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Expression of glutamate dehydrogenase and aspartate aminotransferase in eucalypt ectomycorrhizas

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SUMMARY

Previous studies of nitrogen-assimilating enzymes in ectomycorrhizal associations of the Pinaceae (Norway spruce, Douglas fir) and the Fagaceae (beech) have suggested that the host plant may regulate the expression of some fungal enzymes. To improve our understanding of the regulation of nitrogen-assimilating enzymes in ectomycorrhizal plants, we have extended this work to a third plant family, the Myrtaceae.

Glutamate dehydrogenases, specific either for NAD (NAD-GDH) or NADP (NADP-GDH), and aspartate aminotransferase (AAT) were investigated by enzyme assays and electrophoretic patterns on polyacrylamide gels. Non-mycorrhizal roots of four species of *Eucalyptus* (*E. diversicolor*, *E. globulus*, *E. nitens* and *E. regnans*) were characterized by a high activity of NAD-GDH, while only NADP-GDH was found in *Hebeloma westraliense* in pure culture. In associations of these eucalyptus with *Hebeloma westraliense*, both NAD- and NADP-GDHs were detected. By contrast, NAD- and NADP-GDHs found in the free-living mycelium of *Laccaria laccata* were not detected in the associated ectomycorrhizas.

In ectomycorrhizas of five species of eucalypts (*E. diversicolor*, *E. globulus*, *E. grandis*, *E. regnans* and *E. urophylla*) associated with either *Laccaria laccata*, *Scleroderma verrucosum* or *Pisolithus tinctorius*, fungal AAT, which was very active in free-living mycelia, was not detected in the symbiotic tissues. In contrast, the two root AAT isoenzymes remained active in the mycorrhizas and were even stimulated.

These results suggest that fungal gene expression is moderated by the host plant but also depends on the fungal associate. Factors which may contribute to the observed differences in enzyme activity between *Hebeloma* and *Laccaria* are discussed.

Key words: Aspartate aminotransferase, *Eucalyptus* ectomycorrhizas, glutamate dehydrogenases, *Hebeloma westraliense*, *Laccaria laccata*, *Pisolithus tinctorius*, *Scleroderma verrucosum*, nitrogen assimilation.

INTRODUCTION

Ectomycorrhizas are symbiotic associations between vascular plants, especially trees, and fungi, which contribute greatly to the growth of the host plant. The beneficial effect is mainly due to the fact that the fungus provides the tree with water and nutrients, especially nitrogen and phosphorus, while the fungus, for its part, obtains carbohydrates from the host (Harley & Smith, 1983).

In many forests, accumulation of organic material leads to acidification of the soil where little nitrification occurs and trees are thus forced to rely on ammonium as their primary nitrogen sources (Brand, Kehoe & Connors, 1986; Read, Leake & Langdale, 1989). In most vascular plants, including woody species, ammonium is assimilated through the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Miflin & Lea, 1980; Oaks & Hirel, 1985). There is little evidence for the participation of glutamate dehydrogenase (GDH) in nitrogen assimilation in roots which possess an NAD-dependent GDH involved mainly in the oxidation of glutamate in response to a deficiency of carbon (Robinson *et al.*, 1991). In the ectomycorrhizal fungus *Pisolithus tinctorius*, the results obtained by Kershaw & Stewart (1992) are also consistent with the consecutive action of GS and GOGAT.

However, in many fungi, including several ectomycorrhizal species such as *Cenococcum geophilum* (Genetet, Martin & Stewart, 1984), *Hebeloma* sp. (Chalot *et al.*, 1991) and *Laccaria laccata* (Martin, 1991; Brun *et al.*, 1992), nitrogen assimilation

appears to proceed via the simultaneous activity of GDH and GS. The possibility that, in ectomycorrhizas, fungal and plant cells have different nitrogen assimilation pathways, raises issues concerning their coexistence and interaction. Preliminary results have shown that the activity and amount of the fungal NADP-GDH polypeptide are strongly suppressed in beech ectomycorrhizas (Martin *et al.*, 1986; Dell *et al.*, 1989). By contrast, in other experiments where the host plants were Norway spruce or Douglas fir, fungal NADP-GDH remained fully active in ectomycorrhizas (Dell *et al.*, 1989). As the same fungal genus was used in both types of association, it appears that changes observed depended on the plant associate. In all three cases, the fungal aspartate aminotransferase (AAT), which was very active in free-living mycelium, was not detected in the symbiotic tissues, whereas the root AAT isoenzymes remained active (Chalot *et al.*, 1990*b*).

So far, the work has been largely confined to a single deciduous tree belonging to the Fagaceae (*Fagus sylvatica* L.) and to a few coniferous trees belonging to the Pinaceae [*Picea excelsa* (Lam.) Link. and *Pseudotsuga menziesii* (Mirb.) Franco]. Although only a few types of ectomycorrhiza were investigated, it is tempting to ascribe these different regulatory properties to much larger groups such as the angiosperms and gymnosperms.

