

Use of ^{15}N stable isotope to quantify nitrogen transfer between mycorrhizal plants

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Abstract

Aims

Mycorrhizas (fungal roots) play vital roles in plant nutrient acquisition, performance and productivity in terrestrial ecosystems. Arbuscular mycorrhizas (AM) and ectomycorrhizas (EM) are mostly important since soil nutrients, including NH_4^+ , NO_3^- and phosphorus, are translocated from mycorrhizal fungi to plants. Individual species, genera and even families of plants could be interconnected by mycorrhizal mycelia to form common mycorrhizal networks (CMNs). The function of CMNs is to provide pathways for movement or transfer of nutrients from one plant to another. In the past four decades, both ^{15}N external labeling or enrichment (usually expressed as atom%) and ^{15}N naturally occurring abundance ($\delta^{15}\text{N}$, ‰) techniques have been employed to trace the direction and magnitude of N transfer between plants, with their own advantages and limitations.

Important Findings

The heavier stable isotope ^{15}N is discriminated against ^{14}N during biochemical, biogeochemical and physiological processes, due to a greater atomic mass. In general, non- N_2 -fixing plants had greater

$\delta^{15}\text{N}$ values than N_2 -fixing ($\sim 0\text{‰}$) ones. Foliar $\delta^{15}\text{N}$ often varied by 5 to 10‰ in the order: non-mycorrhizas/AMs > EMs \geq ericoid mycorrhizas. Differences in $\delta^{15}\text{N}$ (‰) or ^{15}N (atom%) values could thus provide N transfer information between plants. A range of between 0 to 80% of one-way N transfer had been observed from N_2 -fixing mycorrhizal to non- N_2 -fixing mycorrhizal plants, but generally less than or around 10% in the reverse direction. Plant-to-plant N transfer may provide practical implications for plant performance in N-limited habitats. Considering that N translocation or cycling is crucial, and the potential benefits of N transfer are great in both agricultural and natural ecosystems, more research is warranted on either one-way or two-way N transfers mediated by CMNs with different species and under field conditions.

Keywords: ^{15}N enrichment • ^{15}N natural abundance (^{15}N) • ^{15}N stable isotope • common mycorrhizal networks (CMNs) • nitrogen transfer

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INTRODUCTION

Mycorrhizal symbiosis

The word *mycorrhiza*, literally ‘fungus root’, is derived from the Greek words *mykes* (fungus) and *rhiza* (root). Mycorrhizas are highly evolved mutualistic associations between soil fungi and plant roots (Brundrett 2002; Brundrett *et al.* 1996; Smith and Read 2008; van der Heijden *et al.* 2006, 2008). Approximately 90% of land plants associate with soil fungi (Basidiomycetes, Ascomycetes and Zygomycetes) to form mycorrhizas, and six types of mycorrhizas [arbuscular (AM), arbutoid, ecto- (EM), ericoid, monotropoid and orchid] have been categorized

by their distinct morphological characteristics (Table 1, Brundrett 2002; Smith and Read 2008; Wang and Qiu 2006). Dual AM and EM associations have also been found in some plant species, including *Abies*, *Acacia*, *Adenostoma*, *Alnus*, *Casuarina*, *Dicymbe*, *Eucalyptus*, *Pinus*, *Pseudotsuga*, *Populus*, *Quercus*, *Salix*, *Tsuga*, *Uapaca* and certain legumes (see He *et al.* 2003; McGuire *et al.* 2008).

The most common and most economically important mycorrhizas are AM and EM. The origins and evolution of AM fungi goes back 350–450 million years when plants were making the transition from aquatic to terrestrial habitats (Remy *et al.* 1994; Simon *et al.* 1993). AMs are associated with

~300 000 plant species from all plant divisions including Bryophytes, Pteridophytes, Gymnosperms and Angiosperms, except most brassicaceous vegetables (Brundrett 2002; Smith and Read 2008; Wang and Qiu 2006). There are six AM genera: *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora*, containing ~200 species that belong to one fungal order (*Glomales*) in the division *Zygomycota* (Morton and Benny 1990). The fungal hyphae often penetrate inside the walls of root cortical cells to form specific 'little tree-shaped' fungal structures called arbuscules, which serve as the main sites of nutrient exchange between the plant and the fungus. The majorities of these species are ubiquitous and non-host specific, but have no demonstrated saprobic ability and thus only reproduce asexually in the presence of living plant partners.

In contrast to AM fungi, EM fungi are facultative biotrophs with ~7750 species (most Basidiomycetes and Ascomycetes) in the division *Dikaryomycota*, but their partners are mostly limited to woody trees (Brundrett *et al.* 1996; Molina *et al.* 1992; Wang and Qiu 2006). EM fungi have regularly septate hyphae and can reproduce without living plant partners, but many of them are host specific for the same plant genus. The morphological characteristics of EMs are also dramatically different from those of AMs (Peterson and Bonfante 1994); EM roots are thicker and have altered branching patterns, which could easily be distinguished from a non-mycorrhizal root by color. The fungal hyphae penetrate root tissues, but not the walls of root cortical cells like in the AM, forming a unique, highly branched network, called the Hartig net. The Hartig net constitutes the plant–fungus interface, where solutes are translocated between the partners: carbohydrates from plant to fungus and inorganic nutrients from fungus to plant (Allen 1992; Marschner and Dell 1994; Read 1991; Smith and Read 2008; Smith *et al.* 1994).

