NEW APPROACHES TO ENHANCED AMMONIUM ASSIMILATION IN PLANTS

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INTRODUCTION

Humanity requires intensive agriculture for support a growing population that is expected to double by the end of the next century. Consequently, the production and application of nitrogen fertiliser has grown exponentially (Eichler and Schulze, 1998).

Increased concentrations of nitrate have been observed in groundwater in many agricultural regions (Hackten-Broeke and van der Putten, 1997; McCracken et al., 1994) since nitrate, as a mobile anion, is susceptible to leaching (Raven et al., 1992). Overall, the additions to groundwater probably represent only a small fraction of the increased nitrate transported in surface waters. However, nitrate has a long residence time in many aquifers, meaning that groundwater quality is likely to continue to decline as long as human activities are having substantial impacts on the N cycle. Considerable losses of N from soil may also be caused by gaseous emissions which are affected by different fertiliser types in various ways (Smith et al., 1997; Freney, 1997). The largest NH$_3$ emissions have been measured when urea or when sludge were surface applied to soils with low proton buffering capacities (Sommer et al., 1997). Other gaseous N losses also occur during the process of nitrification and denitrification (Nielsen et al., 1996).

For sustained productivity and prevention of N related pollution problems, fertiliser N management needs to be improved by reducing losses of N via various mechanisms. There are ways to slow the growth of fertiliser use and also to reduce the mobility and, hence, the regional and global im-

pacts of the N that is applied to the fields. One is to increase efficiency of utilisation, requiring new data for many existing managements. Another is to reduce the use of nitrate as a fertiliser.

The use of ammonium as a fertiliser has called most attention because of its potential to reduce nitrate losses by leaching. Indeed various field studies have shown increased N use efficiency and positive yield responses when ammonium based fertilisers were used (Sommer et al., 1997; Martins-Loução and Cruz, 1999).

Since the application of ammonium fertilisers causes pH decreases in the bulk soil after nitrification, the availability and uptake of soluble nutrients such as P and also Fe, Mn, Cu and Zn can be considerably improved (Marschner, 1995). Therefore, the application of ammonium fertilisers may be a useful tool to prevent nutritional disorders such as Fe chlorosis (Sommer et al., 1997).

This paper is not an attempt to review management practices to reduce N fertiliser inputs. Instead, it is rather to identify and illustrate, using recent available data on carob (Ceratonia siliqua L.), general approaches which may be used to explore plant adaptation to ammonium availability in the soil. It will concentrate particularly on adaptation of plants such as carob which grow under non-uniform supplies of nutrients, particularly nitrogen. Hence, it will give some examples indicating the extent and importance of morphological and physiological adaptations as well as plant/fungus interactions in regulating ammonium assimilation.
SOIL N STATUS AND N UPTAKE

Until 1950 nitrogen (N) acted as a growth-limiting element in most ecosystems. Generally, non-agricultural soils were low in both exchangeable nutrients and organic matter and were characterised by tight nutrient cycles (Miller et al., 1991). Most of the N input in these systems derived from the biological turnover or decomposition of organic matter in the soil. The nitrogenous end product of this process was ammonium ($\text{NH}_4^+$), which was rapidly removed from the soil solution due to uptake by plant roots, uptake by micro-organisms, adsorption on the surface of soil colloids and by binding chemically to organic substances. The rate of decomposition determined the release of energy and nutrients and ultimately the primary production of the ecosystem.

Due to the high atmospheric nitrogen deposition of the last decades (Golding and Dean, 1998; Norby, 1998), the “closed” N cycle, in which inputs and outputs of the system are small in relation to the amounts cycling between soil and vegetation, has become an “open” flow system, in which input and output of nitrogen are the dominant factors, and internal cycling is less important (Magdoff et al., 1997).

Agriculture is the biggest anthropogenic source of nitrogen released into the ecosystems. When nitrate-based fertilisers are applied, leaching of nitrate may contaminate waters and cause eutrophication (Pilbeam and Hutchison, 1998). When urea or organic fertilisers are used, there is always ammonia volatilisation with subsequent deposition of ammonia and ammonium.