However, these investigations should be extended to the other large ectomycorrhizal flowering plant families (Dipterocarpaceae, Myrtaceae) before conclusions concerning the role of the host plant on enzyme expression in the fungal partner can be drawn. In order to improve our understanding of the regulation of the nitrogen-assimilating enzymes, we have focused this study on the family Myrtaceae and the genus *Eucalyptus*. There are a number of advantages in choosing this genus. Firstly, ectomycorrhizas can be synthesized easily *in vitro* (Malajczuk, Lapeyrie & Garbaye, 1990) and the sequence of the different steps of formation of mycorrhizas can be followed reliably (Horan, Chilvers & Lapeyrie, 1988). Secondly, inoculation procedures have been developed (Kuek, Tommerup & Malajczuk, 1992) that permit the mechanized inoculation of nursery plants from seeds. This allows production of reproducible nursery material for experimental purposes. Thirdly, eucalypt mycorrhizas have a commercial importance in plantations in the subtropical and temperate zones of many countries. Consequently, there is some knowledge of the fungi that form ectomycorrhizas with subtropical as well as temperate species of eucalypts (Bougher, Tommerup & Malajczuk, 1991, 1993).

The work reported here analyses the expression of GDHs and AAT in four species of temperate eucalypts and two species of subtropical eucalypts. Ectomycorrhizas were either produced in a glasshouse or were obtained from a commercial nursery.

MATERIALS AND METHODS

Fungal isolates and production of plant material

Fungal cultures were obtained from the mycorrhizal laboratory of the CSIRO Division of Forestry in Perth, Western Australia. The isolates were *Laccaria laccata*, strain ME 26, *Hebeloma westraliense*, strain ME 1, *Scleroderma verrucosum*, strain MH 13 and *Pisolithus tinctorius*, strain MH 57. Mycelium was grown on cellophane over agar in Petri dishes at 21 °C. Three media were used, the modified Melin Norkrans (MMN) agar (Marx, 1969), a modified Pachlewski agar and a mineral nutrient agar. The Pachlewski agar, in which dipotassium tartrate was substituted for diammonium tartrate, contained 25 mM N as $(NH_4)_2SO_4$ and 3·5 mM P as $NaH_2PO_4.2H_2O$, the carbohydrates being glucose (20 g l^{-1}) and maltose (5 g l^{-1}). The mineral nutrient agar contained 20 g l^{-1} glucose and 0·08 mM P as KH_2PO_4 and K_2HPO_4, and had the same nitrogen composition as the Pachlewski medium. The pH was adjusted to 6 with KOH prior to autoclaving. Final pH after autoclaving was 5·6. Enzyme activities were determined when the fungal cultures were in the exponential growth phase, after 10–15 d incubation. Enzyme activities and banding patterns on gels were similar for all three media, and no distinction is made between them. Stipes of *Laccaria laccata* were occasionally collected under eucalypts and their GDH isoform profiles were compared with those of mycelia cultivated in pure culture.

Ectomycorrhizal and non-mycorrhizal roots were obtained from either a commercial nursery near Manjimup, Western Australia or from a glasshouse trial. In the former case, seeds of *Eucalyptus diversicolor*, *E. globulus* spp. *globulus*, *E. nitens* and *E. regnans* were sown into a peat/perlite potting mix in trays, concurrently with bead inoculum (Kuek *et al.*, 1992) of *Laccaria laccata* or *Hebeloma westraliense*. Uninoculated trays received only the seeds. Plants were grown on raised nursery benches outdoors and were fertilized twice weekly with a dilute nutrient fertilizer containing all essential plant nutrients. After three months, the plants were removed from the trays and young ectomycorrhizal and non-mycorrhizal roots were sampled under the microscope.

Glasshouse-grown plants were produced as described by Bougher, Grove & Malajczuk (1990). Briefly, plastic pots, lined with polyethylene bags, were filled with 2 kg of steam-pasteurized yellow sand and basal nutrients were mixed through the soil. Pots were watered to field capacity (10 % w/w) and were allowed to equilibrate for one week before planting. A factorial design consisting of three host species (*E. diversicolor*, *E. grandis*, *E. urophylla*) × two nitrogen sources (NH_4-N, NO_3-N) × two rates of phosphorus [6 mg P kg^{-1} sand-P6 plants, 40 mg P kg^{-1} sand-P40 plants supplied as $Ca(H_2PO_4)_2.H_2O$] ×

three inoculation treatments (nil, *Scleroderma verrucosum*, *Pisolithus tinctorius*) × three replicates was set up as a randomized complete block. The lower P rate was used to maximize mycorrhizal development. The higher P rate was optimal for growth of non-mycorrhizal plants. Seeds were surface-sterilized with 2% NaOCl for 10–15 min and washed in 3 changes of sterile water. One week after germination on the mineral nutrient agar, they were transferred to the surface of 3-wk-old *S. verrucosum* and *P. tinctorius* cultures growing on MMN agar in polycarbonate jars. Uninoculated plants were transferred to jars without the fungus. After 7 d in a growth cabinet (16 h day: 25 °C, 100 μE m^{-2} s^{-1}; 8 h night: 20 °C), seedlings were transferred to the pots (4 per pot) and maintained in a heated glasshouse (23–28 °C).

Nitrogen was supplied as either $(NH_4)_2SO_4$ or $Ca(NO_3)_2$ in solution at weekly intervals to a total of 200 mg N/pot. Plants were thinned to two plants per pot at 3–4 wk after transfer.

At 12–14 wk after planting, representative plants were taken at random and the roots washed free of sand. Mycorrhizal and non-mycorrhizal roots were collected on ice under the microscope.

