Commentary

Taxonomic and functional diversity in arbuscular mycorrhizal fungi – is there any relationship?

During the past decade there has been increasing interest in the role of biodiversity and its importance for the functioning of ecosystems. Several studies have shown that plant species richness and composition affect a number of important ecosystem characteristics, including plant productivity and ecosystem stability (Loreau *et al.*, 2001). While these earlier studies focussed on plants and the significance of plant diversity, there is currently increased interest in the ecological significance of microbial diversity. Microbes are essential components of the Earth's biota since they catalyse unique and indispensable transformations in the biogeochemical cycles of the biosphere, and provide plants and animals with essential nutrients – arbuscular mycorrhizal fungi (AMF) fall into this group, associating with the majority of land plants, providing them with nutrients and protection from a number of environmental stresses (Smith & Read, 1997). Many studies have shown that plant communities vary in AMF diversity – for example, agricultural soils usually have low AMF diversity and harbour only a few species, contrasting with perennial communities such as grasslands or tropical forests, which contain complex AMF communities with higher diversity (Clapp *et al.*, 2002). Still, it is unclear whether such differences in AMF diversity are of ecological importance and whether they influence plant growth, nutrition and ecosystem functioning. In this issue (pp. 357–364), a study by Munkvold *et al.* shows that things are certainly not simple – indeed, the findings may change our way of thinking about the ecological function, evolution and species concept of AMF.

One arbuscular mycorrhizal fungal species can be functionally diverse

Munkvold *et al.* inoculated Cucumber (*Cucumber sativus*) plants with different isolates of the same AMF species that were collected from several countries, including Bolivia, Denmark, Finland, Switzerland and the UK. Four different AMF species were studied: *Glomus mosseae* (13 different isolates), *G. claroideum* (five isolates), *G. caledonium* (four isolates) and *G. geosporum* (two isolates). Subsequently, intra- and interspecific functional diversity of these four *Glomus* species was investigated. Munkvold *et al.* showed that differences in growth and P-uptake of Cucumber plants varied as much between isolates as between different AMF species. Thus, one AMF species can be functionally diverse, which raises the question as to whether a relationship exists between taxonomic and functional diversity in AMF.

Taxonomic diversity and the species concept

Until now, approximately 150 different AMF species in four families have been described based on the morphological characteristics of the spores. Molecular studies that used ribosomal DNA gene sequences largely agreed with this classification and showed that AMF are a separate, monophyletic phylum within the fungi (Schüssler *et al.*, 2001). The isolation of AMF ribosomal DNA from field plant roots revealed unexpectedly large AMF diversity (e.g. Helgason *et al.*, 2002). These studies suggest that many more AMF species exist than have currently been described.

AMF are ancient fungi that are thought to be asexual clonal organisms (Sanders, 2002). Hence different AMF species cannot be described as biological or ecological species (Smith & Read, 1997). The biological species concept is therefore problematic and this could explain why such large intra- and interspecific variability occurs within AMF 'species'. Accordingly, it may be better to define AMF taxa rather than AMF species (D. J. Read, pers. comm.). Similar problems have been observed for prokaryotes (which are largely asexual). The species concept for prokaryotes is based on a number of characteristics including phylogenetic (rDNA), biochemical and physiological characteristics (Rossello-Mora & Amann, 2001). Such an approach might be useful for AMF. One characteristic that may be helpful for identifying AMF 'species' is to test for the occurrence of hyphal fusions and anastomosis. Giovannetti *et al.* (2003) observed that hyphae from spores of the same isolate fused and that exchange of material occurred. However, no anastomosis occurred between hyphae of different isolates, species and genera, indicating that these AMF have a different identity.