The chemical nature of the soil nitrogen pool depends on the quantity and quality of the nitrogen input and on the balance of ammonification, nitrification, immobilisation and denitrification processes in the soil (Gessler et al., 1998), which are governed by the C/N ratio present in the soil (Woldendorp and Läänebroek, 1989). Hence, soil mineral nitrogen may vary spatially and temporarily due to its mobility, but also due to physical and/or biological processes and agricultural practices (Lainé et al., 1998).

Plant species differ in their preference for nitrogen forms, depending on the chemical species available in the soil. Arctic species whose roots are in contact with high concentrations of amino acids, preferentially grow and absorb amino acids, while many other plants grow on mineral nitrogen (Chapin et al., 1993). Species from habitats with high nitrate availability, as well as the majority of crops (maize, wheat, tomato, etc.) show preference for nitrate (Atkin 1998). Woody plants such as spruce and pine (Kronzucker et al., 1997), carob (Cruz et al., 1993a and b) and crops like strawberries (Elmer, 1999) grow better with ammonium. However, the majority of plant species grow best in a mixture of ammonium and nitrate, the optimum depending on temperature, light and nutrient status. Several tree species shift from a preference for nitrate to one for ammonium at an age of several years (Elmer, 1999).

The preference of plants for nitrate or ammonium may be associated with external and/or internal factors. External factors are related to changes in soil pH, availability of other nutrients and microbial changes in the rhizosphere (Marschner and Eltrop, 1996). A high input of ammonium leads to: 1) leaching of potassium, magnesium and calcium from the soil, often resulting in increased ratios of $\text{NH}_4^+$/cations and $\text{NH}_4^+$/NO$_3^-$ (Asman et al., 1998); 2) increased rates of acidification leading to a decrease in soil pH, which can be responsible for the increase in the concentration of toxic ions, such as aluminium, in the soil solution and/or to the increase of the availability of phosphate and manganese to the plant root systems (Marschner and Eltrop, 1996); 3) changing the microbial populations of the rhizosphere (Elmer, 1999).

Some of the internal factors (Gerendas et al., 1997; Martins-Louçao and Cruz, 1999) determining plant preference for either ammonium or nitrate are related to: 1) plant genome 2) ability of the root apoplast to maintain pH; 3) dependence on carboxylate concentrations; 4) dependence on inorganic cation concentrations; 5) availability of carbon skeletons; 6) changes in starch concentration; 7) accumulation of free ammonia or ammonium; 8) changes in polyamine and phytohormone metabolisms.

Changes in these factors can account for the so-called ammonium (a term not easily defined), which is probably dependent more on ammonium concentrations in the soil than on plant genotype (van Sprang and Jansen, 1997).

How root uptake capacity adapts to changes in
N concentrations and sources in order to cope with nitrogen demands for growth is largely unknown (Robinson, 1994; Lainé et al., 1998), but both morphological and physiological modifications are involved (Taylor, 1998). The mechanism of nitrogen uptake is regulated by a source-sink effect and depends on growth, reproductive demands and needs for maintenance, storage and defence. When nitrogen is present in limiting concentrations, the mechanisms of feedback regulation allow the development of maximal rates of uptake (Drew and Saker, 1986; Cruz et al., 1993 c). These maximal rates can be achieved by increasing the number of transporters per unit of root surface, and/or increasing the affinity of the uptake system for the substrate (Cruz et al., 1993 c). Plants can also develop efficient down-regulation of uptake with increasing tissue nutrient status (Cruz et al., 1993 d and e) avoiding costs of extra nutrient uptake and storage.

Ammonium is commonly suggested as a likely candidate for feedback regulation of nitrate uptake and/or reduction (Lee and Drew, 1989; Jackson and Volk, 1995). However Saravitz et al. (1994) demonstrated inhibition of ammonium uptake in the presence of exogenous nitrate, and showed that it involves a stimulation of efflux rather than an inhibition of influx. NH₄⁺ efflux may be the result of direct cytoplasmic NH₄⁺ efflux or the efflux of NH₄⁺ generated endogenously from nitrogenous compounds. Work on carob also suggests this last possibility, since the amount of NH₄⁺ efflux largely exceeds the endogenous NH₄⁺ concentrations (Fig. 1; Cruz et al., 1993 f). These findings show that the capacity for roots to utilise either nitrate or ammonium is dependent on N status of the plant and support the hypothesis that plants must have a homeostasis mechanism which controls internal N concentrations. Such mechanisms might be related to specific signals, namely organic compounds, carbohydrates or hormones, being able to change N-use efficiency and partitioning between root and shoot.