Extraction of native enzymes

Detached ectomycorrhizas, non-mycorrhizal roots and mycelium were placed in cold 50 mM K-phosphate buffer, pH 7·0 containing 10% glycerol, 10 mM glutamate, 10 mM $MgSO_4$, 10 mM DTT, 42 mM 2-mercaptoethanol, 1 mM PMSF and 1 mM Na-EDTA. After blotting and weighing, 50–300 mg were ground over ice using a mortar and a pestle in 1–2 ml of the above extraction buffer to which had been added 3% (w/v) soluble polyvinylpyrolidone (PVP) and insoluble polyvinylpolypyrrolidone (PVPP) (10% f.wt of material). The macerates were centrifuged at 40000 ***g*** for 10 min in the presence of 300 μl of DEAE-Sephadex resin (A 50 Pharmacia) previously swollen in the extraction buffer containing 1 M NaCl. Preliminary experiments showed that enzyme activities were in many cases increased when macerates were passed through, or centrifuged in the presence of, an anion-exchange resin, which probably to a large extent removed pigments and phenolic compounds. The supernatants were used as crude extracts for enzyme assays and slab-gel electrophoresis.

Determination of enzyme activities

Enzyme activities were determined by following the reduction of NADH or NADPH at 340 nm in a Beckman DU 40 spectrophotometer. Glutamate dehydrogenase (GDH) was assayed by reductive amination of α-ketoglutarate (Botton, Msatef & Godbillon, 1987). Extracts (50–300 μl) were incubated at 30 °C in reaction mixtures containing 8·7 mM α-ketoglutarate, 122 mM $(NH_4)_2SO_4$ and 0·18 mM NADPH or NADH in 1·15 ml 100 mM K-phosphate buffer, pH 7·2. Aspartate aminotransferase (AAT) was assayed for oxaloacetate and glutamate production using a coupled assay reaction catalysed by NAD-malate dehydrogenase (Khalid *et al.*, 1988). Extracts (100–300 μl) were incubated at 30 °C in reaction mixtures containing 20 mM aspartate, 10 mM α-ketoglutarate, 0·2 mM NADH, 80 mM pyridoxal 5-phosphate and 1 unit malate dehydrogenase in 1·15 ml 100 mM Tris-HCl buffer, pH 8. Enzyme activities were expressed in nkat g^{-1} f.wt of material (1 nkat corresponds to the conversion of 1 nmole of substrate per second).

Electrophoresis

Gel electrophoresis was carried out according to Davis (1964). Approximately 20 μl of each extract was applied to 6% polyacrylamide slab gel (8 × 6 cm). Electrophoresis was run for about 2 h at 80 V (constant voltage). The bands of NADP-GDH and NAD-GDH activity were located using a tetrazolium assay system (Blumenthal & Smith, 1973) to follow the deamination of L-glutamate. Incubation mixtures contained 30 mM L-glutamate, 0·35 mM NADP or NAD, 25 mM nitroblue tetrazolium and 12·5 mM phenazine methosulphate in 100 mM Tris-HCl buffer, pH 8. Staining for AAT activity was a two-step procedure including incubation of the gel for 30 min in a mixture containing 0·73 mM α-ketoglutarate, 1·33 mM aspartate and 0·08 mM pyridoxal 5-phosphate prepared in 0·1 M Tris-HCl buffer, pH 8, followed by incubation in a fast violet blue solution (3 mg ml^{-1}) for about 10 min. Purple bands formed at the position of AAT activity.

Molecular weights of the native enzymes were determined by using Biorad 4–20% polyacrylamide gradient gels calibrated with the following protein standards: urease I (545 kDa), urease II (272 kDa), bovine serum albumin (132 kDa), chicken egg albumin (45 kDa) and α-lactalbumin (14·2 kDa).

RESULTS

Formation of mycorrhizas and plant growth

There was extensive development of young mycorrhizas at the pot wall/potting mix interface in the nursery trays. *Hebeloma* and *Laccaria* mycorrhizas were similar across the host species and to those described previously for *Eucalyptus marginata* (Malajczuk, Dell & Bougher, 1987) and *E. camaldulensis* (Bougher *et al.*, 1991). The uninoculated plants were non-mycorrhizal at harvest.

In pot cultures, pyramidal clusters of *Scleroderma* and *Pisolithus* mycorrhizas formed in the crown root region of P6 plants. Mycorrhizas did not form in the inoculated P40 or the uninoculated plants. Gen-

Table 1. *Enzyme activities (mean ± SE) in mycorrhizal and non-mycorrhizal eucalypt roots and in the ectomycorrhizal fungi* Hebeloma westraliense, Laccaria laccata, Scleroderma verrucosum *and* Pisolithus *tinctorius*