In recent years, the ribosomal, DNA-based taxonomy of AMF has been used to describe AMF communities in plant roots in the field. Several studies revealed that only a few centimetres of plant root contained complex and diverse AMF communities consisting of different AMF species. These studies also showed that different plant species associate with different AMF communities (e.g. Vandenkoornhuyse *et al.*, 2002). In addition, molecular identification tools make it now possible to characterise AMF communities in plant organs or at sites that were previously hard to sample. For

example, Scheublin *et al.* (2004) observed specialised AMF communities in root nodules from a number of leguminous plants. Overall, these studies say something about the distribution of AMF sequence types and fungal biology. However, important issues are: (1) whether such differences in AMF communities are of functional importance for plant growth, nutrition and community composition; and (2) whether such differences can be used to predict variation in biomass production and nutrient uptake in plants.

Functional diversity

It has been known for a long time that there is functional diversity within AMF and that the different taxa vary from each other with respect to stimulating plant growth and nutrition (Smith & Read, 1997). Functional diversity of AMF also has implications for plant community structure and plant diversity. In one study, plant community structure changed when different AMF taxa were present. This could be explained by the fact that different plant species received different amounts of benefit from different AMF (van der Heijden *et al.*, 1998). Moreover, a positive relationship between plant diversity, plant productivity and AMF diversity was found. The lowest productivity was found in grassland macrocosms without AMF or with only a few AMF while higher plant diversity and productivity were observed in grassland macrocosms with 8 or 14 different AMF species (van der Heijden *et al.*, 1998).

While earlier studies only compared a few isolates or species, the work by Munkvold *et al.* and also Hart & Reader (2002a) used a more systematic approach as they tested the effects of 24 and 21 AMF isolates on plant growth, respectively. The results of Munkvold *et al.* show that there is high functional diversity within a single AMF species with respect to P-uptake and plant growth stimulation. The results of Hart & Reader (2002a) also show that AMF families differ in the benefit they confer to their host plants (even though there is large variation within and between AMF species and genera). Furthermore, Koch *et al.* (2004) obtained 16 different isolates of one AMF species, *Glomus intraradices,* from one site. They observed that these isolates (which were genetically different from each other) varied from each other in that they produced different amounts of hyphae. Overall, these studies show that there is considerable functional diversity within AMF and that variation within one AMF species can be bigger than between different AMF species or even genera. This functional diversity is important for the growth of individual plants and for the composition of plant communities.

Links between taxonomic and functional diversity

There has been much debate in the ecological literature about whether a relationship exists between taxonomic diversity and functional diversity. This discussion has been particularly relevant for explaining effects of biodiversity on ecosystem functioning (Loreau *et al.*, 2001). The results of Munkvold *et al.* (2004) and Hart & Reader (2002a), question whether a relationship between taxonomic and functional diversity exists in AMF. Interestingly, sequences of a few AMF species have been obtained from almost any ecosystem that has been sampled (Opik *et al.*, 2003) and several AMF species appear to have a global distribution. For example, sequences of *Glomus intraradices* and *Glomus* sp. UY1225 have been found in European forest, European grassland and in a tropical rainforest in Central America. The flora of Europe and Central America is completely different and evolved in different directions when the continents separated some 200 million years ago. Similar evolutionary processes would be expected for AMF, at least, if they coevolved with their environment after their occurrence about 400 million years ago. Hence it is surprising to find the same AMF species in Europe and Central America. Moreover, AMF are thought to have limited dispersal capacities (their whole life-cycle is completed below ground) and it is unlikely that isolates of AMF species with a global distribution have recently been in contact with each other. Thus, it is possible that such AMF species are functional diverse. This, together with the observations of Munkvold *et al.* (2004) and Hart & Reader (2002a) challenges existing AMF taxonomy and suggests that taxonomic groups within AMF species should be defined.

Munkvold *et al.* studied different isolates and species of one AMF genus, *Glomus*. However, it has been hypothesized that functional diversity is dependent on the level of taxonomic resolution (Hooper *et al.*, 2002). Adding increasing numbers of genotypes or isolates of a particular species will probably add some degree of functional diversity. However, adding different genera or families will probably add more functional variation. Hart & Reader (2002b) proposed that a link exists between functional traits of AMF (size of the hyphal network) and taxonomy at the family level. They observed that AMF isolates that belonged to the *Gigasporaceae* produced more external hyphae compared with *Glomaceae* and *Acaulosporaceae*. This may indicate that plants colonized by the *Gigasporaceae* have access to a larger soil volume and acquire more nutrients. Although criticized (see Olsson *et al.*, 2003) and not necessarily in agreement with other studies (e.g. Jakobsen *et al.*, 1992), this notion is important and should be tested again in a systematic way for a large number of isolates. Interestingly, Hart & Klironomos (2002) showed that the variation in growth response of *Plantago lanceolata* was high in treatments comprising different AMF genera but lower in treatments containing different isolates from the genus *Glomus*. It is therefore likely that AMF taxa that belong to different genera possess functionally diverse traits.