RESPONSES TO AMMONIUM NUTRITION

Morphological adaptations

Root systems are highly adaptable, and their growth and development involves complex interactions between both the soil environment and the shoots.

Mineral nutrient supply can strongly affect root growth, morphology and the distribution of root systems in the substrate. This effect is particularly marked with nitrogen, less distinct with phosphorous and absent with other nutrients, except for magnesium (Marschner, 1995). In the responsive zone (where concentration range of nutrients limits plant growth), increasing nitrogen supply enhances both root and shoot growth.

In soil-grown plants high concentrations of nitrogen are associated with the development of high root surface areas. The effect is more pronounced with the supply of ammonium than with nitrate (Marschner and Eltrop, 1996). Some work has suggested that the root distribution of some crops is more associated with the ammonium:nitrate ratio than the total nitrogen concentration in the soil (Bloom et al., 1993). Nitrate-fed carob plants have more biomass in the lower part of the root system than those fed with ammonium. Ammonium-fed plants tend to produce shorter (Fig. 2) and thicker (Fig. 3) roots (Cruz et al., 1997).

The reasons for the differing effects of nitro-
gen forms on root morphogenesis are not fully understood: differences in both pathways of assimilation in the roots and in plant hormonal balance are probably involved. Cell division rates in the root apical meristem are regulated by the redox state of the cytosol. A positive correlation between reduced glutathione (GSH), ascorbate levels and cell division has been observed (Sánchez-Fernández et al., 1997). Since levels of reduced GSH are dependent on the levels of reduced NAD(P)H in the cytosol (via the redox shuttle), it is tempting to hypothesise that the form of nitrogen absorbed and assimilated may influence root cell meristem division and thus root morphology, as a consequence of differential demands for reductant during localised assimilation of ammonium and nitrate.

Another factor contributing to the differences observed between the root morphologies of nitrate- and ammonium-fed plants is the concentration of sucrose in young tissues. Sucrose concentration may affect the metabolism of existing meristems, or the initiation of new lateral roots (Williams and Farrar, 1992), and it is also known that ammonium and nitrate acquisition induce variations in the energy requirements and in plant carbohydrate status. The changes in carob root architecture were observed to be associated with increasing concentrations of carbohydrates in the roots, in particular of sucrose. More root branching was observed in plants with greater sucrose concentrations in roots (Fig. 4). The observed differences in carob root architecture seem to be a consequence of the concentration of soluble carbohydrates. These results are in agreement with the hypothesis that sucrose acts as a chemical signal regulating the cell division in roots (Taylor and Bloom, 1998).

It seems clear that plants living in the presence...
nutrient and plant species investigated, as well as edaphic and environmental condition. Plants have evolved complex systems to absorb ammonium and nitrate from the rhizosphere and assimilate them into organic compounds. The metabolic cost of nitrate absorption and assimilation is far greater than that of ammonium because nitrate must first be reduced to nitrite and then to ammonium (Salsac et al., 1987).

High-affinity transport systems involving active transport enable root cells to accumulate ammonium and nitrate when external concentrations are low (Cruz et al., 1995; Crawford, 1995). Low affinity transport systems involving active transport of nitrate and passive transport of ammonium enable the absorption of inorganic nitrogen at higher concentrations (Cruz et al., 1993).

The age of root tissues may influence root capacity for ion absorption, as well as the relative importance of the high and low affinity transport systems for inorganic nitrogen. Studies on the absorption of ammonium along the roots indicated that there is a gradient of ammonium uptake, with higher rates near the root apex and lower uptake rates on the older part of the roots. In carrot seedlings, the absorption of ammonium near the root apex is dependent on metabolic activity, while in more mature root zones the passive component of ammonium uptake is more important (Cruz et al., 1997).

