungi (Ho & Trappe, 1980). Failure of mycorrhizal roots of beech to take up or reduce nitrate has been demonstrated by Carrodus (1967) and by Smith (1972). In contrast, the ericoid endophyte clearly has the ability to utilize nitrate albeit at a slow rate, at least in the early stages of growth. This confirms the observation of Pearson & Read (1975). The lag in utilization of nitrate could be attributable to the requirement for induction of nitrate reductase activity. Mycorrhizal infection provides for significant enhancement of plant yield on nitrate at one harvest and this, in turn, is reflected in higher plant nitrogen contents. It is clear that infection would be of importance for plants in any circumstances where active nitrification was occurring in the soil.

The often pronounced effects of endophyte growth upon pH of the growth medium might also be of importance in the natural environment. In heathland environments ammonium, together with basic and neutral amino compounds, are the predominant sources of simple nitrogen compounds (Read & Bajwa, 1985) and it could be predicted from the present results that their assimilation would lead to acidification of soil. This is a phenomenon which has been observed in the field under ericaceous plants (Grubb & Suter, 1971) and is of considerable importance both for microbial and plant ecology.

This study confirms that the mycorrhizal fungus is likely to play an important role in the nitrogen nutrition of plants with ericoid mycorrhizas. The fungus provides access to both organic and mineral sources which are otherwise either unused or only little used by the plant. Such benefits are likely to be of great ecological significance, particularly as it is increasingly evident that amino-acids are quantitatively important as nitrogen sources in acidic organic soils. A further feature which may be of significance to both partners in the mycorrhizal association is that the demands of the fungus on host carbon reserves are likely to be reduced in proportion to the quantity of carbon assimilated from soil in the form of amino-acids.

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