The taxonomic and evolutionary relatedness between organisms are often depicted in phylogenetic trees. Studies with prokaryotes have shown that phylogenetic trees based

on rDNA do not concur with trees that are based on functional genes responsible for some metabolic pathways (Pace, 1997). The results presented by Munkvold *et al.* may indicate that this is also true for AMF and that functional diversity is independent of taxonomic diversity. Clearly more research is needed here.

Perspectives

What are the consequences of these findings? First, molecular studies that describe AMF communities in plant roots tell us something about fungal identity, but it is unclear whether this information can be used to explain functional differences in plant growth and community composition. The fact that AMF isolates with similar identity induce different plant growth responses is not particularly promising. Second, the taxonomy and phylogeny of AMF is based on spore morphology and on ribosomal gene sequences. These characteristics may be essential for fungal identity, but this may be unrelated to any benefits that are given to the plant. Future studies should therefore include genes that are likely to be responsible for functional diversity (e.g. diversity of phosphate transporter genes). Moreover, the species concept in AMF needs more attention, and should probably be revised. It may be necessary to define taxonomic groups below the species level. Third, it is important to consider that two completely different organisms are being compared. Functional diversity is determined by assessing variability in plant response while taxonomic diversity is based on fungal gene sequences. The question is whether these different organisms can be compared. Fourth, while the study of Munkvold *et al.* used isolates from different geographical areas, subsequent studies should use fungal isolates and plant genotypes from the same community, as has already been done in the study by Helgason *et al.* (2002) with one isolate per AMF species. Future studies should also inoculate plants with mixtures of several AMF, reconstruct the AMF community that is found in the field and subsequently monitor plant performance. Such experiments will reveal whether species diversity in plant roots is interlinked with functional diversity.

Many studies have shown that there is high functional diversity in AMF. An important issue is whether the plant is utilizing this functional diversity. It is still unclear whether plants are able to select effective AMF, or combinations of AMF taxa, that are most beneficial in terms of stimulating plant growth and enhancing fitness. In conclusion, the study by Munkvold *et al.* indicates that there is no relationship between taxonomic and functional diversity, at least not at lower taxonomic resolution (AMF species). Functional diversity of AMF is likely to be important for plants, but the significance of taxonomic diversity is unresolved. Future studies should test whether different AMF genera or families differ from each other and if they provide different types of benefits to plants and ecosystems.

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Key words: arbuscular mycorrhizal fungi, biodiversity, Cucumber (*Cucumber sativus*), ecosystems, evolution, microbial diversity, plants.

Letters

Competition or complementation: the iron-chelating abilities of nicotianamine and phytosiderophores

Efficient acquisition of iron (Fe) in graminaceous plant species relies on the synthesis and release of phytosiderophores, which are low-molecular weight chelators with functional carboxy-, amino- and hydroxy-groups for hexadentate metal coordination (Sugiura & Nomoto 1984). Metal-phytosiderophores formed in the rhizosphere are subsequently taken up by YS1-type membrane proteins, that energize root uptake by the cotransport of metal-phytosiderophores with protons (Schaaf *et al.*, 2004). As Fe(III)-phytosiderophores dominate Fe(III) chelation over hydroxide formation in the pH range between 3.5 and 8.5 (von Wirén *et al.*, 1999) and as they are mainly found in root exudates or the xylem sap (Kawai *et al.*, 2001), phytosiderophores may adopt a major role in extracytosolic Fe chelation.