Together, the relative importance of the passive and the active components of ammonium uptake along the roots and the development of a root system with shorter roots (Fig. 5) are important physiological and morphological mechanisms in the acclimation to a root medium with increased ammonium concentrations.

A root system with short roots seems to permit a better regulation of ammonium uptake than one with long roots, and consequently increase N uptake, by increasing the number of root tips in which the rates of nitrogen uptake are larger (Fig. 5). Mechanisms involved in the ammonium assimilation pathway also have important physiological impacts on plant growth and development (Hirel et al., 1997). Glutamine synthetase (GS) has a pivotal role in assimilation of ammonium, and its activity can be enhanced under conditions where more N is taken up, either as nitrate or ammonium (Hirel et al., 1997). This activity is higher in

of high ammonium concentrations may modify their root morphology in order to achieve a better regulation of ammonium uptake, since ammonium cannot accumulate in the cells without slowing plant growth (Salsac et al., 1987; Magalhães and Huber, 1991). Nevertheless, root elongation can be advantageous for plants in areas with abundant precipitation, where nitrate can be leached to deeper layers of the soil, or in Mediterranean areas, where available water is in the deeper parts of the soil (Rhizopoulous and Davies, 1991).

**Physiological adaptations**

Information on the spatial distribution of ion absorption along roots is essential for understanding mineral nutrition and plant growth. Some researchers have claimed that nutrient absorption takes place only at the apical regions of the root axes or branches (Bar-Yosef et al., 1972), whereas others believe that absorption takes place over the entire root surface (Taylor and Bloom, 1998; Colmer and Bloom, 1998). Experimental evidence has supported both hypotheses, depending on the

Figure 4. Root dry weight and number of root tips as a function of root sucrose concentration. Root dry weight was determined in plants grown for 12 weeks with either nitrate or ammonium as nitrogen source. Sucrose concentration was calculated from the difference in reducing sugar concentration before and after incubation with invertase. Values represent the means of 3 experiments with 10 replicates each. The relation between sucrose and root dry weight can be written by the equation $y=0.037x+0.265$. Vertical bars represent standard deviation.
ammonium-fed carob plants than in nitrate-fed ones. This may be due to the fact that ammonium uptake needs immediate assimilation while nitrate taken up can be either reduced or accumulated (Cruz et al., 1993d). Growth analysis demonstrates a linear correlation between GS activity and total dry weight (Fig. 6). Ammonium-fed plants show a considerable growth advantage over nitrate-fed ones. This means that carob plants are able to increase ammonium assimilation in order to overcome problems of ammonium accumulation.

Genetic manipulations of GS and glutamate synthase (GOGAT) activities have induced a high nitrogen use efficiency in legume and non-legume plants (Hirel et al., 1997), but also led to early senescence and premature flowering when plants were grown on an ammonium-rich medium, due to accelerated plant development (Vincent et al., 1997). A negative correlation between biomass production of legume plants and over-expression of GS activity was observed (Migge et al., 1998). To date the use of both mutants and transgenic technology in the investigation of ammonia assimilation is still limited but it offers an important tool for exploring the role played by GS in the organs of different plant species, providing a key towards a further manipulation of plant nitrogen use efficiency in the future.

Plant/fungus interactions

Our discussion thus far has centred on the direct acquisition of mineral nutrients by roots, but it is important to realise that this process may be modified by the association of mycorrhizal fungi or N2-fixing microorganisms with the root system (Filek et al., 1997). Although a legume, carob does not form a symbiotic association with N2-fixing bacteria (Martins-Loução, 1990), but does host arbuscular mycorrhiza (Correia and Martins-Loução, 1996), whereby the fungi enter the root through either the epidermis or the root hair, then spread throughout the regions between cells and even penetrate individual cells of the cortex. Within the cells, the fungal hyphae branch giving rise to particular structures called arbuscules. The arbuscules appear to be sites of nutrient transfer between the fungus and the host plant (Smith et al., 1997).

