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Assimilation of mineral nitrogen and ion balance in the two partners of ectomycorrhizal symbiosis: Data and hypothesis

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Summary. Assimilation pathways of mineral nitrogen and ion balances of the two partners of ectomycorrhizal symbiosis (fungi and woody plants) are reviewed. Data are presented about the partners both in pure culture and in mycorrhizal association. The two forms of mineral nitrogen, ammonium and nitrate, differ in their mobility in the soil, their transport into the cells, their uptake rates by plants and their assimilation pathways. These metabolic differences are related to differences in adjustment of ion balances and carbon metabolism under conditions of nitrate or ammonium nutrition. The data obtained on the partners of ectomycorrhizal symbiosis are discussed from this point of view and the observations composed with those on herbaceous angiosperms.
Key words. Nitrate; ammonium; nitrate reductase; ion balance; organic acids; carbon metabolism; ectomycorrhizal association; woody plants; ectomycorrhizal fungi.

Ammonium and nitrate are the major mineral nitrogen sources used by plants. The uptake and the assimilation of these nutrients cause modifications of the ion balance of tissues, with the production of H^+ and OH^- ions. Mechanisms for the metabolic adjustment of ion balance in herbaceous plants have been reviewed elsewhere [71, 87]. In this article, the recent findings about the capacities for and the consequences of the use of these two mineral nitrogen sources by ectomycorrhizal fungi and forest trees are presented and discussed.

Assimilation pathways of ammonium and nitrate

Uptake of the two nitrogen forms. The two ions, ammonium and nitrate, differ in their mobility in the soil [34] and also in their rate of uptake by plants.

Reduced mineral nitrogen generally occurs in the form of NH_4^+: the pK of NH_3/NH_4^+ is 9.25, and at the neutral or acidic pH usually found in natural conditions, > 99% of the total ammonia is in the form of NH_4^+ [11]. The plasma membrane is more permeable to NH_3 than NH_4^+, and the neutral form can be taken up via a non-specific passive diffusion. The transport of NH_4^+ is also a passive process along the electrical potential difference ($\Delta\psi$) across the membrane, created by the functioning of proton pumps and leading to a $\Delta\psi$-dependent uniport [39]. It is probable that NH_4^+ transport is mediated by a specific carrier because it is saturable, with K_m values of the order of micromolar. Ammonium entry is under genetic control in most bacteria and fungi studied [39].

As opposed to the entry of ammonium, the entry of NO_3^- into the cell is discouraged by the electrical potential difference across the plasma membrane. Thus, nitrate transport is active and probably occurs by means of a membrane carrier [16, 89].

Given the different nature of the two types of transport, it is to be expected that the ammonium uptake rate will be higher than that of nitrate in herbaceous plants, mycorrhizal or non-mycorrhizal forest trees or ectomycorrhizal fungi (table 1). However, woody plants absorb both NH_4^+ and NO_3^- at low rates as compared to herbaceous plants and mycorrhizal fungi.

The two partners of ectomycorrhizal symbiosis accumulate low amounts of nitrate in the vacuoles. In ectomycorrhizal fungi, the maximal levels are about 10 µmol · g f.wt^{-1} [70]. Similarly, in coniferous trees, nitrate does not appear to accumulate to a great extent [48, 52, 79, 82]. Young maritime pines (*Pinus pinaster*), 1–5-months old, grown in 5 mM NO_3^-, had nitrate levels between 0.5 and 2.3 µmol · g root f.wt^{-1}. This corresponds to concentrations of approximately 3 mM (70–80% moisture content); only traces of NO_3^- were found in needles [82]. After 24 h, the roots of 35-day-old Austrian pines (*Pinus nigra nigricans*) had accumulated 10 µmol NO_3^- · g f.wt^{-1} from a 5-mM NO_3^- solution; nitrate could not be detected in the needles [52]. In contrast, in herbaceous plants, the NO_3^- content can be greater than 100 µmol · g f.wt^{-1} [5, 33]. In many species vacuolar accumulation of NO_3^- and K^+ contributes to osmotic potential (e.g., underwater stress). It is clear that in woody plants NO_3^- cannot play a similar role.

Assimilation of the two nitrogen forms. Although there are few experimental data on the partners of the ectomycorrhizal symbiosis, the biochemical pathways of mineral nitrogen assimilation seem to be identical to those of the other plants (fig. 1).

Nitrate reductase (NR) activity has been measured mainly in vivo in woody plants (table 2) because of problems

Table 1. Mineral nitrogen uptake rates (µmol · g d.wt^{-1} · h^{-1}) for woody plants, ectomycorrhizal fungi, ectomycorrhizal roots and herbaceous angiosperms

Plant species	NH_4^+	NO_3^-
Host-plants for ectomycorrhizal fungi		
Pseudotsuga menziesii	3.8–6 [74]	0.6–0.8 [75]
Picea sitchensis	4.0–6.0 [74]	0.6–1.0 [75]
Tsuga heterophylla	1.8–4.0 [74]	0.8 [75]
Pinus radiata	7.0 [25]	
Pinus nigra nigricans	2.5 [50]	
Ectomycorrhizal fungi		
Hebeloma crustuliniforme	310 [46]	39 [47]
Laccaria laccata	190–360 [47]	
Pisolithus tinctorius	210–600 [47]	
Cenococcum graniforme	19 [29]	43 [29]
Mycorrhizal roots		
*Fagus sylvatica**	14-32 [13]	0–1.0 [8]
Hebeloma crustuliniforme and		
Pseudotsuga menziesii	7–9 [74]	0.6–1.4 [75]
Picea sitchensis	6.5–9 [74]	0.6–1.4 [75]
Tsuga heterophylla	3.8–7.8 [74]	
Herbaceous plants		
Triticum aestivum	100.5 [62]	
Glycine max	50–83 [90]	
Zea mays		50 [89]
Hordeum vulgare	75 [8]	60 [8]

* Fungal symbiont has not been identified.