Nicotianamine (NA), which occurs in all higher plants, is structurally similar to phytosiderophores and serves as a precursor in their biosynthetic pathway. Fe trafficking within the plant has been shown to have a strong dependency on NA; this has been demonstrated by severe symptoms of Fe deficiency in mutant or transgenic plant lines with low NA levels (Ling *et al.*, 1999; Takahashi *et al.*, 2003). NA has been shown to chelate not only Fe(III) but also Fe(II) (Beneš *et al.*, 1983), in particular at higher pH values, which may allow its function as an intracellular Fe(II) scavenger thereby protecting the cell from Fe(II)-mediated oxidative damage (von Wirén *et al.*, 1999).

The occurrence of phytosiderophores and NA in graminaceous plant species thus raises the question whether Fe(III) may undergo a chelate exchange from phytosiderophores to NA, as proposed by von Wirén *et al.* (1999). These authors concluded on the basis of titration and capillary electrophoresis studies that the phytosiderophore deoxymugineic acid (DMA) chelates Fe(III) preferentially at more acidic pH values (pH 3–6), while NA is a more competitive chelator at pH values > 6.

Reichman & Parker (2002) reanalysed the previously published data and claimed to have identified information which partially rejects the above hypothesis and has important implications for Fe physiology and transport in plants. The two aspects which were the subject of criticism were (i) the competition between NA and DMA for Fe(III) at pH 7.0, and (ii) the relative affinity of NA for Fe(II) and Fe(III). We address these two aspects in sequence.

Nicotianamine outcompetes deoxymugineic acid for iron(III) chelation at neutral pH values

By considering the formation of a single negatively charged complex $[Fe(III)\text{-}DMA(H_{-1})]^{-}$, along with the neutral complex $[Fe(III)\text{-}DMA]^{0}$, Reichman & Parker (2002) calculated a speciation plot, in which Fe(III)-DMA formation dominated over the pH range 2.5–9, thus prohibiting the occurrence of an Fe(III)-NA complex, when NA was present at equimolar

Table 1 pKa values and binding constants for iron(III) for nicotianamine and deoxymugineic acid

	pKa^1	pKa^2	pKa^3	pKa^4	pKa^5	pKa^6	log K_1	log K_2	log K_3
DMA	< 1.5	1.94	3.42	9.12	9.66			24.86	18.24
NA	< 1.5	2.35	3.27	9.64	10.05	7.73	26.53	21.01	11.58

The pKa^1 values are too low to be reliably measured. The constants, K_1 to K_3, represent the equilibrium constants for the following equations: $H^+ + Fe^{3+} + L^{3-} = HFeL^+$:$K_1$; $Fe^{3+} + L^{3-} = FeL$:K_2; $Fe^{3+} + L^{3-} = H^+ + FeL(H_{-1})^-$:$K_3$, where L^{3-} represents DMA or NA with the three carboxyl groups deprotonated.