	Enzyme capacity (nkat g^{-1} f. wt)		
	NAD-GDH	NADP-GDH	AAT
Eucalyptus nitens (non-mycorrhizal roots)	1·05 ± 0·25	0·12 ± 0·01	5·67 ± 1·32
E. globulus (non-mycorrhizal roots)	1·84 ± 0·42	0·24 ± 0·12	8·28 ± 2·01
E. diversicolor (non-mycorrhizal roots)	1·93 ± 0·24	0·36 ± 0·03	7·14 ± 0·97
E. regnans (non-mycorrhizal roots)	0·67 ± 0·12	0·17 ± 0·02	10·06 ± 3·04
E. grandis (non-mycorrhizal roots)	0·78 ± 0·16	0·25 ± 0·10	4·67 ± 1·08
E. urophylla (non-mycorrhizal roots)	0·88 ± 0·27	0·34 ± 0·04	5·34 ± 0·98
Hebeloma westraliense (pure culture)	0·12 ± 0·03	5·61 ± 1·24	14·88 ± 3·28
Eucalyptus nitens/H. westraliense mycorrhiza	1·85 ± 0·35	2·08 ± 0·30	14·85 ± 2·15
E. globulus/H. westraliense mycorrhiza	1·92 ± 0·41	1·51 ± 0·25	12·50 ± 3·01
E. diversicolor/H. westraliense mycorrhiza	2·18 ± 0·48	0·86 ± 0·17	15·72 ± 4·57
E. regnans/H. westraliense mycorrhiza	0·67 ± 0·23	n.d.	18·15 ± 4·29
Laccaria laccata (pure culture)	2·15 ± 0·32	3·17 ± 0·81	20·12 ± 5·21
Eucalyptus nitens/L. laccata mycorrhiza	1·97 ± 0·46	0·12 ± 0·02	12·56 ± 3·40
E. globulus/L. laccata mycorrhiza	n.d.	n.d.	15·67 ± 5·12
E. diversicolor/L. laccata mycorrhiza	2·12 ± 0·67	0·21 ± 0·01	18·24 ± 3·14
E. regnans/L. laccata mycorrhiza	0·65 ± 0·28	0	17·10 ± 3·01
Scleroderma verrucosum (pure culture)	2·87 ± 0·61	0·37 ± 0·06	21·95 ± 4·38
Eucalyptus grandis/S. verrucosum mycorrhiza	2·04 ± 0·72	0·26 ± 0·08	31·24 ± 5·45
E. urophylla/S. verrucosum mycorrhiza	1·33 ± 0·64	0·30 ± 0·05	16·47 ± 4·11
E. diversicolor/S. verrucosum mycorrhiza	1·52 ± 0·73	0·45 ± 0·03	14·76 ± 6·05
Pisolithus tinctorius (pure culture)	5·33 ± 0·76	0·04 ± 0·01	11·79 ± 3·21
Eucalyptus grandis/P. tinctorius mycorrhiza	2·03 ± 0·41	0·17 ± 0·04	22·10 ± 5·23
E. urophylla/P. tinctorius mycorrhiza	1·82 ± 0·38	0·35 ± 0·20	13·24 ± 4·04
E. diversicolor/P. tinctorius mycorrhiza	3·75 ± 0·84	0·31 ± 0·32	12·13 ± 3·11

Results of 2–3 determinations from 3-month-old seedlings. n.d., not determined.

erally, inoculation of P6 plants with *Scleroderma* and *Pisolithus* increased growth by 50–200 % compared to P6 uninoculated plants. Mycorrhizal plants were intermediate in size between the uninoculated P6 and P40 plants. Uninoculated plants supplied with NO_3-N were smaller than plants supplied with NH_4-N. The effects of N form on ectomycorrhizal development and plant growth will be reported separately. As there was no qualitative difference in GDH and AAT activities on acrylamide gels for ectomycorrhizas from pots supplied with the two N forms, no distinction is made between them here.

Activity of the ammonium-metabolizing enzymes

As shown in Table 1, non-mycorrhizal roots were characterized by high activity of NAD-GDH and low activity of NADP-GDH. In pure culture of *Hebeloma westraliense*, only NADP-GDH was detected in appreciable amounts. In the *Eucalyptus/H. westraliense* mycorrhizal associations, both NAD-GDH and NADP-GDH were generally detected at comparable activities. This distribution suggests that the NADP-GDH in the mycorrhizas is in the fungal compartment.

In the free-living mycelium of *Laccaria laccata*, both NAD-GDH and NADP-GDH were expressed, while in the *Eucalyptus/L. laccata* mycorrhizas only NAD-GDH activity was appreciable. These results, based on enzyme activities, suggest that the fungal NADP-GDH may be repressed in the mycorrhizal tissues.

Both *Scleroderma verrucosum* and *Pisolithus tinctorius* in pure culture showed much NAD-GDH activity, while NADP-GDH was hardly detected, especially with *Pisolithus tinctorius*. In the associations of eucalypts with these fungi, only the NAD-specific enzyme was detected in significant amounts.

AAT activity was present in both fungal and plant tissues. However, AAT activity was much higher in mycorrhizal than in non-mycorrhizal roots in all associations synthesized in the glasshouse and field nursery.