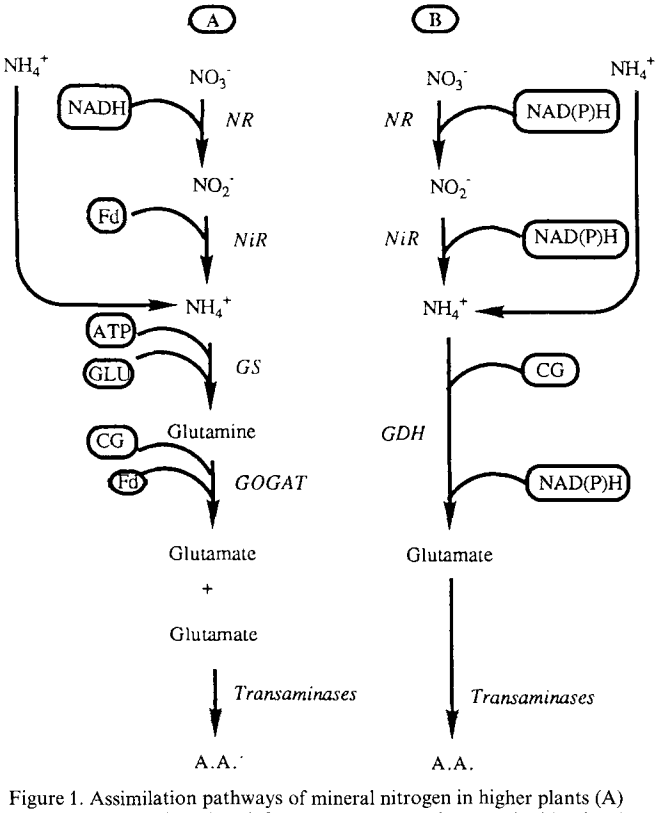

Figure 1. Assimilation pathways of mineral nitrogen in higher plants (A) and fungi (B). Fd (reduced feredoxine during photosynthesis); GLU (glutamate); CG (2-oxoglutarate); NR (nitrate reductase); NiR (nitrite reductase); GS (glutamine synthetase); GDH (glutamate dehydrogenase); GOGAT (glutamate oxoglutarate aminotransferase).

with in vitro measurement due to enzyme inhibition during extraction. In the hosts of ectomycorrhizal fungi that have been studied, NR is very variable in the roots, very low in the shoots and the activity is always less than the activity found in the different organs of herbaceous plants (table 2). NR activity is always higher in the roots than in the shoots, in contrast to the findings in herbaceous angiosperms (table 2). It is probable that NR of trees is NADH-dependent, as in most higher plants[3]. Indeed, nitrate reductase extracted from leaves, stems and roots of peach trees (*Prunus persica*) shows a specificity for NADH and is inducible by its substrate, nitrate[72].

In maritime pines previously grown without nitrate, in vivo NR activity is inducible in the roots and shoots (fig. 2). Maximal activity is reached after 48 h in roots and after 24 h in shoots. Excision of the organs does not modify the induction kinetics of NR activity in shoots although the amount of the anion (^{15}N labelled) arriving in leaf cells is greatly increased (table 3). The data in table 3 enable us to calculate in situ nitrate reductase activities from accumulated quantities of reduced ^{15}N, which are respectively 0.19 and 0.57 $\mu mol \cdot h^{-1} \cdot g\ f.wt^{-1}$ for excised roots and shoots. As compared to in vivo NR activity measurements, the maximal capacity of reduction in roots is not reached (potential NR activity of 1.0 $\mu mol \cdot h^{-1} \cdot g\ f.wt^{-1}$ after 24 h of induction) whereas for the aerial parts the assay in vivo (between 0.4 and 0.5 $\mu mol \cdot h^{-1} \cdot g\ f.wt^{-1}$ is similar to the in situ nitrate reduction rate. On the other hand, if the nitrate content reaches levels higher than the reduction capacity, this leads to an accumulation of the anion in leaf cells which is rarely observed in the intact plant (table 3)[82]. These results suggest that in maritime pines, the maximal reduction capacity in the shoots is limited and does not depend on the amount of the anion arriving in these organs. In contrast, in roots, the reduction capacity is strongly inducible, and nitrate uptake is limiting.

The first NR activity measurements on mycorrhizal fungi were performed in vivo on spores from different strains of *Glomus mossae*[35] and on mycelia of 7 ectomycorrhizal species[36]. The activities measured varied between 0.47 and 2.22 $\mu mol\ NO_2^- \cdot h^{-1} \cdot g\ d.wt^{-1}$, values which are low as compared to those obtained for herbaceous plants (table 2). However, the in vivo measurement used in this work probably underestimates the actual reduction capacity for these fungi, because the method is based on blocking the nitrite reductase (NiR) by inhibiting the generation of reducing power for this enzyme[21,32]. In fungi, the preferential electron donor for both NR and NiR might be NADPH (fig. 1)[3,28].