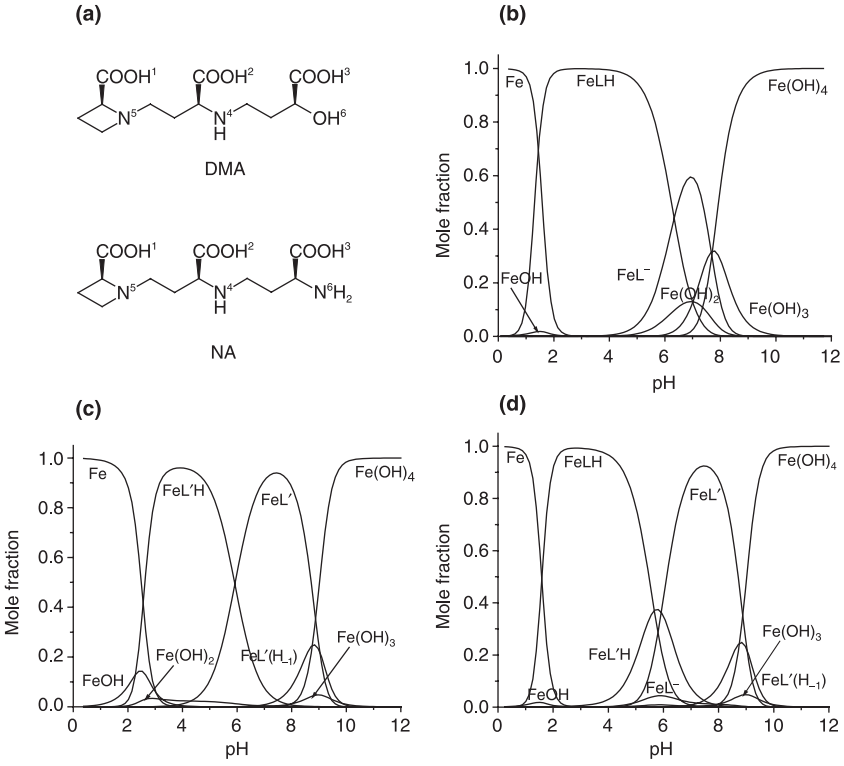

Fig. 1 (a) Structures of deoxymugineic acid (DMA) and nicotianamine (NA). Superscript numbers indicate the assignment of the pKa values given in Table 1. (b)–(d) Computer simulations of the pH dependence of the Fe(III) complexes of NA and DMA: (b) 1 μM Fe +10 μM DMA (= L); (c) 1 μM Fe +10 μM NA (= L′); (d) 1 μM Fe +10 μM DMA (= L) +10 μM NA (= L′).

concentrations (Reichman & Parker, 2002, Fig. 2B). It was then concluded that DMA outcompetes NA over the entire physiologically relevant pH range, in contrast to that proposed by von Wirén *et al.* (1999).

Since the analysis of chelate formation reported by von Wirén *et al.* (1999) was based on experimentally determined pKa values and iron(III) affinity constants, we first reassessed the pKa values for both NA and DMA and also determined the affinity constants for Fe(III) by potentiometric titration. Our previously determined values of the Fe(III) affinity constants were based on spectrophotometric analysis of competition studies with maltol.

Employing chemically synthesised compounds the same sequence of pKa values for both NA and DMA was obtained as previously reported (von Wirén *et al.*, 1999) (Table 1). DMA has four measurable pKa values whereas NA has five. This difference is due to the terminal amino group of NA, that protonates at a pKa of 7.73, while the terminal hydroxyl group of DMA does not dissociate even at very high pH values (Table 1, Fig. 1a). There are different protonation states for both NA-Fe(III) and DMA-Fe(III) complexes and these possess different net charges, namely for DMA [Fe(III) DMA]^0, [Fe(III) DMA(H_{-1})]$^-$ and [Fe(III) DMA·(H_{-1})OH]$^{2-}$. We have now measured the equilibrium constants relating to each of these species for both NA and DMA by potentiometric titration as previously reported (von Wirén *et al.*, 1999) (Table 1). With DMA we found evidence for the protonated species [Fe(III) DMA]^0 over the pH range 2–7 which agrees with the earlier data produced by von Wirén *et al.* (2000). We found no evidence for the hydroxylated species [Fe(III) DMA·(H_{-1})OH]$^{2-}$. We also investigated the possibility of dimer formation of the type reported for HEDTA (Gustafson & Martell, 1963) and EDTA (Schuger *et al.*, 1969; 1972) and could find no evidence for the existence of such species at μM concentrations and

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Table 2 Affinity constants of nicotianamine and deoxymugineic acid for iron(III)

	Determined from potentiometric titration	Determined from spectrophotometric analysis of competition with maltol	
	pFe^{3+}	pFe^{3+}	Log K_{Fe}^{3+}
DMA	15.5	15.4	18.1
NA	17.1	17.1	20.6

pFe^{3+} determined for $[iron]_{total} = 10^{-6}$ M, $[phytosiderophore]_{total} = 10^{-5}$ M; pH 7.0; log K_{Fe}^{3+} determined by competition with maltol.

below. This analysis is in agreement with the electrophoretic determination of Fe(III)-DMA species that showed a gradual shift from the negatively charged to the neutral species when lowering the pH from 7 to 5 (von Wirén *et al.*, 2000, Fig. 3). Unfortunately, this latter study was apparently not considered by Reichman & Parker (2002). Thus the speciation plot for Fe(III)-DMA is relatively simple with the two Fe complexes [Fe(III) DMA·(H_{-1})]$^{1-}$ and [Fe(III) DMA]^0 dominating over the pH range 2–8 (Fig. 1b). The pKa value for this deprotonation process is 6.3.