Electrophoretic patterns of the enzymes

Glutamate dehydrogenases. In non-mycorrhizal roots gel electrophoresis on 6 % acrylamide confirmed the presence of NAD-GDH, revealed as a major band at R_f of 0·26 in *Eucalyptus diversicolor* and 0·32 in *E. nitens*, *E. globulus* and *E. regnans* (Figs 1 and 2). These NAD-GDH bands were slightly stained with NADP, presumably because of a partial dephosphorylation of this cofactor during the staining process. Estimates of the molecular weights of the native NAD-GDHs from eucalypts, by poly-

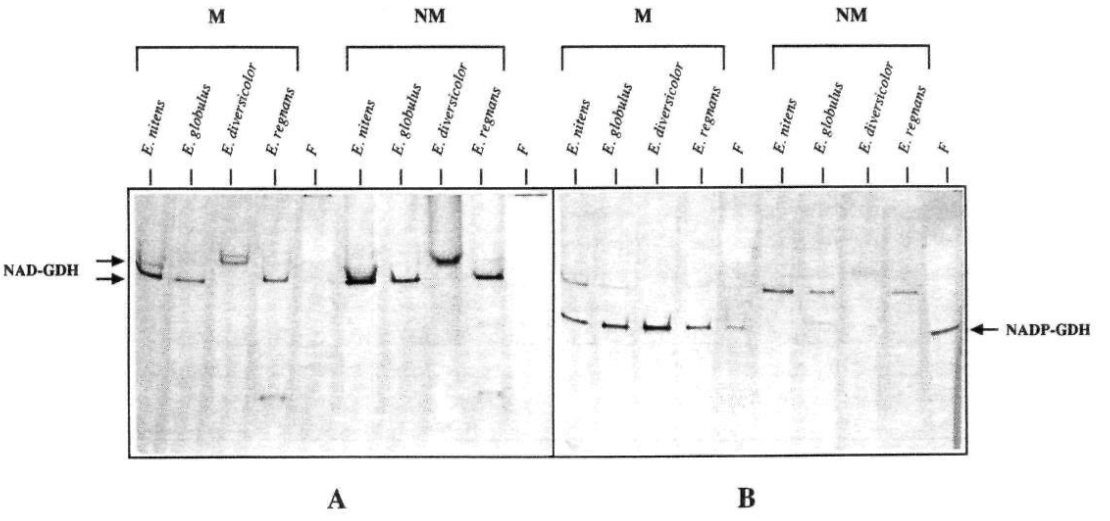

Figure 1. Uniform (6%) polyacrylamide gel electrophoresis of NAD-GDH (**A**) and NADP-GDH (**B**) extracted from *Hebeloma westraliense* in pure culture (F), non-mycorrhizal roots (NM) and ectomycorrhizas (M). Gels were stained for NAD-GDH (**A**) and NADP-GDH (**B**). Each well was loaded with about 25 μg of proteins (crude extract). NAD-GDH was also slightly detected with NADP as a cofactor in the deamination reaction on gels.

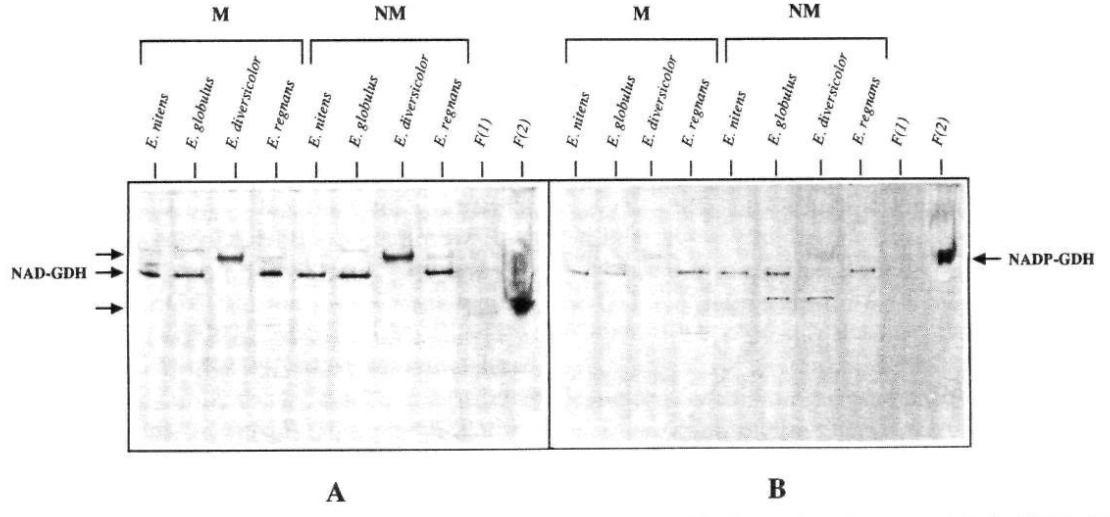

Figure 2. Uniform (6%) polyacrylamide gel electrophoresis of NAD-GDH (**A**) and NADP-GDH (**B**) extracted from *Laccaria laccata* (F), non-mycorrhizal roots (NM) and ectomycorrhizas (M). F(1) is an enzymic extract from a stipe, several weeks old, of *L. laccata* collected under eucalypts; F(2) is an enzymic extract of mycelium of *L. laccata* cultivated in a Petri dish. Gels were stained for NAD-GDH (**A**) and NADP-GDH (**B**). Each well was loaded with about 25 μg of proteins (crude extract). NAD-GDH was also slightly detected with NADP as a cofactor in the deamination reaction on gels.

acrylamide gradient gel electrophoresis, were 410 kDa for *E. diversicolor* and 375 kDa for *E. nitens*, *E. globulus* and *E. grandis*.

No NAD-GDH bands were found in the mycelium of *Hebeloma westraliense* cultivated in pure culture and only the plant NAD-GDH was detected in ectomycorrhizas (Fig. 1A). The free-living mycelium of *H. westraliense* showed a single NADP-GDH band with an R_f of 0·44 which was also found in the *Eucalyptus/Hebeloma* ectomycorrhizas (Fig. 1B).