The nitrate reductase was extracted and its activity measured in vitro in the ectomycorrhizal Basidiomycete *Hebeloma cylindrosporum*[69]. Grown on nitrate medium (KNO_3 6 mM), thalli have on average a NR activity of 5.0 $\mu mol\ NO_2^-$ produced $\cdot h^{-1} \cdot g\ f.wt^{-1}$, which corresponds to an activity of 50 $\mu mol\ NO_2^-$ produced $\cdot h^{-1} \cdot g\ d.wt^{-1}$ (d.wt = 10% of f.wt), a value of the same order of magnitude as is measured in higher plants. In contrast to the NR of *Neurospora crassa*, which accepts NADH as an electron donor[43], the nitrate reductase of *H. cylindrosporum* used exclusively NADPH[69]. Condi-

Table 2. In vivo measurements of nitrate reductase in shoots or roots of woody or herbaceous species. The plants were non-mycorrhizal and the activities are expressed in $\mu mol \cdot NO_2^- \cdot h^{-1} \cdot g\ d.wt^{-1}$.

Plant species	Roots	Shoots
Herbaceous plants		
Triticum aestivum	8[64]	
	18[2]	50[2]
Spinacia oleracea		40[58]
Glycine max	35[37]	
Woody plants		
Pseudotsuga menziesii	13.3[36]	
	0.7[4]	0.43[4]
Pinus contorta	3.28[4]	1.57[4]
Pinus banksiana	0.15[49]	0.02[49]
Pinus radiata	1.93[1]	Not measurable[1]
Eucalyptus regnans	2.71[1]	0.87[1]
* *Picea abies*		1.52[86]
* *Pinus halepensis*		0.64[86]
* *Pinus ponderosa*		2.76[86]
* *Pinus sylvestris*		3.36[86]

* Given values are calculated from author's data obtained after feeding the shoots with KNO_3 5 mM and assuming that d.wt is 25% of f.wt.

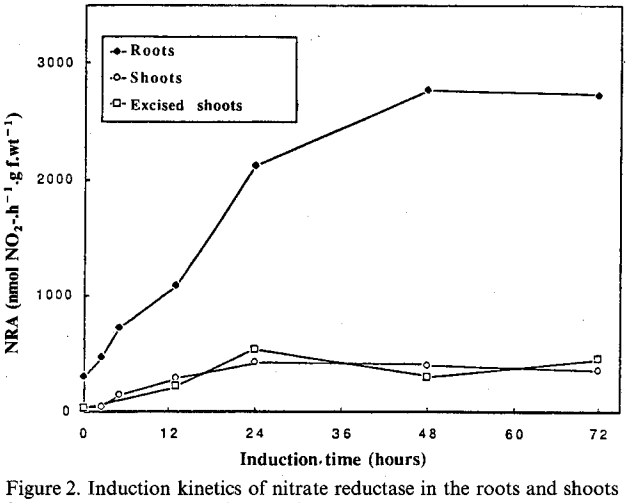

Figure 2. Induction kinetics of nitrate reductase in the roots and shoots from young non-mycorrhizal maritime pines (*Pinus pinaster*). Plants or excised shoots are incubated in a solution containing KNO_3 10 mM and $CaCl_2$ 1 mM. Nitrate reductase activity (NRA) is measured in vivo (from Scheromm[81]).

Table 3. Accumulation of ^{15}N-nitrate and reduced ^{15}N by roots and shoots of non-mycorrhizal maritime pines (*Pinus pinaster*) incubated for 24 h in a solution containing KNO_3 (10 mM) and $CaCl_2$ (1.0 mM). Organs were intact or excised. The values in the brackets are confidence intervals for p = 0.05 (from Plassard[68]).

	Intact plants		Excised organs	
	Roots	Shoots	Roots	Shoots
Accumulated nitrate	1.27	0.14	1.44	9.39
($\mu mol \cdot g\ f.wt^{-1}$)	(0.3)	(0.03)	(0.33)	(4.02)
Accumulated reduced ^{15}N	6.89	1.20	4.61	13.81
($\mu mol \cdot g\ f.wt^{-1}$)	(0.87)	(0.77)	(2.97)	(3.72)

Table 4. Comparison of nitrate reductase activities of *Neurospora crassa* and *Hebeloma cylindrosporum* grown in different conditions. NRA: specific nitrate reductase activity in vitro (μmol $NO_2^- \cdot h^{-1} \cdot$ mg protein^{-1}).

Medium	NRA in fungal species	
	Neurospora crassa[a]	*Hebeloma cylindrosporum*[b]
Without NO_3^-	nd[c]	2.25
NH_4^+	0.0	1.84
NH_4^+ + organic acid	nd[c]	0.13
NO_3^-	0.75	1.34

[a] Values for *Neurospora crassa* are from Nason and Evans [65].
[b] Data for *Hebeloma cylindrosporum* are from Scheromm [81].
nd[c]: no data available.

tions necessary for the appearance of the activity differ for the two fungal species (table 4). In *N. crassa* mycelia, NR activity is inducible by its substrate, nitrate, whereas in *H. cylindrosporum* thalli, this anion is not indispensible to the appearance of the enzyme (table 4). If ammonium is supplied as sole nitrogen source, *H. cylindrosporum* shows very slow growth; rapid growth can be reestablished by the addition of organic acids to the ammonium medium [84] (see fig. 4). Taken together, these results suggest that the NR activity of *H. cylindrosporum* would be depressed by nitrogen deficiency or when a source of nitrogen which is badly assimilated is supplied to the thalli [85].