In mycorrhizal associations, fungi are essential connections for plants to take up soil nutrients such as N, P, Zn, Fe and water, and to tolerate soil drought, toxic metals and pathogens; in return, the fungi use ~20% of the plant's net photosynthetic C gain and even vitamins for their belowground functions (Smith and Read 2008). In addition, EM and ericoid mycorrhizas are able to use organic N sources (Chalot and Brun 1998; Lipson and Nasholm 2001; Nasholm and Persson 2001; Nasholm *et al.* 1998, 2009; Rains and Bledsoe 2007; Wallenda and Read 1999). The fungi also play vital roles in soil structure, plant performance, and plant biodiversity and productivity (Brundrett 2002; Leake *et al.* 2004; Simard and Durall 2004; Smith and Read 2008; van der Heijden *et al.* 2006, 2008).

Common mycorrhizal networks

Plants often grow close together, either as single-species populations or primarily in multiple-species communities. On the one hand, a single plant can form mycorrhizas with many fungi in intra- or interspecific combinations. On the other hand, hyphae of a single fungal species can interconnect many plants as many mycorrhizal fungi exhibit little host specificity (Smith and Read 2008). As a consequence, a common mycorrhizal network (CMN) could form within and between plant roots (Newman

1988). The CMNs, interconnecting individual species, genera and even families of plants in natural communities, are usually woven into an even larger network of fungi and roots (Newman 1988; Read 1997), forming 'wood-wide-web' of symbiotic mycelia, a term that first appeared in the journal *Nature* (see the cover of Volume 388, 7 August 1997). Perry (1998), Wilkinson (1998) and Selosse *et al.* (2006) have given thoughtful discussions on the evolutionary ecology of mycorrhizal networks.

Studies of CMNs began in the early 1980s (e.g. Chiariello *et al.* 1982; Francis and Read 1984; Heap and Newman 1980a,b). Mycorrhizal links between plants have been observed visually under laboratory conditions (Finlay and Read 1986a,b; Francis and Read 1984; Heap and Newman 1980a,b; Newman 1988; Newman *et al.* 1992, 1994) and by autoradiography (Chiariello *et al.* 1982; Finlay and Read 1986a; Francis and Read 1984; Hirrel and Gerdemann 1979; Lerat *et al.* 2002; McKendrick *et al.* 2000; Read 1991; Read *et al.* 1985; Wu *et al.* 2001). However, at present neither AM nor EM networks have directly been examined in natural ecosystems, due to their cryptic, fragile and microscopic nature, although there are indirect evidences that plants from the same or the different populations share a common hyphal network (Booth 2004; He *et al.* 2006; Kennedy *et al.* 2003; McGuire 2007; Onguene and Kuyper 2002; Ronsheim and Anderson 2001).

The function of CMNs is to provide pathways for movement or transfer of nutrients including C (Simard *et al.* 1997), N (He *et al.* 2004, 2005), P (Smith *et al.* 2001), arsenic (P analog), cesium and rubidium (K analogs) (Meding and Zasoski 2008), water (Egerton-Warburton *et al.* 2007) or even genetic material (Giovannetti *et al.* 2004). Transfer of NH_4^+ or NO_3^- between N_2 -fixing plants and non- N_2 -fixing plants was mediated by AM CMNs (Bethlenfalvay *et al.* 1991; Frey and Schuepp 1993; Johansen and Jensen 1996; Moyer-Henry *et al.* 2006) or EM CMNs (Arnebrant *et al.* 1993; Ekblad and Huss-Danell 1995; He *et al.* 2004, 2005). Nitrogen gradients between N-rich donors and N-limited receivers may be a driving force for unidirectional N transfers via CMNs (Bethlenfalvay *et al.* 1991; Frey and Schuepp 1993).

Nitrogen benefits from N_2 -fixing to non- N_2 -fixing plants

Nitrogen, with the highest 78.0% in the atmosphere, is not readily available to plants. Some plant species is able to directly utilize atmospheric dinitrogen (N_2) by mutualistic symbioses with N_2 -fixing prokaryotes. This is termed N_2 fixation, mainly formed between soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* or *Sinorhizobium* and leguminous plants, or between the genus *Frankia* and some nonleguminous actinorhizal plants. It is estimated that N_2 fixation could range from ten(s) to a few hundred kg/ha/year in legumes and actinorhizal plants (Dixon and Wheeler 1983; Herridge *et al.* 2008; Huss-Danell 1997; Pawlowski 1999; Peoples and Herridge 1990, 1999; Schwintzer and Tjepkema 1990; Sprent 2001). Originally, all N is derived from the reduction of N_2 by N_2 fixation, and soil N (inorganic

and/or organic) is initially converted from such fixed N by soil microbial processes (Brady and Weil 2007; Paul 2006).