These results contrast with those obtained with *Laccaria laccata*. The symbiont in pure culture exhibited an NAD-GDH band at R_f 0·40 (Fig. 2A) and an NADP-GDH band at R_f 0·30 (Fig. 2B), which were not detected in the associated ectomycorrhizas. Consequently the significant activities of NAD-GDH detected in the eucalypt/*Laccaria* ectomycorrhizas were obviously due to the plant enzyme.

NADP-GDH bands were not detected on polyacrylamide gels from *Scleroderma verrucosum* and *Pisolithus tinctorius*, either when the fungi were cultivated in pure culture or when they were associated with eucalypt roots. This is obviously due to the very low activity of the enzyme in the fungal cells (Table 1). By contrast, NAD-GDH bands were

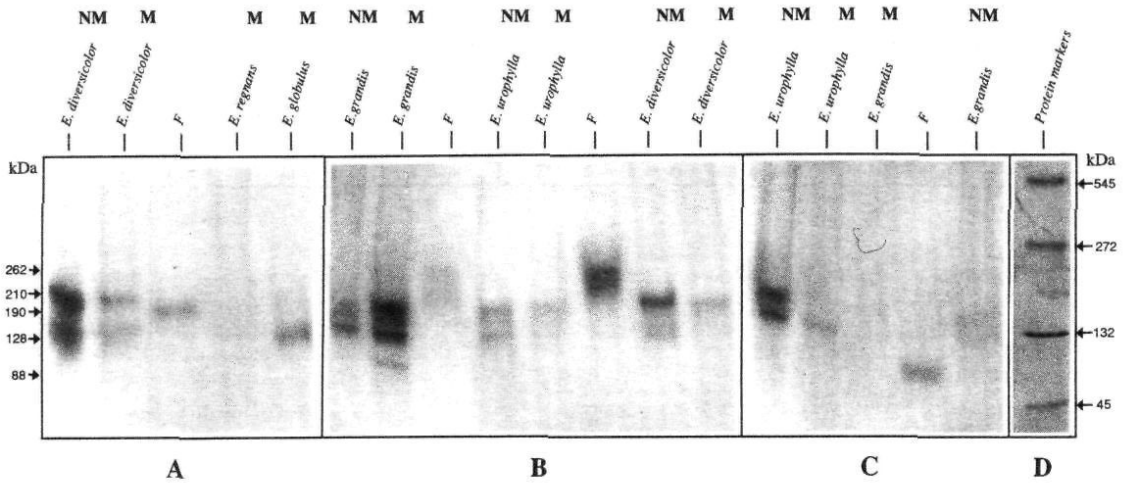

Figure 3. Electrophoretic patterns on gradient gels of AAT extracted from non-mycorrhizal roots (NM), ectomycorrhizas (M), and free-living fungi (F). The ectomycorrhizal fungi were *Laccaria laccata* (**A**), *Scleroderma verrucosum* (**B**) and *Pisolithus tinctorius* (**C**). Protein markers represented in (**D**) were urease I (545 kDa), urease II (272 kDa), bovine serum albumin (132 kDa) and chicken egg albumin (45 kDa). Each well was loaded with approximately 40 μg protein, except for (left to right) wells 2, 4, 8 and 16, which were loaded with 28 μg of protein. Protein markers (8 μg) were stained with Coomassie blue.

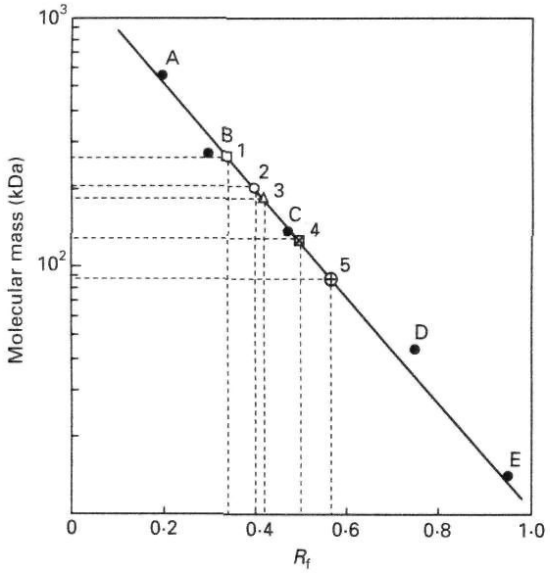

Figure 4. Molecular mass estimation of AAT from eucalypt ectomycorrhizas and ectomycorrhizal fungi. Molecular masses of standard marker proteins were plotted as a function of R_f derived from polyacrylamide gradient gel electrophoresis. The standard proteins were: (A) urease I (545 kDa), (B) urease II (272 kDa), (C) bovine serum albumin (132 kDa), (D) chicken egg albumin (45 kDa) and (E) α-lactalbumin (14·2 kDa). The dashed lines mark the mobility and the corresponding molecular mass of the enzyme. 1, *Scleroderma verrucosum* (262 kDa): 2, first isoform of eucalypt (210 kDa); 3, *Laccaria laccata* (190 kDa); 4, second isoform of eucalypt (128 kDa); 5, *Pisolithus tinctorius* (88 kDa).

detected in the free-living mycelium of both fungi (not shown). However, the expression of these fungal NAD-specific GDHs in the *Eucalyptus* ectomycorrhizas has, so far, not been determined. Indeed, in our standardized conditions, NAD-GDH of *S. verrucosum* had approximately the same electrophoretic mobility on gels as those found in the host roots. As for *P. tinctorius*, the NAD-GDH bands detected in the *Eucalyptus* ectomycorrhizas were too faint to establish whether the fungal enzyme remains active in the association.