Ammonium, from the reduction of nitrate or from the external medium, is finally incorporated into carbon skeletons in order to produce amino acids according to two pathways: the glutamate dehydrogenase (GDH) pathway in fungi [59, 61] and the glutamine synthetase (GS)/glutamate oxoglutarate aminotransferase (GOGAT) pathway in higher plants [61, 66] (fig. 1).

In forest trees, ammonium assimilation seems to occur mainly by the GS/GOGAT pathway, as demonstrated by the detection of glutamine synthetase and glutamate synthase activities in *Fagus* ectomycorrhizas [55, 56], or extraction and measurements of GS in needles, stems and roots of jack pines (*Pinus banksiana*) [93].

On the other hand, GDH is the main enzyme of ammonium assimilation in most fungi [59] (fig. 1). The reaction catalyzed by GDH is reversible, and many fungi possess two forms of GDH: an NAD-dependent GDH, thought to operate primarily in glutamate catabolism, and an NADP-dependent GDH, thought to be primarily involved in glutamate synthesis. In fact, an NADP-dependent glutamate dehydrogenase has been extracted and purified from the ectomycorrhizal ascomycete *Cenococcum graniforme* [53]. This enzymatic activity has also been measured in vitro in *Pezizella ericae* [76] and *Hebeloma sp.* mycelia [20]. Labelling experiments with $^{15}NH_4^+$ carried out on mycelia of *Cenococcum graniforme* demonstrate that the primary ammonium assimilation is catalyzed by the sequential activity of GDH and GS [30]. Ammonium ion assimilation leads to the synthesis of large amounts of glutamine, alanine and arginine [51]. These amino acids represent the bulk of the free amino acids found in the mycelia of ectomycorrhizal fungi [23, 24, 30, 42]. However, when the fungus is associated with the root in mycorrhizas, the expression of the fungal NADP-dependent GDH seems to be largely controlled by the host-plant [20, 55] (see Martin and Hilbert, this review).

Nitrate and ammonium nutrition

As the transport of NH_4^+ through the membrane is a passive process and there is no proof of a significant accumulation of this cation in the cells, its uptake is closely linked to the biosynthesis of ketoacids involved in its assimilation. These ketoacids are the product of anaplerotic pathways which maintain the concentration of Krebs cycle intermediates to ensure energy production. Ectomycorrhizal fungi are able to fix CO_2 when grown in vitro with ammonium as a nitrogen source [27, 54, 57]. Biosynthesis of amino acids has been studied by nuclear magnetic resonance spectroscopy (NMR) following the incorporation of ^{13}C glucose by *Cenococcum graniforme* and *Sphaerosporella brunnea* mycelia. For the two species, carbon-13 labelling of C_2, C_3 and C_4 of glutamate, glutamine and arginine indicate the simultaneous participation of oxaloacetate and acetyl-CoA pools in ammonium assimilation. However, the high level of label in carbon atoms 2 and 3 indicates that the dominant flux of labelled pyruvate for ammonium assimilation involves anaplerotic fixation of CO_2 although the enzyme which catalyzes this dark fixation has not been identified [54, 57]. There are three enzymes known for anaplerotic CO_2 fixation (fig. 3); phophoenolpyruvate carboxykinase and pyruvate carboxylase activities have been detected in certain non-mycorrhizal filamentous fungi but phosphoenolpyruvate carboxylase, widespread in higher plants, has never been detected in the fungal species studied [14].

In an ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum* Romagn., the growth of the thalli is very low on an ammonium medium as compared to that obtained on a nitrate medium (fig. 4A). The addition of organic acids from the Krebs cycle (malate, citrate and succinate) reestablishes rapid growth (fig. 4B). Growth difficulties of *H. cylindrosporum* on ammonium medium are probably due to an insufficient supply of the ketoacids necessary in order to assimilate NH_4^+. As organic anions are actually absorbed by the thalli [81], these compounds

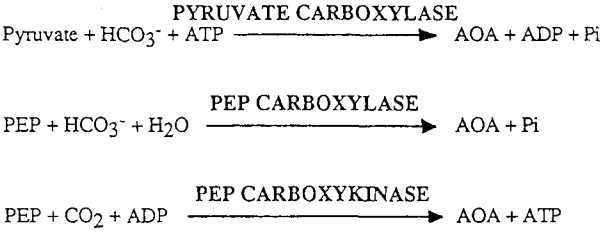

Figure 3. Enzymes involved in anaplerotic CO_2 fixation; PEP (phosphoenol pyruvate); AOA (oxaloacetate).

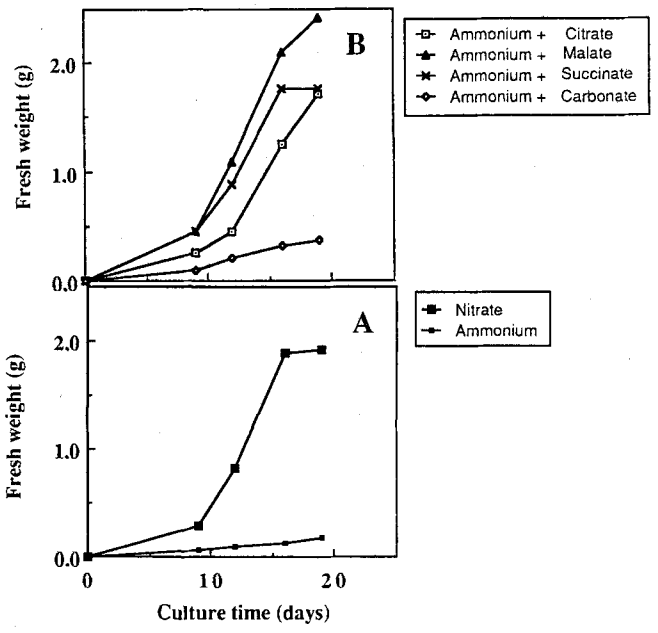

Figure 4. Growth of *Hebeloma cylindrosporum* thalli on nitrate or ammonium media. *A* Thalli grown on nitrate or ammonium; *B* Thalli grown on ammonium in the presence of an organic acid (citrate, malate, succinate) or in the presence of $CaCO_3$ (from Scheromm[81]).

