# Belowground pathways for nitrogen transfer from a tropical legume tree to an associated fodder grass

Riina Jalonen

Department of Forest Sciences
Faculty of Agriculture and Forestry
University of Helsinki

Academic dissertation

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## ABSTRACT

Legume trees which form symbiosis with $N_2$-fixing bacteria can help replenish and maintain soil fertility on tropical agricultural lands by supplying nitrogen to the system. However, the mechanisms of N transfer from the trees to associated crops are not well understood. The role of root exudation and common mycelial networks of mycorrhizal fungi (CMN) in interplant N transfer was analysed in this study. A cut-and-carry agroforestry system comprising a legume tree (*Gliricidia sepium*) and a fodder grass (*Dichanthium aristatum*) was used as a model system. Nitrogen transfer was measured by labelling the tree with $^{15}$N. Variation in the isotopic composition of the N sources from the tree and its effects on N transfer estimates was analysed using experimental methods and mathematical modeling. Both plant species were observed to form symbiosis with the same subgroup of *Rhizophagus intraradices* in the field, indicating favourable conditions for the formation of CMN. In pot culture *D. aristatum* obtained up to 14% of its N from *G. sepium* via belowground pathways over 10 weeks, which was mainly accounted for N exudation. Nitrogen transfer via CMN contributed up to 2.5% of N in *D. aristatum* and corresponded to 18% of total N transferred. Nitrogen transfer via CMN increased with arbuscular colonisation of the N donor and with decreasing N concentration of the N recipient. Transfer estimates varied manifold depending on the assumed isotopic composition of transferred N, which highlights the need for careful estimation of the isotopic ratios of the actual N sources. The results suggest a significant role for root exudates and CMN in transferring N from legume trees to the associated crops, as opposed to the common perception of tree prunings and mulching as the primary N sources to the crops. Design and management options of agroforestry systems could be reviewed to foster belowground N transfer and improve the sustainability of farming systems.

**Keywords***: root exudation, common mycelial networks (CMN), *Gliricidia sepium*, *Dichanthium aristatum*, *Rhizophagus intraradices*, $^{15}$N labelling

## RÉSUMÉ

Les arbres légumineux qui forment symbioses avec les bactéries fixatrices d'azote peuvent aider à maintenir ou à rétablir la fertilité des sols dans les agroécosystèmes tropicaux, en fournissant de l'azote au système. Cependant, les mécanismes de transfert d'azote des arbres aux plantes associées n'ont pas bien connus. Dans cette étude, on a analysé le transfert d'azote souterrain via les exsudats racinaires et les réseaux des champignons mycorhiziens (angl. *Common Mycelial Networks*, CMN). Un système agroforestier de production du fourrage, composé d'un arbre légumineux (*Gliricidia sepium*) et d'une herbe fourragère (*Dichanthium aristatum*), a été utilisé comme système modèle. Les arbres et l'herbe étaient régulièrement taillés ou coupée et la biomasse était exportée de la parcelle. Le transfert d'azote a été mesuré en utilisant le marquage artificiel $^{15}$N des feuilles de l'arbre. La variation de la composition isotopique des sources azotées de l'arbre et ses effets sur le transfert d'azote ont été analysés par des méthodes expérimentales et la modélisation. On a observé que *Gliricidia sepium* et *D. aristatum* forment des symbioses avec le même sous-groupe du champignon mycorhizien *Rhizophagus intraradices* sur la parcelle agroforestière, ce qui indique des conditions favorables pour la formation de CMN. Dans une expérimentation en pot de dix semaines, *D. aristatum* a obtenu jusqu'à 14% de son azote de *G. sepium* par le transfert souterrain, ce qui a été associé principalement à l'exsudation racinaire de l'arbre. Le transfert via le CMN a apporté jusqu'à 2.5% de l'azote de *D. aristatum*, ce qui correspond à 18% de l'azote total transféré. Le transfert d'azote via le CMN a été positivement corrélé avec la colonisation des arbuscules dans l'arbre donneur d'azote, et négativement corrélé avec la concentration d'azote dans la plante associée. L'évaluation du transfert dépend fortement de l'estimation de la composition isotopique des sources azotées, ce qui souligne l'importance de cerner avec fiabilité les teneurs en $^{15}$N de ces sources. Les résultats indiquent que les exsudats racinaires et le CMN ont un rôle clé dans le transfert d'azote des arbres légumineux aux plantes associées. Cette étude améliore ainsi le cadre conceptuel actuel de la problématique traitée, d'après lequel l'engrais vert (p. ex. recyclage dans le sol des tailles de l'arbre) est considéré la seule source de transfert d'azote. De nouvelles options pour la conception et la gestion des systèmes agroforestiers pourraient être envisagées afin d'optimiser le transfert souterrain d'azote et assurer la durabilité de ces systèmes.