The main effect of mycorrhization on plant N status was in N_2 -fixing plants (Hayman 1986; Li et al. 2009; Vassilev et al. 2001) since common host genes are necessarily required for both nodulation and mycorrhizal formation in those plants (Albrecht et al. 1999; Ané et al. 2004; Sprent and James 2007) for their high-N-demanding requirements (McKey 1994; Sprent 1994, 2001). The N_2 -fixing plant can fertilize the soil directly and neighboring plants indirectly through above- and belowground litter inputs, leachates from leaves and exudates from roots. When legumes are intercropped with nonlegumes, an increase in both growth and yield of the legumes is often found, and the intercrops are more productive than intercrops of either two legume or two nonlegume species (Chalk 1998; Fujita et al. 1992; Herridge et al. 2008; Li et al. 2007; Malezieux et al. 2009).

Compared to monocultural cropping systems, these benefits obtained by the nonlegume crops may be due to transfer of the symbiotically fixed leguminous N, either (i) through release from nodulated roots, decomposition of dead nodule, root tissue and aboveground litter in the soil (Chalk 1998; Dubach and Russelle 1994; Ledgard 2001; Ledgard and Steele 1992; Paynel et al. 2001; Shen and Chu 2004; Sierra and Desfontaines 2009; Sierra and Nygren 2006); (ii) through mycorrhizal hyphal uptake and translocation (Kapulnik and Douds 2000; Marschner and Dell 1994; Miller and Allen 1992; Smith and Read 2008); or (iii) through common AM linkages between legumes and non-legume plants (Bethlenfalvay et al. 1991; Frey and Schuepp 1992, 1993; Johansen and Jensen 1996; Moyer-Henry et al. 2006). Similar observations have also been made between EM N_2 -fixing *Alnus glutinosa* (European alder) or *A. incana* (Grey alder) and EM non- N_2 -fixing *Pinus contorta* (Lodgepole pine), *P. sylvestris* (Scots pine) (Arnebrant et al. 1993; Ekblad and Huss-Danell 1995), or EM *Casuarina cunninghamiana* (river she-oak) and EM *Eucalyptus maculata* (spotted gum) (He et al. 2004, 2005). Thus N_2 -fixing plants (N donors) and non- N_2 -fixing plants (N receivers) provide good systems for investigating N transfer between plants.

Nitrogen transfer between mycorrhizal plants

Nitrogen transfer from one plant (donor) to another (receiver) is of fundamental importance in N_2 -fixing plant-based agricultural and natural ecosystems (Chalk 1998; Graham and Vance 2003; Forrester et al. 2006; Fujita et al. 1992; Stern 1993). The process of N deposition from one plant and subsequent uptake by another plant is termed N transfer (Jensen 1996). Nitrogen transfer from the N_2 -fixing N donor to the non- N_2 -fixing N receiver is estimated on the assumption that equal proportions of non-labeled and externally labeled N are transferred. Numerous studies over the past three decades have demonstrated that plant-to-plant N transfer from the donor to the receiver is not restricted to mass flow and diffusion through soil pathways and can take place directly through mycorrhizal hyphae in

CMNs that interconnect roots. Nevertheless, recapture of decomposed and exuded plant and fungal materials by the receiver may simultaneously occur in the soil.

In many cases, N is transferred from N_2 -fixing mycorrhizal to non- N_2 -fixing mycorrhizal plants via a CMN (unidirectional or one-way N transfer) (He et al. 2003; Newman 1988). A range between 0 to 80% of one-way N transfer has been observed from N_2 -fixing mycorrhizal to non- N_2 -fixing mycorrhizal plants by numerous studies (Table 2, see He et al. 2003; Newman 1988). In a few cases, N is also transferred from non- N_2 -fixing mycorrhizal plants to N_2 -fixing mycorrhizal plants via a CMN, but the percentage of N transfer is less than or around 10% (He et al. 2004, 2005; Johansen and Jensen 1996; Li et al. 2009; Moyer-Henry et al. 2006; Shen and Chu 2004). Thus, the N_2 -fixing plant is not always warranted to be the N donor. Furthermore, several studies showed that growth of the recipients had been improved by the net N gains through CMNs in the recipients (He et al. 2004, 2005; Johansen and Jensen 1996; Moyer-Henry et al. 2006; Newman 1988).

METHODS FOR INVESTIGATING NITROGEN TRANSFER BETWEEN PLANTS

^{15}N stable isotope

Nitrogen has 15 radioactive isotopes— ^{10}N , ^{11}N , $^{11\text{m}}\text{N}$, ^{12}N , ^{13}N , ^{16}N , ^{17}N , ^{18}N , ^{19}N , ^{20}N , ^{21}N , ^{22}N , ^{23}N , ^{24}N and ^{25}N —and two stable isotopes— ^{14}N and ^{15}N (http://en.wikipedia.org/wiki/Isotopes_of_nitrogen). The half-life time in the radioactive isotopes ranges from the shortest 2×10^{-22} s (^{10}N) to the longest 11 min (^{12}N), which makes them unsuitable for most biological investigations. Of the two stable isotopes, ^{14}N is more abundant and ^{15}N is rare, accounting for $\sim 99.6337\%$ and $\sim 0.3663\%$ of atmospheric N, respectively. Since the ratio of $^{15}\text{N}/^{14}\text{N}$ (0.0036765) in the atmosphere is a constant, atmospheric N_2 is used as the standard for ^{15}N analysis by continuous-flow isotope ratio mass spectrometer (Knowles and Blackburn 1993; Mariotti 1983; Unkovich et al. 2001). Theoretically, any biochemical, biogeochemical or physiological process will discriminate against ^{15}N , compared to ^{14}N , due to the greater atomic mass of ^{15}N . Differences in ^{15}N atom percentage (atom%) or natural abundance ($\delta^{15}\text{N}$, ‰) between two individuals thus provide N movement information in an experimental system.