Aspartate aminotransferases. By using polyacrylamide gradient gels, two distinct isoforms of AAT were usually detected in non-mycorrhizal roots of eucalypts (Fig. 3). The two root isoforms were estimated to be 128 and 210 kDa (Fig. 4).

The *Laccaria laccata* AAT was detected as a single band estimated to be 190 kDa (Fig. 4). This fungal band was not found when *L. laccata* was associated with *E. diversicolor*, *E. regnans* and *E. globulus* (Fig. 3A). Similarly, the single bands found respectively in *S. verrucosum* (262 kDa) and in *P. tinctorius* (88 kDa) were not detected when the fungi were associated with *E. grandis*, *E. urophylla* and *E. diversicolor* (Fig. 3B, C). This strongly suggests the absence of the fungal AAT form in eucalypt ectomycorrhizas.

Although the AAT staining method used in this work is not sensitive enough to quantify the different isoforms, it is clear that formation of mycorrhizas very likely modifies plant banding patterns. Indeed, the staining intensity of the 128 kDa band found in non-mycorrhizal roots of *E. diversicolor* and *E. urophylla* appeared, by comparison with the other plant isoform, to be reduced in the roots associated with *L. laccata* and *S. verrucosum*, respectively (Fig. 3A, B). The same was true for the 210 kDa band, which was not detected in the *E. urophylla*/*P. tinctorius* association (Fig. 3C). Synthesis of new plant isoforms during the formation of mycorrhizas also cannot be ruled out. In this experiment, a third AAT band of about 90 kDa, not detected in non-

mycorrhizal roots, was clearly revealed in the *E. grandis*/*S. verrucosum* association (Fig. 3B). However, many artefacts, such as polymerization, depolymerization and loss of activity of a particular isoform, can occur during electrophoresis, and further investigations are required before any definite conclusion can be drawn.

DISCUSSION

Previous results have suggested that fungal gene expression is moderated by the host plant. Indeed, evidence from enzymic reactions in crude extracts, electrophoretic patterns and immunological tests using antibodies raised against purified NADP-GDH have consistently shown that the accumulation of the GDH polypeptide is strongly suppressed in beech ectomycorrhizas. By contrast, the fungal NADP-GDH was active in ectomycorrhizas of several coniferous tree species (Martin *et al.*, 1986; Dell *et al.*, 1989). The present results clearly show that, in eucalypt associations, expression of the fungal GDH polypeptides also depends on the fungus. In the *Eucalyptus*/*Hebeloma westraliense* association, NADP-GDH was present, while in the *Eucalyptus*/*Laccaria laccata* association both fungal NADP-GDH and NAD-GDH were repressed. In the early studies, work was confined to the expression of the NADP enzyme alone. The present results also indicate that the fungal NAD-GDH enzyme is modified in the ectomycorrhizas.

The expression of fungal NADP-GDH has previously been demonstrated in spruce/*Hebeloma* sp. ectomycorrhizas (Dell *et al.*, 1989), and experiments with ^{15}N confirmed that the enzyme operated to assimilate nitrogen, with approximately one-third of the ammonium entering the glutamate-amino N and the remainder entering the glutamine-amide N (Chalot *et al.*, 1991). Consequently, it is not surprising to detect fungal NADP-GDH in *Eucalyptus*/*H. westraliense* ectomycorrhizas. It is somewhat more surprising to note the absence of NADP-GDH in the association between eucalypts and *L. laccata*. Indeed, immunogold labelling techniques have shown that the fungal enzyme was present in Douglas fir/*L. laccata* ectomycorrhizas (Brun, 1992). In that case, the fungus was the strain S-238 isolated from *Tsuga mertensiana* in the USA, and a few preliminary culture tests in the presence of different nitrogen sources have shown that this strain was slightly different from the Australian *L. laccata* used in this experiment. The use of a different host plant, in addition to another strain, might explain such a discrepancy. It would be of value to use the antibodies raised against NADP-GDH of S-238 to confirm the absence of the enzyme in eucalypt ectomycorrhizas.

The physiological differences between *Hebeloma* and *Laccaria* are not fully understood. The two genera are not closely related and form ectomycorrhizas that are anatomically distinct. *Hebeloma* gives rise to superficial ectomycorrhizas with a mycelium loosely adpressed to the host cells with a similar structure to those initiated by *Hysterangium* and *Cortinarius*, while the mycelium of *Laccaria* is more closely associated with the plant, as there is an extensive Hartig net between the radially expanded epidermal cells. Detailed anatomical studies of this association have already been published by Malajczuk *et al.* (1987). Immunological labelling of NADP-GDH of *Cenococcum geophilum* in spruce and beech ectomycorrhizas has clearly indicated that the amount of the enzyme decreased progressively from the peripheral cells of the sheath to the most internal cells of the Hartig net, irrespective of the plant taxa (Chalot, Brun & Botton, 1990*a*). Although we cannot generalize, as an even distribution of this enzyme has also been found in young ectomycorrhizas of Douglas fir/*Laccaria laccata* (isolate S-238) (Brun *et al.*, 1994), it is tempting to ascribe to the plant a part of the enzymic repression which can differentially affect the fungus according to the degree of fungal dependence on the host. The mechanism of repression so far remains unknown although glutamine accumulated in the fungal sheath has been regarded as a possible repressor of NADP-GDH synthesis (Martin *et al.*, 1993). However, we have no information demonstrating correlative accumulations in the different eucalypt ectomycorrhizal associations.