Enhanced nutrient uptake by mycorrhizal roots is partly a consequence of the growth of extraradical mycelia, which are able to absorb from beyond the depletion zones that surround the
roots. Their principal role in nutrient uptake has been shown to be the facilitation of P uptake by plants (George et al., 1995). However, there is increasing evidence of a major contribution by arbuscular mycorrhiza (AM) fungi to the uptake and assimilation of other nutrients (Faure et al., 1998). The ability of mycorrhizal roots to utilise nitrogen sources has usually been attributed in most cases to an indirect effect associated with improved phosphorous nutrition (George et al., 1995). Nevertheless, recent reports have focused on the use of NO$_3^-$, NH$_4^+$ and amino acids by external hyphae of AM fungi (Tobar et al., 1994; Azcón et al., 1996; Johansen et al., 1996; Hawkins and George, this book).

AM colonisation is very high in roots of carob seedlings growing in soil and is independent of nitrogen concentrations in the soil solution. However, colonisation is restricted to the root region between 2 and 10 cm from the root apex. Arbuscules are more abundant in the region between 2 and 6 cm from the root apex, exactly the region of the root where AM infection increased nitrate and ammonium uptake (Fig. 7).

Results obtained with carob (Fig. 8) show that in relation to control plants (growing in soil without fertilisation and with no mycorrhizal infection), the addition of fertiliser (nitrate and ammonium in a proportion of 1:1) increased dry weight production by about 6.8%, while the inoculation with AM fungi increased dry weight production by 60%, clearly showing the advantages of promoting mycorrhizal infection. The lower increase in dry mass production when fertiliser was added together with mycorrhizal infection may arise from the fact that mycorrhizal infection is inhibited when high levels of nutrients are present in the soil (Magdoff et al., 1997). Similar results were obtained for roots of Allium cepa infected with AM where an increased nitrogen use efficiency was found, particularly under drought stress conditions (Azcón and Tobar, 1998).

It is interesting that fertilisation did not significantly change the plant leaf area, while the mycorrhizal infection doubled the leaf area in relation to control plants (Fig. 9). Again the mycorrhizal effect was more evident when no fertiliser was added to the soil. Differences in shoot development between non-mycorrhizal and mycorrhizal plants may be related to different flux and turn-

![Figure 7. Uptake rates for nitrate and ammonium along roots of 6-month-old carob seedlings inoculated or non-inoculated with mycorrhizal fungi. N concentration in the root medium was 3 mM. Values represent the means of 40 replicates. Columns followed by the same letter are not significantly different at p<0.05.](image)

over of nitrogen compounds, due to factors such as C:N ratio of the plant material (Ferrario-Méry et al., 1997), and changes in hormonal balance due to arbuscular mycorrhiza, leading to an enhancement of the cytokinin:absisic acid ratio. This has been observed in the Medicago/Glomus mycorrhizal symbiosis (Goicoechea et al., 1997). An increase in the cytokinin: absisic acid ratio also increases the load of nitrogen compounds on the root xylem parenchyma cells, allowing a greater shoot development (Lips, 1997). In carob plants nitrate is preferentially (80%) assimilated in the roots, due to xylem loading problems (Cruz et al., 1993d). Mycorrhizal infection seems to increase nitrate loading of the xylem (results not shown), which implies changes in the compartmentation of nitrate reductase activity between roots and shoots. Different proportions of nitrate assimilation in roots and shoots were observed by Azcón
and Tobar (1998), and may be implicated in the responses of mycorrhizal plants to environmental factors.

There is still a lack of a mechanistic model for explaining isotopic plant variation with or without mycorrhiza (Azcon et al., 1998) and the relation of whole plant δ^{15}N to N sources in the soil needs to be approached with caution (Handley et al., 1998). However, the data from a glasshouse experiment indicate that AM infection increases the importance of ammonium uptake relative to that of nitrate (Table 1). δ^{15}N values of the whole plant are closer to those of ammonium in mycorrhizal than in non-mycorrhizal plants. More efficient utilisation of ammonium by mycorrhizal plants was also observed by Hogberg (1990) and Turnbull et al. (1995) and may lead to increased H⁺ secretion into the rhizosphere. This can increase the bioavailability of compounds not readily soluble