In general, ^{15}N isotopic composition can provide information on (i) N inputs through N_2 fixation by free-living and symbiotic organisms, (ii) inputs of fertilizer N, (iii) the extent of N cycling and (iv) sources of N available for plant growth (Boddey et al. 2000; Dawson et al. 2002; Evans 2001; Handley and Scrimgeour 1997; Höglberg 1997; Nadelhoffer and Fry 1994; Robinson 2001). As a consequence, the $\delta^{15}\text{N}$ levels in vegetation usually reflect the ^{15}N abundance of N sources available to the plants (Shearer and Kohl 1986). Over the past four decades, both ^{15}N labeling or enrichment studies (usually expressed as atom%) (Chalk 1998; He et al. 2003; Hogh-Jensen

2006; Ledgard *et al.* 1985; Newman 1988; Stern 1993) and ^{15}N natural abundance ($\delta^{15}\text{N}$) studies (Binkley *et al.* 1985; Kohls *et al.* 1994; Moyer-Henry *et al.* 2006; Sierra and Nygren 2006; van Kessel *et al.* 1994) have been employed to measure N transfer between plants.

^{15}N labeling or enrichment method

In the ^{15}N labeling or enrichment method, an external N source (up to 99.90 at% ^{15}N enrichment) is used to trace N transfer or N metabolism over short-term periods (days to months). Measurement of isotopic effects is straightforward because there is a large enrichment of ^{15}N over background. Several sources of error have been identified (Herridge *et al.* 2008; Knowles and Blackburn 1993; Malezieux *et al.* 2009; Unkovich *et al.* 2001). The application rate of ^{15}N -enriched fertilizer must be low enough so that elevated N in the soil does not inhibit N translocation or cycling.

Nitrogen transfer has been quantified in three ways: percentage of N_{transfer} (% N_{transfer} , equations 1–3), amount of N transferred (mg/plant, equation 4) and percentage of NDFT (% NDFT, N in the N receiver derived from transfer, equations 4 and 5) (Giller *et al.* 1991; Johansen and Jensen 1996; Ledgard *et al.* 1985).

$$\% N_{\text{transfer}} = \frac{{}^{15}\text{Ncontent}_{\text{receiver}} \times 100}{({}^{15}\text{Ncontent}_{\text{receiver}} + {}^{15}\text{Ncontent}_{\text{donor}})} \quad (1)$$

$$\text{where } {}^{15}\text{Ncontent}_{\text{plant}} = \text{atom}\% {}^{15}\text{N excess}_{\text{plant}} \times \text{total N}_{\text{plant}} / \text{atom}\% {}^{15}\text{N excess}_{\text{labeled N}} \quad (2)$$

$$\text{and atom}\% {}^{15}\text{N excess}_{\text{plant}} = \text{atom}\% {}^{15}\text{N}_{\text{plant after labeling}} - \text{atom}\% {}^{15}\text{N}_{\text{plant background}} \quad (3)$$

$$N_{\text{transferred}} = \% N_{\text{transfer}} \times \text{total N}_{\text{donor}} / (100 - \% N_{\text{transfer}}) \quad (4)$$

$$\% \text{NDFT} = N_{\text{transfer}} \times 100 / \text{total N}_{\text{receiver}} \quad (5)$$

In addition, % NDFT values can also be calculated as follows (Tomm *et al.* 1994):

$$\% \text{NDFT} = \left(\frac{\text{atom}\% {}^{15}\text{N excess}_{\text{receiver}}}{\text{atom}\% {}^{15}\text{N excess}_{\text{donor}}} \right) \times 100 \quad (6)$$

Equations (5) and (6) are equal if the 0.3663% ^{15}N of atmospheric N_2 is subtracted from the measured atom% ^{15}N in equation (5).

Two-way (bidirectional) or net N transfer can be calculated as the difference between N transfer from plant A to plant B, and the reverse. Then, net N transfer could benefit one of the two plants, when that plant received more N than it donated through the transfer (He *et al.* 2004, 2005; Johansen and Jensen 1996; Li *et al.* 2009; Moyer-Henry *et al.* 2006; Newman 1988).

However, the use of externally added ^{15}N to quantify N transfer has some limitations, especially in fertile soils. First, compared to the large volume of soil N simultaneously available to plants with the ^{15}N -labeled external N, the amount of N transferred from N donor to N receiver may be much less than the amount of N taken up from the soil. Secondly, the root depth and pattern of N uptake of the N receiver may be different in intercrop and pure system situations, which may cause the ratio of unlabeled to labeled N to be different in different growth systems. Both of them can invalidate the assumptions for estimating N transfer.