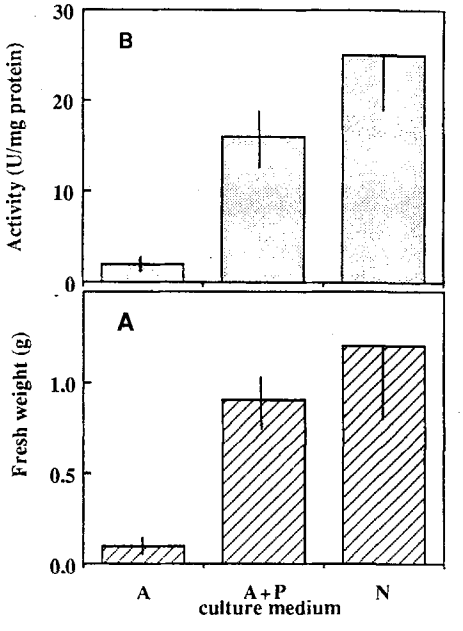

Figure 5. Growth and PEP carboxykinase activity of *Hebeloma cylindrosporum* thalli grown on ammonium (A), ammonium + pyruvate (A + P) or nitrate (N). One unit of enzymatic activity is defined as the quantity of enzyme catalyzing the oxidation of one nanomole of NADH per minute. Confidence intervals are given for $p = 0.05$ (from Scheromm[81]).

probably supply the carbon skeleton for mineral nitrogen assimilation in this species. In fact, a phosphoenolpyruvate (PEP) carboxykinase activity was detected[84] and measured in vitro. Enzyme activity was found to be very weak on the ammonium medium and to become significant only when nitrate was supplied as a nutrient or after the addition of an organic acid (pyruvate) to the NH_4^+ medium (fig. 5)[84]. These data therefore suggest that there is a close relationship between growth and PEP carboxykinase activity in the thalli of *Hebeloma cylindrosporum*, and that this activity could be a limiting factor in the use of NH_4^+ by this fungal species.

Roots of mycorrhizal or non-mycorrhizal forest trees are capable of incorporating ^{14}C into their tissues from labelled bicarbonate[12, 13, 27]. This dark fixation is stimulated in the presence of ammonium in the medium and glutamine, glutamic acid and aspartic acid are the predominant ^{14}C-labelled metabolites in non-mycorrhizal roots of *Pinus contorta*, *Pinus ponderosa* and *Pinus taeda*[27], and in mycorrhizal roots of *Fagus sylvatica*[13]. However, we have no data on the anaplerotic CO_2 fixation in woody plants.

In contrast to NH_4^+, NO_3^- is absorbed by the intermediary of an active transport. Its reduction, which is expressed by the equation:

$$NO_3^- + 8e^- + 8H^+ \longrightarrow NH_3 + 2H_2O + OH^-$$

should bring about a considerable increase in the cell pH. Two mechanisms ensuring the stability of this pH have been described for higher plants[71]: 1) the excretion of OH^- from the root cells towards the external medium: biophysical pH-stat, and 2) the synthesis of a carboxyl function and a proton by β-carboxylation: biochemical pH-stat.

The ion balances were determined for two ectomycorrhizal fungal species which grow well on a nitrate medium, *H. cylindrosporum* and *Pisolithus tinctorius*[60]. The observed excess of anion over cation uptake (table 5) must be equivalent to the net anion efflux in the medium[91], and the stoichiometry between anion efflux and nitrate assimilation can be calculated: the value is 0.94 for *H. cylindrosporum* and 0.73 for *P. tinctorius*.

In *H. cylindrosporum* the presence of a PEP carboxykinase activity under conditions of nitrate nutrition (fig. 5) could have an important role in β-carboxylation and make possible the synthesis of organic anions in response to the production of OH^- due to nitrate reduction. However, it has been determined that 0.99 molecules of OH^- are excreted per molecule of accumulated reduced nitrogen[68] (fig. 6). The charge balance is therefore maintained mostly by the function of the biophysical pH-stat in this fungal species.

On the other hand, in *P. tinctorius*, the direct measurement of the secreted OH^- yielded is 0.285 ± 0.08 OH^- molecule per assimilated and accumulated nitrate ion[68]. This is only about 40% of the anion efflux needed to maintain the charge balance in the medium. In this species, nitrate nutrition results in an excretion of pigments. The absorption spectrum of the culture medium has a maximum of 320 nm[68] and the measurement of the optical density of the medium as this wave length is well correlated with nitrogen accumulation in the thalli

Table 5. Amounts of anions and cations absorbed and accumulated by *Hebeloma cylindrosporum* or *Pisolithus tinctorius* thalli grown for 20 days in a nitrate medium (KNO_3 6 mM). For each line, means (n = 10) with the same letter are not significantly different for p = 0.05 (from Plassard[67]).