Table 1. δ^{15}N (%) of the soil and inorganic nitrogen soil fractions at the beginning of the experiments in sterile and non-sterile soils; and δ^{15}N of the whole plant after 6 months of growth. Plastic pots containing 1.5 l of soil collected under carob trees were autoclaved 3 times at 100 °C for 1 h (Myc-) or not (Myc+). The amounts of nitrate- and ammonium-N were first determined colorimetrically, and appropriate amounts of soil, equivalent to 100 µg N of ammonium-N and nitrate-N, were extracted with nano-pure water. The resulting extracts were subjected to sequential diffusion (Sorensen and Jensen, 1991) prior to isotopic analysis of the extracted nitrate- and ammonium-N. Soils were finely ground in a ball mill and weighed into tin cups for combustion in a continuous flow isotope ratio mass spectrometer (CF-IRMS, Europa Scientific 20-20 and Tracer Mass) as described in Handley et al. (1997). After 6 months of plant growth in either non-sterilised (Myc+) or sterilised (Myc-) soil, the plants were harvested and whole plant δ^{15}N was determined in a continuous flow isotope ratio mass spectrometer (CF-IRMS, Europa Scientific 20-20 and Tracer Mass).

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<td>N Soil</td>
<td>0.7</td>
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<td>NO₃⁻ -N Soil</td>
<td>-2.5</td>
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<td>NH₄⁺ -N Soil</td>
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<td>Whole plant</td>
<td>3.1</td>
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under specific chemical conditions of the soil (Ortas et al., 1996). The use of N stimulates the uptake of phosphorous by the plant, especially when ammonium is present in the root medium (Marschner and Eltrop, 1996). This mechanism is of special importance in calcareous soils in which phosphorous availability is limited due to its ready precipitation as calcium phosphate.

The δ \(^{15}\)N of total soil N (Table 2) is usually slightly greater than that of atmospheric N\(_2\) though \(^{15}\)N may be less abundant where the soil organic matter is dominated by a large amount of recently added litter (Hopkins et al., 1998). Since mycorrhizal plants have a higher fraction of \(^{15}\)N than non-mycorrhizal plants (Table 1), a depletion of the soil in \(^{15}\)N should be expected due to mycorrhizal plant growth. Nevertheless the opposite is observed. The growth of mycorrhizal plants leads to an enrichment of the soil in \(^{15}\)N from a δ \(^{15}\)N of 0.7 to 2.1 % in six months (Table 2). So, the enrichment of the soil in \(^{15}\)N cannot be due to the growth of mycorrhizal plants. This seems to demonstrate that mycorrhizal development interferes not only with the processes of nitrogen uptake by plants, but also with other soil processes such as nitrification and denitrification, processes that account for the enrichment of the soil in \(^{15}\)N (Hopkins et al., 1998).

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**FINAL REMARKS**

The results presented here show clearly that carob can benefit from the presence of ammonium in the root medium. Like carob, most plants can adapt to ammonium nutrition by morphological and physiological modifications or by promoting the association with microorganisms. The effects of ammonium on plant growth are mainly dependent on ammonium concentrations, and also on plant species. It is therefore important to explore physiological and molecular tools for manipulation of ammonium assimilation by plants. This requires a better understanding of the morphological and physiological responses of plant species to ammonium.

AM infection allows plants to produce more biomass and use more ammonium than non-mycorrhizal plants. In addition, arbuscular mycorrhizal fungi confer greater tolerance to toxic metals (Munyanziza et al., 1997); root pathogens (Klig and Jakobsen, 1998); drought (Munyanziza et al., 1997); high soil temperatures (Bendavidval et al., 1997); adverse pH and transplant shock (Munyanziza et al., 1997). They also contribute to the formation of soil aggregates (Kling and Jakobsen, 1998). Considering that most high-input agricultural practices are detrimental to mycorrhizal fungi (Schweiger and Jakobsen, 1998; Bendavidval et al., 1997), a low-input sustainable agriculture should be promoted.

In addition, the introduction of spores of the best mycobiont and breeding for genotypes with a more efficient mycorrhizal symbiosis might enhance production in soils where nutrients (mainly nitrogen and phosphorous) restrict crop production. Besides promoting crop production, mycorrhizas may reduce nutrient losses to the environment.


New approaches to enhanced ammonium assimilation in plants


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