^{15}N natural abundance method

As a general rule, non- N_2 -fixing plants had a greater $\delta^{15}\text{N}$ values than N_2 -fixing ones. For example, $\delta^{15}\text{N}$ values were +4.7 to +8.3‰ in non- N_2 -fixing *Eucalyptus* trees in Brazil and Thailand, whilst -1.4 to +1.9‰ in N_2 -fixing *Casuarina* seedlings growing in laboratory (Tjepkema *et al.* 2000; Yoneyama *et al.* 1990, 1993). In Australia, field *Eucalyptus* $\delta^{15}\text{N}$ values were +0.3 to +1.8‰ but only -0.6 to -0.8‰ in field *Acacia* (Schmidt *et al.* 1999). As a general rule a 5–10‰ variation of foliar $\delta^{15}\text{N}$ values (Evans 2001; Handley and Scrimgeour 1997; Handley *et al.* 1998; Hobbie and Hobbie 2006, 2008; Högborg 1997; Robinson 2001) was related to mycorrhizal types in the order: non-mycorrhizal/AM > EM \geq ericoid mycorrhizal (Michelsen *et al.* 1998). Variations in natural abundance signatures likely result from discrimination against the heavier ^{15}N over the lighter ^{14}N isotope during N uptake and transfer from mycorrhizal fungi to host plants (Hobbie and Hobbie 2006, 2008; Hobbie *et al.* 2000; Schmidt and Stewart 1997). Although small, these differences between isotopic compositions of the source and the sink (plant or soil) reflect the magnitude of naturally occurring ^{15}N variation during N translocation in the long-term periods (years to decades) (Binkley *et al.* 1985; Hobbie and Hobbie 2006, 2008; Kohls *et al.* 1994; Moyer-Henry *et al.* 2006; Sierra and Nygren 2006; van Kessel *et al.* 1994). Differences in ^{15}N could thus form a basis for tracing N transfer between plants.

^{15}N natural abundance is expressed as delta (δ) in parts per thousand ‰, per mil; Knowles and Blackburn 1993):

$$\delta^{15}\text{N}(\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}}) - 1] \times 1,000 \quad (7)$$

where R is the ratio of $^{15}\text{N}/^{14}\text{N}$ (atom%) of the sample and standard, and the standard is atmospheric N_2 ($\delta^{15}\text{N} = 0$; Mariotti 1983). The $\delta^{15}\text{N}$ value (0‰) of atmospheric N_2 (0.00366295 ^{15}N abundance) is the standard for $\delta^{15}\text{N}$ analysis (Mariotti 1983).

The whole plant $\delta^{15}\text{N}$ can be calculated by (Handley and Scrimgeour 1997):

$$\delta^{15}\text{N}_{(\text{whole plant})} = [(\delta^{15}\text{N}_{(\text{shoot})} \times \text{mg N in shoots}) + (\delta^{15}\text{N}_{(\text{roots})} \times \text{mg N in roots})] / (\text{mg N in whole plant}) \quad (8)$$

Table 1: characteristics of the six types of mycorrhizal associations (modified from Brundrett 2002, 2009; Brundrett et al. 1996; He et al. 2003; Rinaldi et al. 2008; Smith and Read 2008)

Characteristics	Mycorrhizal types					
	AM	EM	Arbutoid	Ericoid	Monotropoid	Orchid
Fungal organisms	Glomeromycota (~200 spp.)	Asco, Basidio (~7 750 spp.)	Asco, Basidio	<i>Hymenoscyphus</i> , <i>Oidiodendron</i> , selected Asco (~15 spp.)	Selected Basidio	<i>Rhizoctonia</i> and selected Basidio (~10 spp.)
Plants	Vascular plants	Gymnosperms, Angiosperms	Ericales	Ericales	Monotropaceae	Orchidaceae
Chlorophyll	+	+	+/-	+	-	+/-
Root structures						
Arbuscules	+	-	-	-	-	-
Fungal sheath	-	+ (-)	+	-	+	-
Hartig net	-	+	+	-	+	-
Hyphae coils	+/-	-	+	+	-	+
Hyphae in cells	+	- (+)	+	+	+	+
Septate hyphae	- (+)	+/-	+	+	+	+
Vesicles	+/-	-	-	-	-	-
Nutritional interactions						
Fungi → plants	Mineral nutrients	Mineral nutrients	Probably mineral nutrients	Nutrients, especially NH ₄ ⁺ and organic N	Carbohydrates and mineral nutrients	See footnote
Plants → fungi	Carbohydrates	Carbohydrates	Probably carbohydrates	Carbohydrates	Unknown	Unknown

Asco, Ascomycota; Basidio, Basidiomycota; +, present; -, absent; +/-, present or absent; (+), sometime present; (-), sometime absent. Two nutritional interactions exist from a symbiont to a host in Orchid mycorrhizas: juvenile achlorophyllous hosts, carbohydrates and mineral nutrients; adult photosynthetic hosts, probably mineral nutrients only.

Quantification of N transfer could be the same as in the ¹⁵N enrichment method if %N transfer had firstly been obtained as follows (He 2002):

A: one-way N transfer from the N₂-fixing donor to the non-N₂-fixing receiver:

$$\% N_{\text{transfer}} = \left(\delta^{15}\text{N}_{\text{receiver}_{\text{control}}} - \delta^{15}\text{N}_{\text{receiver}_{\text{mycorrhizal treatment}}} \right) / \delta^{15}\text{N}_{\text{receiver}_{\text{control}}} \times 100 \quad (9)$$

B: one-way N transfer from the non-N₂-fixing donor to the N₂-fixing receiver:

$$\% N_{\text{transfer}} = \left(\delta^{15}\text{N}_{\text{receiver}_{\text{mycorrhizal treatment}}} - \delta^{15}\text{N}_{\text{receiver}_{\text{control}}} \right) / \delta^{15}\text{N}_{\text{receiver}_{\text{control}}} \times 100 \quad (10)$$

However, the negative $\delta^{15}\text{N}$ values of the nodulated N₂-fixing reference plant (cultivated with N-free solution) had to be added into the $\delta^{15}\text{N}$ values of the receivers in order to avoid negative % N_{transfer} values in the equation 10.