	Fungal species			
	Hebeloma cylindrosporum		*Pisolithus tinctorius*	
	Absorption ($\mu eq \cdot g\ f.wt^{-1}$)	Accumulation	Absorption ($\mu eq \cdot g\ f.wt^{-1}$	Accumulation
Potassium	50.9[b]	50.9[b]	129[a]	129
Calcium	4.6[c]	4.6[c]	27[a]	27[a]
Magnesium	14.4[b]	14.4[b]	57[a]	57[a]
Sodium	6.7[b]	6.7[b]	15[a]	15[a]
Total inorganic cations (C)	76.6[c]	76.6[c]	228[a]	228[a]
Phosphorus	25.9[b]	25.9[b]	55[a]	55[a]
Chloride	3.7[a]	3.7[a]	10[a]	10[a]
Sulfate (S)	25.6[b]	nm*	45[a]	nm*
Nitrate (N)	256.7[b]	0.7[c]	448.6[a]	1.6[c]
Total inorganic anions (A)	316.9[b]	30.3[d]	558.6[a]	66.6[c]
Organic anion accumulation (C-A)		43.6[b]		161.4[a]
Excess anion uptake (A-C)	240.3		330.6	

nm*: not measurable.

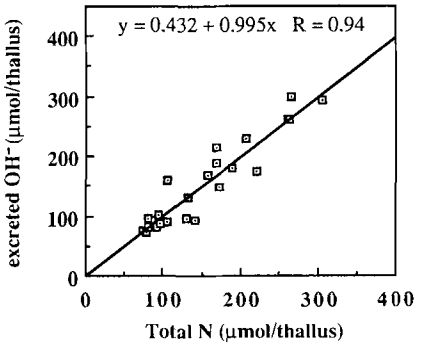

Figure 6. Relationship between the quantities of OH^- excreted in the culture medium by *Hebeloma cylindrosporum* thalli grown on nitrate (6 mM) and the accumulation of total N in the mycelia (from Plassard[68]).

(fig. 7). The difference between the calculated quantity of excreted anions and the quantity of OH^- secretion actually measured indicates the existence of a biochemical pH stat. In view of the correlation between pigment formation and nitrogen accumulation, and in view of the fact that phenol compounds can have dissociation constants sufficiently low to be ionized at neutral pH, it is tempting to speculate that the pigments excreted by *P. tinctorius* are involved in this pH-stat mechanism. Identification of the compounds is needed to further investigate this possibility.

In mycorrhizal or non-mycorrhizal trees, nitrate nutrition is possible[4,41,82,92], and the woody plants are almost always considered to reduce nitrate mainly in their roots[9,49,52,67,73]. In fact, a study with ^{15}N-labelled nitrate revealed that the roots of intact young maritime pines were found to reduce on the average 90% of the absorbed anion[82,83]. In woody plants, both nitrate or ammonium nutrition results in the accumulation of reduced nitrogen in the roots, and in the export of reduced nitrogen from the roots towards the shoots. For example, Margolis and Vézina[48] indicate that the concentrations and the composition of amino acids of the xylem sap of yellow birch (*Betula alleghaniensis*) grown on NO_3^- or NH_4^+ are very similar.

In maritime pines, the levels of total mineral cations are not significantly different in the shoots of pines grown on ammonium or nitrate[82]. As there is no nitrate accumulation, and as the nitrate absorbed is assimilated in the roots, organic anions must compensate for the charge of the cations transported in the xylem sap and accumulated in shoots. The capacity for synthesis of organic anions seems to be high in the maritime pine irrespective of the mineral nitrogen source[82]. Indeed, it can be higher than is needed to compensate for nitrate assimilation, which requires at most as much organic anion as the amount of reduced nitrogen in the plant. For example, in the case of maritime pines grown for 5 months in axenic conditions, the sum of organic anions is – until the third month of growth – higher than that of reduced nitrogen atoms (table 6). In this woody species, synthesis of these anions is probably partly independent of the reduction of nitrate. The measurement of net fluxes of anions and cations in young plants of *Pseudotsuga menziesii* incubated for 4 h in an ammonium medium made it possible for Bledsoe and Rygiewicz[6] to establish an ion balance

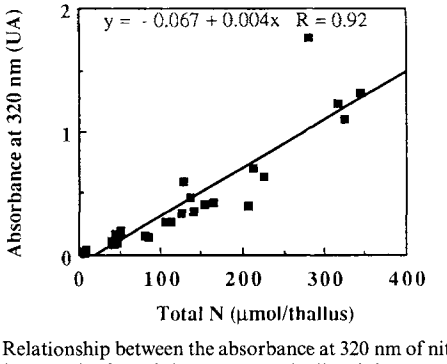

Figure 7. Relationship between the absorbance at 320 nm of nitrate medium after the growth of *Pisolithus tinctorius* thalli and the accumulation of total N in the mycelia. The absorbance of the unknown compound is expressed in arbitrary units (UA) with the optical density at 320 nm = 10^4 UA (from Plassard[68]).

Table 6. Amounts of organic anions and reduced nitrogen in non-mycorrhizal maritime pines (*Pinus pinaster*) grown axenically for 5 months in nitrate medium. Organic anion accumulation is estimated by the difference C-A as in table 5 (from Scheromm[81]).