With NA there are three major Fe(III) species. As derived from the binding constants (Table 1) and the associated speciation plot, [Fe(III)-NA·H]$^{1+}$ dominates at pH 2–6, while [Fe(III)-NA]^0 dominates over the pH range 6–9 (Fig. 1c). This is consistent with with the neutral charge of the Fe(III)-NA complex at pH 7.0 when determined by paper electrophoresis (von Wirén *et al.*, 1999). The hydroxylated species [Fe(III)-NA·(H_{-1})]$^{1-}$ is only present at more alkaline pH values (above 8.0). As with DMA we could find no evidence for the existence of dimer complexes at concentrations below 1 μM.

The affinity constants of both NA and DMA for Fe(III) have been determined by two independent methods, namely potentiometric titration and spectrophotometric analysis of the competition of each ligand with maltol. As there are several Fe(III) complex species for NA and mugineic acid (MA), the competition analyses give an apparent formation constant (K_{Fe}^{3+}). With these Fe(III) affinity constants it is possible to calculate the pFe^{3+} values independently. Both pairs of values are in good agreement (Table 2).

On the basis of this new conductiometric data (Table 1) we have recalculated the speciation of Fe(III) in the simultaneous presence of NA and DMA. Consideration of the negatively charged [Fe(III)-DMA(H_{-1})]$^{1-}$, that was omitted in von Wirén *et al.* (1999), will effectively decrease the relative competitiveness of Fe(III)-DMA over Fe(III)-NA. Over the 5.5–8.5 pH range it is clear that NA is predicted to dominate the co-ordination of Fe(III) (Fig. 1d), which supports the earlier analysis reported by von Wirén *et al.* (1999), where both DMA species have not been considered separately.

Relative affinity of nicotianamine for iron(II) and iron(III)

A major point of Reichman & Parker's (2002) criticism pertained to the experimental approach made by von Wirén *et al.* (1999), in which capillary electrophoresis was employed to verify the relative ability of NA to form Fe(II) or Fe(III) complexes. Reichman & Parker (2002) raised the issue that Fe(III)-NA complexes were not detected by capillary electrophoresis, by virtue of the rapid formation of Fe(III)-hydroxides. This difference, however, is clearly indicated in Fig. 5 of von Wirén *et al.* (1999), where it is demonstrated that the pFe^{3+} value for NA at pH 7.4 is only marginally greater than the Fe(III)-hydroxide solubility curve, whereas the corresponding pFe^{2+} value is very well resolved from the Fe(II)-hydroxide solubility curve. Thus, although the affinity constant for Fe(III) is larger than that for Fe(II) ($10^{18.4}$ vs $10^{12.8}$, respectively), competition with the hydroxide anion at pH 7.4 is much more effective for Fe(III) than for Fe(II). Since Fe(III)-hydroxide formation can certainly occur in the cytoplasm, our experimental conditions properly reflect the situation in planta and correctly emphasize the fact that Fe(II) possesses a relatively higher stability when complexed by NA.

Reichman & Parker (2002) developed another argument based on theoretically derived redox potentials of NA-complexed Fe(II) and Fe(III) which led to the conclusion that thermodynamic considerations can satisfactorily explain the high stability of Fe(II), while kinetic aspects as in von Wirén *et al.* (1999) are not needed. This statement turns out to be purely speculative. First, Reichman & Parker's (2002) computation of redox potentials considered [Fe(III)-DMA]$^{1-}$ and [Fe(III)-DMA·OH]$^{2-}$ as the most important complexes, which is in contrast with the predicted and experimentally determined species at neutral pH (von Wirén *et al.*, 2000). Second, Reichman & Parker's (2002) theoretically derived reduction potentials do not agree with experimentally derived values from Sugiura & Nomoto (1984), as they indicate in their correspondence 'the reasons for this discrepancy are not known'. Third, kinetic considerations are of outmost importance when the formation of DMA complexes with different metal ions are observed. For example, the efficient formation of Cr(III)-DMA complexes is severely restricted by its slow kinetics. Thus, metals that undergo faster complex formation will dominate even if their formation constants are weaker (Hider, 1984).