The ¹⁵N natural abundance method depends on the fact that the soil N pool is enriched in ¹⁵N compared to the atmosphere due to soil processes since N mineralization discriminates against the heavier ¹⁵N isotope (Dijkstra et al. 2006; Yoneyama 1996). However, in ¹⁵N natural abundance stud-

ies, ¹⁵N discrimination over background is small, making measurement of naturally occurring isotopic effects more difficult. Meanwhile, there must be a sufficiently large difference (usually ~3–4‰) between soil $\delta^{15}\text{N}$ and atmosphere $\delta^{15}\text{N}$ in order to measure dilution effects (see Boddey et al. 2000; Evans 2001; Shearer and Kohl 1986; Yoneyama 1996; Robinson 2001). But it would take years or decades to distinguish those ¹⁵N differences in big trees in the field. This ¹⁵N natural abundance technique is similar to the ¹⁵N enrichment analysis because a reference plant is also required to account for fractionation or discrimination due to metabolic processes and to avoid the tedious assay of soil spatial and temporal variation in $\delta^{15}\text{N}$. Use of the naturally occurring ¹⁵N variation to investigate the role of mycorrhizas in N transfer is increasing (Dawson et al. 2002; Evans 2001; He et al. 2003; Hobbie and Hobbie 2006, 2008; Hogg-Jensen 2006; Moyer-Henry et al. 2006; Pate et al. 1993; Sierra and Nygren 2006).

Nitrogen transfer between plants: ¹⁵N enrichment versus ¹⁵N natural abundance

Over the past 40 years, the ¹⁵N enrichment technique has widely been used to investigate N translocation or cycling