Culture time (months)	Organic anions (μeq · plant^{-1})	Reduced nitrogen (μmol · plant^{-1})
1	33.7	29.7
2	202.5	94.2
3	396.6	300.1
4	292.3	392.1
5	349.8	369.3

Table 7. Organic anion composition of roots of three-month-old maritime pines (*Pinus pinaster*). The plants were first grown in gnotobiotic conditions without nitrogen supply and incubated for 10 days in hydroponic conditions on nitrate (N) or ammonium (A) medium (from Scheromm[81]).

Organic acid (μmol · g f.wt^{-1})	Culture medium N	A
Quinic	43.0	77.2
Shikimic	10.0	3.1
Citric	4.9	nd
Malic	4.6	nd

nd: not detectable.

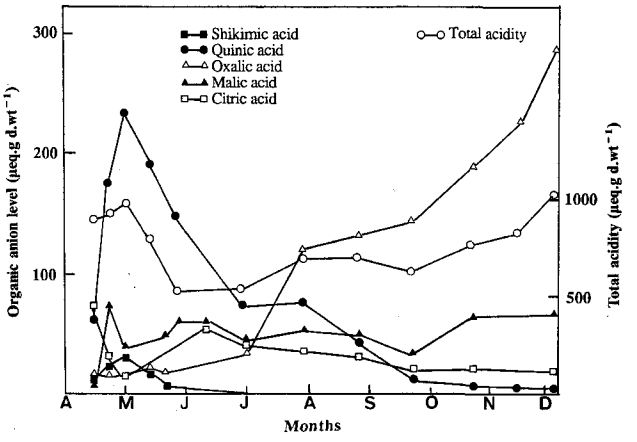

Figure 8. Evolution of organic anion levels and of total acidity in the leaves of *Quercus pedunculata* during an annual cycle (redrawn from Boudet[10]).

for the non-mycorrhizal roots of this plant. The values of proton excretion and of organic anion synthesis are very high as compared to those usually measured on agricultural species. However, according to the authors, these values are not representative of long-term mineral nutrient acquisition, but only of the time during which NH_4^+ uptake occurs[6].

In many woody plants, the alicyclic acids, quinic acid and shikimic acid, rather than the acids of the Krebs cycle, are the most abundant organic anions[10,17,18,80] (table 7). These compounds, precursors of aromatic acids and therefore of lignin, are synthesized from PEP produced by glycolysis and from erythrose-4-phosphate formed in the pentose pathway. This biosynthetic route is therefore independent of the β-carboxylation reactions generally associated with NO_3^- reduction. The predominance of alicyclic acids might be connected to the adaptation of many woody plants to ammonium nutrition[78]. However, the pH of the liquid extracted by crushing pine (*Pinus nigra* ssp. *nigricans*) or spruce (*Picea abies*) needles, corresponding to the vacuolar sap, is between 3.5 and 4.5[17]. The two alicyclic acids, quinic and shikimic acids, whose pK is 4.21, are therefore stored in the vacuoles often largely in protonated form, and their role in the ion balance of the cell is virtually unknown. In the leaves of oaks (*Quercus pedunculata*), quinic acid is abundant at the beginning of vegetation but decreases rapidly, whereas oxalate accumulates in large amounts (fig. 8). The levels of malate and citrate, which are always lower than that of the preceding acids, are almost invariable (fig. 8). Oxalate accumulation has been considered to be a reflection of nitrate reduction in fungi[45]. This interpretation is untenable for tree leaves in which NO_3^- reduction is almost negligible. A hypothesis to explain this phenomenon could be that the deamination of asparagine and/or aspartate (which can be predominant forms of reduced nitrogen transport from the roots towards the shoots) causes a high production of oxaloacetic acid, a known precursor of oxalic acid[15], in the leaf cells.

The adjustment of ion balance in fungi and trees

In mycorrhizal fungi, the accumulation of mineral cations and anions varies greatly according to the species (table 5), and the contribution of the acids of the tricarboxylic acid cycle (TCA) to the ion balance is not precisely known. In *Paxillus involutus*, nitrate nutrition leads to a stimulation of oxalate synthesis. This oxalate is mainly excreted to the external medium and produces calcium oxalate crystals, resulting from the interaction between the organic acid and calcium ions in the medium[45]. Oxalic acid is synthesized from oxaloacetate resulting either directly from CO_2 fixation by β-carboxylation, or from citrate, isocitrate and glyoxylate[44]. The excretion of this oxalate to the medium therefore corresponds to a particular variant of the biochemical pH stat.

In trees, oxalate could also play an important role in the establishment of the ion balance. Furthermore, pectic substances could be important: in the roots of maritime pines, nitrate nutrition causes a considerable increase in the levels of bivalent cations. Levels are multiplied by 10 and 3 respectively for total calcium and magnesium[82]. In very young maritime pine roots, 40% of calcium and 30% of magnesium are localized in the walls[31]. If we take these proportions into account, almost half of the increase of the calcium levels must be compensated by the negative charges in the walls. The assays of uronic acids performed on roots of dicotyledonous or monocotyledonous plants and coniferous trees (table 8) show that maritime pines have high levels of uronic acids, of the same order of magnitude as, or higher than, those of the dicotyledonous plants studied. However, the measurement of the cation exchange capacity (CEC) of these

Table 8. Uronic acids levels (UA) and cation exchange capacity (CEC) of young roots from different plant species. Data are expressed in meq · 100 g root d.wt^{-1}. Uronic acid levels are from Salsac[77] and CEC from Ghorbal[31].