The lack of any pronounced activity of NADP-GDH in *Pisolithus tinctorius* is in agreement with the findings of Kershaw & Stewart (1992). These authors showed that assimilation of ^{15}N-labelled ammonium by this fungus was entirely consistent with the operation of the GS/GOGAT cycle, glutamate being provided by transamination, and GDH (assumed to be NADP-GDH) playing no role. This also implies that, in *P. tinctorius*, NAD-GDH is not an alternative to synthesis of glutamate as already demonstrated in vascular plants (Robinson *et al.*, 1991). Such a result, indicating the presence of an NAD-GDH in *P. tinctorius*, contrasts with that obtained by Vézina *et al.* (1989), where no enzyme activity was detected in the fungus in pure culture. Although the extraction conditions of the enzyme were different, it is also possible that some *P. tinctorius* strains might have little, if any, NAD-GDH polypeptide.

The present results demonstrate the lack of fungal AAT activity in the eucalypt ectomycorrhizas irrespective of the fungal species, and are in complete agreement with previous data obtained with several ectomycorrhizal associations. Indeed, results from electrophoretic patterns, as well as immunological tests and immunocytochemical labelling using antibodies raised against *Cenococcum geophilum*, were consistent with a strong suppression of synthesis of

the fungal AAT in spruce and beech ectomycorrhizas (Chalot *et al.*, 1990*b*). Experiments using ^{15}N labelling also suggest that AAT is not operative in the fungal compartment of the spruce/*Hebeloma* sp. association, as no labelled aspartate was detected (Chalot *et al.*, 1991). The absence of labelling in aspartate was also observed in *Pinus/Paxillus involutus* ectomycorrhizas by Finlay *et al.* (1988) and these authors, during a study of the labelling of aspartate in root axes, suggested that the presence of plant tissues was necessary to the synthesis of this amino acid. In the association between *Eucalyptus globulus* and *Pisolithus tinctorius*, Rabbani *et al.* (1992) also noticed the nearly complete absence of labelled aspartate in the mycelium collected in the vicinity of the roots which were supplied with ^{14}C-labelled alanine, while the fungus in pure culture accumulated substantial amounts of labelled aspartate.

It is obvious from the enzymological assays that formation of mycorrhizas was accompanied by a significant increase of AAT activity. As the fungal enzyme was repressed in the symbiotic tissues, it is the plant isoforms that should be stimulated. Except for the association between *Eucalyptus grandis* and *Scleroderma verrucosum*, where an additional band was detected, there was no evidence from the banding patterns on gels of an increase in the synthesis of the plant isoforms. Indeed, it was surprising to note the partial losses of some plant bands during formation of mycorrhizas. This apparent discrepancy can be explained by the fact that detection of AAT activity with fast violet blue staining can give only relative rather than absolute quantitative data and, in addition, a strict comparison of the banding patterns would have required all wells to have been loaded with the same AAT activity, which was not possible in this experiment. However, metabolism of ^{14}C-labelled alanine in *E. globulus/P. tinctorius* ectomycorrhizas was indicative of an increased activity of the plant AAT isoforms, as labelled aspartate was detected at significant levels in parts of the seedlings only when the roots were mycorrhizal (Rabbani *et al.*, 1992).

Repression of fungal enzymes, and more especially AAT, in the symbiotic tissues suggests that some amino acids might be translocated from the plant to the fungus. In the experiment carried out by Rabbani *et al.* (1992), in addition to alanine which was provided to the roots, glutamate was another candidate for translocation to the mycelium, but there was no evidence of translocation of aspartate, the main amino acid synthesized by AAT. However, organic acids can also be translocated and a better understanding of the exchange of amino acids between the partners requires additional investigation.

Repression of enzymes in ectomycorrhizas is obviously not restricted to those involved in nitrogen cycling. Sen (1990) reported that an acid phosphatase isoform, detected by electrophoretic analysis in vegetative mycelium of *Suillus bovinus* and *S. variegatus*, was not expressed in *Pinus sylvestris* ectomycorrhizas. Recent investigation with a number of enzymes including esterases, peptidases and several dehydrogenases has generally shown that expression of fungus and host-specific isozyme activities is strongly dependent on the host–fungus species combination (Rosendahl & Sen, 1992).

In conclusion, it appears that ectomycorrhizal fungi employ several pathways which assimilate ammonium, the major nitrogen component of the forest soils, but their high degree of metabolic integration during mycorrhizal development modifies expression and distribution of the N-assimilating enzymes in the symbiotic tissues. Although the present results based on enzyme activities are in general agreement with previous data demonstrating a repression of enzyme biosynthesis in the symbiont, other mechanisms, such as production of metabolically active compounds which would interfere with the enzymic reactions, cannot be ruled out. This emphasizes a need for further work on the regulation of the fungal enzymes in ectomycorrhizas.

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