Table 2: transfer of N from one plant to another via either AM or EM CMNs

Species A	Species B	Linkage direction ^a	Inoculum involved	Element transferred	N _{transfer} %	Reference
AM: CMNs						
<i>Arachis hypogaea</i> (peanut)	<i>Sida spinosa</i> (prickly sida)	A → B	Field soil with roots	¹⁴ NH ₄ ⁺	30.0–	Moyer-Henry <i>et al.</i> 2006
	<i>Senna obtusifolia</i> (sicklepod)	A → B			80.0	
<i>Bromus hordeaceus</i> (soft brome)	<i>Vitis vinifera</i> (grapevine)	A → B	Field soil with roots	¹⁵ NH ₄ ⁺	24.8	Cheng and Baumgartner 2004
<i>Glycine max</i> (soybean)	<i>G. max</i> (non-nodulated)	A → B	Field soil with roots	¹⁴ NH ₄ ⁺	48.0	Moyer-Henry <i>et al.</i> 2006
	<i>S. spinosa</i> (prickly sida)	A → B	Field soil with roots	¹⁴ NH ₄ ⁺	30.0–	Moyer-Henry <i>et al.</i> 2006
	<i>S. obtusifolia</i> (sicklepod)	A → B			80.0	
	<i>Sorghum bicolor</i> (sorghum)	A → B	<i>Glomus mosseae</i>	¹⁴ NH ₄ ⁺	22.5	He 2002
	<i>Zea mays</i> (maize)	A → B	3 <i>Glomus</i> species	¹⁵ NH ₄ ⁺	~5.0	Hamel <i>et al.</i> 1991a,b, 1992
		A → B	Field soil with roots	¹⁵ NH ₄ ⁺ , ³² P	3.0	Eissenstat 1990
<i>Hordeum vulgare</i> (barley)	<i>Pisum sativum</i> (pea)	A → B	<i>G. intraradices</i>	¹⁵ NH ₄ ⁺ , ¹⁵ NO ₃ ⁻	4.0	
<i>Medicago polymorpha</i> (bur clover)	<i>V. vinifera</i> (grapevine)	A → B	Field soil with roots	¹⁵ NH ₄ ⁺	5.5	Cheng and Baumgartner 2004
<i>Oryza sativa</i> (rice)	<i>Vigna radiata</i> (mung bean)	A → B	<i>G. caledonium</i>	¹⁵ NH ₄ ⁺	2.7	Li <i>et al.</i> 2009
<i>Pisum sativum</i> (pea)	<i>H. vulgare</i> (barley)	A → B	<i>G. intraradices</i>	¹⁵ NH ₄ ⁺ , ¹⁵ NO ₃ ⁻	15.0	Johansen and Jensen 1996
<i>Pueraria phaseoloides</i> (kudzu)	<i>Hevea brasiliensis</i> (rubber)	A → B	<i>G. clarum</i>	¹⁵ NO ₃ ⁻ , ³² P	0.3	Ikram <i>et al.</i> 1994
<i>Sorghum bicolor</i> (sorghum)	<i>Glycine max</i> (soybean)	A → B	<i>G. mosseae</i>	¹⁴ NH ₄ ⁺	28.5	He 2002
<i>Trifolium alexandrinum</i> (berseem)	<i>Z. mays</i> (maize)	A → B	<i>G. intraradices</i>	¹⁵ NH ₄ ⁺	4.7	Frey and Schuepp 1992, 1993
<i>Trifolium repens</i> (white clover)	<i>Lolium perenne</i> (ryegrass)	A ↔ B	<i>G. mosseae</i>	¹⁵ NH ₄ ⁺	5.0	Haystead <i>et al.</i> 1988
<i>V. radiata</i> (mung bean)	<i>O. sativa</i> (rice)	A → B	<i>G. caledonium</i>	¹⁵ NH ₄ ⁺	16.1	Li <i>et al.</i> 2009
<i>Z. mays</i> (maize)	<i>T. alexandrinum</i> (berseem)	A → B	<i>G. intraradices</i>	¹⁵ NH ₄ ⁺	0.1	Frey and Schuepp 1992, 1993
EM: CMNs						
<i>Alnus glutinosa</i> (alder)	<i>Pinus contorta</i> (pine)	A → B	<i>Paxillus involutus</i>	¹⁵ NH ₄ ⁺	15.0	Arnebrant <i>et al.</i> 1993
<i>A. incana</i> (alder)	<i>P. sylvestris</i> (pine)	A → B	<i>P. involutus</i>	¹⁵ NH ₄ ⁺	9.0	Ekblad and Huss-Danell 1995
<i>Betula pendula</i> (birch)	<i>Picea abies</i> (spruce)	A → B	<i>Scleroderma citrinum</i>	¹⁵ NH ₄ ⁺ , ¹³ C	0.3	Ek <i>et al.</i> 1996
<i>Casuarina cunninghamiana</i>	<i>C. cunninghamiana</i>	A → B	<i>Pisolithus tinctorius</i>	¹⁴ NH ₄ ⁺	30.5	He 2002
	<i>Eucalyptus maculata</i>	A → B	<i>P. tinctorius</i>	¹⁴ NH ₄ ⁺	32.8	He 2002
	<i>E. maculata</i>	A → B	<i>P. tinctorius</i>	¹⁵ NH ₄ ⁺	10.1	He <i>et al.</i> 2004, 2005
	<i>E. maculata</i>	A → B	<i>P. tinctorius</i>	¹⁵ NO ₃ ⁻	5.3	He <i>et al.</i> 2004, 2005
<i>E. maculata</i>	<i>C. cunninghamiana</i>	A → B	<i>P. tinctorius</i>	¹⁴ NH ₄ ⁺	26.7	He 2002
	<i>C. cunninghamiana</i>	A → B	<i>P. tinctorius</i>	¹⁵ NH ₄ ⁺	39.1	He <i>et al.</i> 2004, 2005
	<i>C. cunninghamiana</i>	A → B	<i>P. tinctorius</i>	¹⁵ NO ₃ ⁻	23.6	He 2002
	<i>E. maculata</i>	A → B	<i>P. tinctorius</i>	¹⁴ NH ₄ ⁺	10.0	He 2002
<i>P. abies</i> (spruce)	<i>B. pendula</i> (birch)	A → B	<i>S. citrinum</i>	¹⁵ NH ₄ ⁺ , ¹³ C	5.3	Ek <i>et al.</i> 1996

including N transfer between plants in agricultural systems (Chalk 1998; Herridge *et al.* 2008; Newman 1988; Stern 1993; Unkovich *et al.* 2001). However, the addition of external ¹⁵N to quantify N transfer has limitations. First, root depth and N-uptake pattern of the N receiver may be dissimilar in single- and mixed-species situations, which may cause a different unlabeled to labeled N ratio in the plant. Secondly, microbial ac-

tivities may be enhanced by the addition of labeled ¹⁵N, resulting in nutrient competition between plants and microorganisms. Meanwhile, products (N₂ or NO) of denitrification may be enriched in ¹⁵N because denitrification discriminates against ¹⁵N. Less than 10 mg N serve [2-chloro-6-(trichloromethyl) pyridine, a nitrification inhibitor] per kg soil could be applied to prevent from denitrification (Laskowski *et al.* 1975). Last, the most

critical source of error comes from the reference plant selection, if the source ^{15}N is different between the reference and the test plant (Knowles and Blackburn 1993; Unkovich and Pate 2001).

Over the past 20 years, the ^{15}N natural abundance has yielded a comparable precision to both the classic N mass balance and the later ^{15}N enrichment technique (Boddey *et al.* 2000; Handley and Raven 1992; Handley and Scrimgeour 1997; Herridge *et al.* 2008; Högberg 1997; Huss-Danell 1997; Knowles and Blackburn 1993; Robinson 2001; Shearer and Kohl 1986; Tjepkema *et al.* 2000; Unkovich and Pate 2001). By contrast, ^{15}N natural abundance has not been widely employed in N-transfer studies. A reason may be that the interpretation of isotopic compositions in mycorrhizal fungi and mycorrhizas is complex, particularly where exists a bidirectional N transport between fungi and plants (He *et al.* 2003; Hobbie and Hobbie 2006, 2008; Martin and Botton 1993; Martin *et al.* 2001; Wallenda *et al.* 2000). The greatest advantage of the natural abundance is its minimal disturbance to the plant–soil system. This may allow for tracing long-term ^{15}N natural variations at both individual and ecological systems. Therefore, the detecting of N transfers by $\delta^{15}\text{N}$ variations between different plants mirror the direct and inherent effects of internal N translocation or cycling. However, it should be noted that ^{15}N abundance in plants or soils might be spatially heterogeneous (Hansen *et al.* 1987; Pate *et al.* 1993; Shearer and Kohl 1986) and the selection of an appropriate reference plant remains difficult as in the ^{15}N enrichment experiment (Herridge *et al.* 2008; Knowles and Blackburn 1993; Unkovich and Pate 2001).