Until such time as there is better consistency between theory and experiment, we believe that arguments based on redox potentials associated with a complicated series of equilibria cannot provide definitive conclusions. On the basis of the concepts presented herein, the transfer of Fe(III) from

DMA to NA would appear to be possible. However the kinetics of the movement of Fe(III) from one hexadentate siderophore to another is exceedingly slow (Hider, 1984) and, therefore, as previously suggested by von Wirén *et al.* (1999) and Sugiura & Nomoto (1984), reduction to Fe(II) before transfer to NA would appear to be a likely mechanism.

Physiological implications

The important point that emerges from this debate is that both von Wirén *et al.* (1999) and Reichman & Parker (2002) agree that NA form stable Fe(II) complexes. To avoid further speculation on metal speciation in plant extracts, future investigations should definitely focus on the direct chemical analysis of metal chelates. Such an analysis has recently been performed with yeast subjected to Ni toxicity, where Ni(II)-NA complexes were detected (Vacchina *et al.*, 2003). As demonstrated recently, NA complexes with Ni(II), Fe(II) and Fe(III) form suitable substrates for membrane transport mediated by the metal phytosiderophore transporter ZmYS1 (Schaaf *et al.*, 2004). Thus apparent Fe deficiency symptoms as observed in the NA-deficient tomato mutant *chloronerva* (Ling *et al.*, 1999) are likely to be related with an intracellular maldistribution of Fe (Becker *et al.*, 1995). Such a role is further supported by an immunochemical localization of NA in vacuoles (Pich *et al.*, 2001). Another sharp contrast to the conclusion by Reichman & Parker (2002) that Fe-NA is unlikely to be employed for long-distance Fe transport, is the finding that NA deficiency in transgenic tobacco plants resulted in malnutrition of flowers with metal micronutrients including Fe. Again, this was best explained by an involvement of NA in intra- and intercellular Fe trafficking (Takahashi *et al.*, 2003). Furthermore, recent studies on a ZmYS1 homolog from Arabidopsis, AtYSL2, indicated that nicotianamine-chelated Fe(II) is a suitable substrate for AtYSL2. Since AtYSL2-GFP fusion proteins are mainly located in the plasma membrane of vascular cells in roots (DiDonato *et al.*, 2004), there is a high probability for Fe(II)-NA acting as an endogenous mobile Fe binding form.

Taken together, in addition to the physico-chemical characterization of Fe-NA chelates, there is plenty of evidence from physiological and genetic studies that further indicate a role of NA in Fe trafficking, also in graminaceous species where DMA and NA occur in parallel (Becker *et al.*, 1995; Pich *et al.*, 2001; Bereczky *et al.*, 2003; Takahashi *et al.*, 2003; Schaaf *et al.*, 2004). NA chelation and subsequent transport or compartmentation offers an attractive opportunity for plant cells for short- and long-distance transport of Fe and other essential metals and to decrease the immediate toxicity of Fenton-reactive free metals or simply to support their tissue and cellular distribution. In view of all this experimental evidence, Reichman & Parker's (2002) criticisms would appear to be unfounded.

Acknowledgements

We thank Prof Dr T Kitahara and Prof Dr S Mori, (University of Tokyo, Japan) for the supply of NA and DMA. We thank the DFG Bonn for financial support to NVW (Grant WI 1728/6-1); the BBSRC for financial support to RCH; and CREST (Care Research for Evolutional Science and Technology) of Japan Science and Technology Corporation to EY.

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Key words: chelation, graminaceous plant species, iron nutrition, iron trafficking, strategy II.