	UA (meq · g d.wt^{-1})	CEC
Dicotyledonous plants		
Vicia faba	28–29	10–17
Lupinus luteus	32	8–11
Helianthus anuus	33–35	
Monocotyledonous plants		
Zea mays	7–13	
Sorghum vulgare	10–11	
Allium cepa	25	
Coniferous trees		
Pinus halepensis	26	16–20
Pinus pinaster	43	10–13

roots indicates that 73% of the charges from uronic acids do not participate in cation exchanges and we can assume that these charges are concealed by methyl groups. As the level of uronic acids does not vary in response to variation of nutrition[38,40], it might be hypothesized that the increase in the wall of calcium, and to a lesser extent of magnesium, is connected to a lower degree of methylation of uronic acids in nitrate nutrition. Finally, phosphate could contribute to ion balance. In maritime pines, the accumulation of P observed in the roots in nitrate nutrition[82] may be due to the functioning of the biophysical pH-stat, and correspond to an antiport $PO_4H_2^-/OH^-$, as the hydroxyl production is increased in the root cells upon reduction of nitrate.

Modifications induced by mycorrhizal association

Mycorrhizal symbiosis can increase uptake rates of ammonium and nitrate by higher plants. High intensity of ammonium uptake by fungal symbionts (cf. table 1) could explain the high levels of NH_4^+ uptake observed for intact[74] or excised[26] mycorrhizal roots. The stimulation of nitrate uptake in mycorrhizal symbiosis is subject to controversy[12,74,75]. France and Reid[26] observed that mycorrhizal roots had a significantly higher nitrate uptake capacity than non-mycorrhizal roots, *Pisolithus tinctorius* being a more efficient fungal partner than *Thelephora terrestris*. Moreover, the ratio between the ammonium and nitrate uptake rate was lower for mycorrhizal plants than for the non-mycorrhizal controls, suggesting a more pronounced effect of mycorrhizal symbiosis in nitrate rather than in ammonium nutrition. According to France (1980, cited in France and Reid[27]), the absorption capacity for the anion, measured in mycorrhizal roots with *Pisolithus tinctorius*, is 7 times higher than that estimated from the absorption capacity of the separated partners, suggesting a synergistic effect due to the association. The association of *Pseudotsuga menziesii* and *Hebeloma crustuliniforme* improves the growth and nitrogen levels of the plants grown in controlled conditions with nitrate or ammonium[7], with a more marked effect of the symbiosis on the utilization of NO_3^-. In this case, the fungal symbiont significantly improves the amounts of NO_3^- absorbed by this plant species[75]. However, the mycorrhizal effect depends on the host plant species because *Hebeloma crustuliniforme* has little effect on the NO_3^- uptake of *Picea sitchensis*. The improvement of phosphorus nutrition after mycorrhizal infection causes a better export of the nitrogen assimilated in the roots towards the leaves[63].

Until now, the measurement of the potential contribution of fungal enzymes to nitrate reduction by forest trees has received little attention. However, the behavior of a species such as the black pine (*Pinus nigra nigricans*) on calcareous soils, where mineral nitrogen probably occurs in the form of nitrate, illustrates the importance of the fungal symbiont. Without mycorrhizal infection, the tree does not develop normally. Mycorrhizal infection reestablishes growth, probably by making nitrate assimilation possible and improving protein synthesis[19]. This observation therefore suggests an important direct role of the mycorrhizal fungus. Recently, Finlay et al.[24] demonstrated that the fungus *Paxillus involutus*, associated with young *Fagus sylvatica* plants, is able to absorb and to reduce nitrate supplied only to the fungus, and to translocate reduced nitrogen towards the host plant. However, maritime pines which are associated with *Hebeloma cylindrosporum* and incubated for 48 h on $K^{15}NO_3$ (10 mM) do not absorb nor reduce more nitrate than non-mycorrhizal plants[83]. Under these experimental conditions, only the phloem transport is increased by the mycorrhizal infection[83].

Ion balance is another point to consider. NH_4^+ assimilation causes proton excretion while nitrate assimilation causes OH^- excretion[77]. Rygiewicz et al.[74,75] observed that mycorrhizal roots excrete less H^+ or OH^-, respectively, per ammonium or nitrate ion absorbed and assimilated, than non-mycorrhizal roots. Mycorrhizas could therefore act as a rhizosphere buffer and in particular facilitate ammonium uptake, which falls very quickly with the decreasing medium pH[22,90]. In an ammonium medium, a hypothesis to explain the decrease of proton excretion by mycorrhizal roots could be that the H^+ liberated in the interface of the Hartig net might be used in the transport of molecules exchanged between the fungal hyphae and the host cells, according to the models elaborated by Smith and Smith[88].

Conclusion

New data on the capacity for mineral nitrogen use of the partners of ectomycorrhizal symbiosis have recently become available. However, there are still not enough specific studies on the means of adjustment of the ion balance in these plants, whether they are considered alone or in association. The biochemical pathways involved in the main physiological functions of fungi and forest trees do not differ from those described for herba-

ceous angiosperms, but the relative importance of the different pathways is certainly not the same. Ion balance is ensured mainly by NO_3^- and organic acids of the TCA cycle in herbaceous plants, and this is never observed in fungi or in trees. In trees, alicyclic acids are important instead, probably as a consequence of the part they play in the biosynthesis of lignin. Thus, for mycorrhizal or non-mycorrhizal woody plants, which generally develop better on NH_4^+ than on NO_3^-, the dissociation between the functions of organic acids in the assimilation of mineral nitrogen and ion balance may be connected with the adaptation of these plants to using NH_4^+ as the sole nitrogen source.

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