PERSPECTIVES AND FUTURE DIRECTIONS

Understanding the directions and magnitude of N transfer between plants through CMNs helps to clarify the agricultural and ecological importance of CMNs (He *et al.* 2003; Leake *et al.* 2004; Newman 1988, 1992; Selosse *et al.* 2006; Simard and Durall 2004; Smith and Read 2008). One-way or two-way AM- or EM-mediated N transfers do occur within the same or between different plant species via either AM or EM networks. Initially, most studies investigated two related questions: First, can N be transferred from one plant to another via a CMN? Secondly, is there any benefit from such N transfers to the recipients? Those questions are obviously important in addressing competition between plants (He *et al.* 2003; Herridge *et al.* 2008; Malezieux *et al.* 2009; Newman 1988; Read 1998; Read *et al.* 1985; Smith and Read 2008). More attention has been given to the first question, and there is little data for the second question. Without understanding two-way or net nutrient transfer between plants, it is difficult to understand the ecological or agricultural importance of CMNs (He *et al.* 2005, 2006; Leake *et al.* 2004; Newman 1988, 1992; Simard and Durall 2004).

Studies are urgently needed to identify net N transfer and to determine whether mycorrhizal hyphae play a direct role. First, the loss of N from roots into the soil followed by its uptake

by mycorrhizal roots would appear to be plant-to-plant N transfer through a CMN. Secondly, when water table is high, diffusion and mass flow in the soil profile may appear to be CMN transfer. It is necessary to separate the root systems of plants and allow only hyphal penetration, but no root contact. In most studies, 25- to 45- μm nylon or stainless metal meshes were used. A polytetrafluoroethylene hydrophobic membrane was applied (Frey *et al.* 1998; Mader *et al.* 1993, 2000). A narrow air gap between plants separated by fine meshes could also prevent interspecific nutrient movement through soil pathways (Faber *et al.* 1991; He *et al.* 2004, 2005; Meding and Zasoski 2008). However, ^{15}N could cross the air gap in hyphae, then leak into the soil and be taken up again as an indirect step following the direct transfer through hyphae. This sort of ^{15}N movement or translocation could be minimized by severing the hyphae within the air gap to see if transfer is disrupted and the flow, once established, stops.

New technologies and ideas are required to address if mycorrhizal-mediated N transfer between plants varies temporally and spatially and what benefit(s) could be given to the recipient through N transfer from the donor. Some perspectives and future directions are as follows:

- Does N transfer occur between any mycorrhizal plants, irrespective of their N_2 fixation characteristics? What determines the magnitude and direction of such N transfers in monocultural and/or mixed-species plant systems?
- Do AM and EM networks behave similarly on N transfer, considering that AM and EM networks are structurally different and that EM fungi can acquire N from organic sources?
- Are there interactions between AM and EM networks on N transfer since some plants do have dual AM/EM associations?
- Can the ^{15}N natural abundance method be employed to detect N transfer, similar to the ^{15}N enrichment technique?
- What kind of N is transferred? Inorganic N, organic N or both? Can quantum dots (fluorescent nanoscale semiconductors; Whiteside *et al.* 2009) be combined with ^{15}N label to trace N transfers if the transferred N is an organic N compound?
- Is there reciprocal C and N transfer? If yes, using $^{13}\text{C}/^{15}\text{N}$ to study C/N transfers between plants could provide clues on C translocation or cycling that plays crucial role on global C/N balance and environmental change.
- Is it possible to employ network theory and computer modeling (Southworth *et al.* 2005) to simulate the direction and distribution of N transfer from one plant to another?
- Can nanoscale secondary ion mass spectrometer (Herrmann *et al.* 2007) and/or other high-mass-resolution image techniques be explored to demonstrate unequivocally that an N transfer occurs through a CMN rather than through the soil?

To study nutrient transfer between mycorrhizal plants, it is important to keep in mind that mycorrhizas are associations involving plants, fungi, other soil microbes and N_2 -fixing microbes in N_2 -fixing plants. It is mycorrhizas and N

availability, not N₂ fixation that, play a vital and decisive role in N redistribution between plants (He 2002, 2005). The lack of convincing data underlines the need for creative, careful experimental manipulations. Nitrogen is crucial to productivity and N translocation or transfer is relevant to global concerns about N excess and N limitation in terrestrial ecosystems (Kaiser 2001; Moffat 1998; Nosengo 2003; Sanchez 2002; Vitousek *et al.* 1997), and such mycorrhizal-mediated N movement between plants could have practical implications for plant performance in both agricultural and natural ecosystems (CSIRO Publishing 1998; Forrester *et al.* 2006; Sprent 2005). Thus, one-way or two-way N transfer with ¹⁵N enrichment and/or ¹⁵N natural abundance method warrants further investigations on many species and under various field conditions.

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