**Mots de clé:** exsudation racinaire, symbioses mycorhiziennes, *Dichanthium aristatum*, *Gliricidia sepium*, *Rhizophagus intraradices*, marquage $^{15}$N.

# ACKNOWLEDGEMENTS

# LIST OF ORIGINAL ARTICLES

The study is based on this extended summary and the following original research articles, which are referred to in the text by their Roman numerals. Articles are reprinted with the kind permission of the publishers.

I **Jalonen, R**., Timonen, S., Sierra, J., Nygren, P. 2012. Arbuscular mycorrhizal symbioses in a cut-and-carry forage production system of legume tree *Gliricidia sepium* and fodder grass *Dichanthium aristatum. Agroforestry Systems*, July 2012, published ahead of print.
doi: 10.1007/s10457-012-9553-1

II **Jalonen, R**., Sierra, J. 2012. Temporal variation of N isotopic composition of decomposing legume roots and its implications to N cycling estimates in $^{15}$N tracer studies in agroforestry systems. *Applied and Environmental Soil Science,* 2012, Article ID 506302.
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III **Jalonen, R**., Nygren, P., Sierra, J. 2009. Transfer of nitrogen from a tropical legume tree to an associated fodder grass via root exudation and common mycelial networks. *Plant, Cell and Environment* 32:1366-1376.
doi: 10.1111/j.1365-3040.2009.02004.x

IV **Jalonen, R**., Nygren, P., Sierra, J. 2009. Root exudates of a legume tree as a nitrogen source for a tropical fodder grass. *Nutrient Cycling in Agroecosystems* 85:203-213.
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# AUTHOR'S CONTRIBUTION

I   The study was planned by Pekka Nygren and Sari Timonen. Riina Jalonen conducted the sampling, analysed the data on mycorrhizal colonisation and interpreted the results. DNA analyses were conducted by S. Timonen who also helped to interpret their results. R. Jalonen was the main author of the paper.

II  The experimental study was planned by Pekka Nygren and Jorge Sierra. Riina Jalonen conducted the experimental study, planned the modeling study together with J. Sierra, developed the model and conducted the simulations. She analysed the data, interpreted the results, and was the main author of the paper.

III The experiment was planned by Pekka Nygren and Jorge Sierra. Riina Jalonen conducted the experiment, analysed the data, interpreted the results together with P. Nygren and J. Sierra, and was the main author of the paper.

IV  The experiment was planned by Pekka Nygren and Jorge Sierra. Riina Jalonen conducted the experiment, analysed the data, interpreted the results together with P. Nygren and J. Sierra, and was the main author of the paper.

# CONTENTS

8

# INTRODUCTION

## Trees as nitrogen providers in agroforestry systems

Soil fertility on tropical agricultural lands is rapidly declining as a result of continuous nutrient removal through crop harvests, shortening cropping cycles and fallow periods, expansion of agriculture to marginal lands, inadequate or inappropriate use of organic or inorganic fertilisers, and lack of incentives for soil conservation practices (Hartemink 2003). Although tropical soils are highly diverse, in many areas they are naturally susceptible to nutritional deficiencies; in Southeast Asia and South America nutrient stress, together with chemical toxicities, affects half or more of the soils (Larson 1986). Depletion of soil fertility reduces agricultural yields and aggravates food insecurity (Sanchez 2002). Nevertheless, in public discussion on food security, the changes in the quality and fertility of soils as the primary physical resource supporting food systems often get shadowed by other issues such as land tenure or climate threats (Stocking 2003).

Nitrogen is one of the most important nutrients in sustaining agricultural production globally. While it is seldom the limiting nutrient in natural ecosystems in the humid tropics, serious N deficits may develop in agroecosystems when N is exported in considerable amounts with crop harvest (Martinelli 1999, Vitousek et al. 2002, Lal 2004).

Farming practices affect the sensitivity of soils to degradation. Practices which integrate soil conservation can help counteract degradation and loss of fertility, and provide alternative or complementary approaches to the use of inorganic fertilisers (Jensen and Hauggaard-Nielsen 2003). Improved fallows or intercropping with legume plants which form symbiosis with $N_2$-fixing bacteria (*$N_2$-fixing plants* in the following), can replenish and maintain soil fertility by contributing to the accumulation of soil organic matter and particularly N (Peoples and Herridge 1990, Giller 2001). For many legume tree species, symbiotic $N_2$ fixation accounts for 30-90% of tree total N under varying natural conditions (Giller 2001). Legume trees may contribute up to 270-550 kg [N] ha$^{-1}$ annually to the N balance of agroecosystems (Sanginga et al. 1994, Jayasundara et al. 1997, Dulormne et al. 2003), and agricultural crops cultivated with $N_2$-fixing trees have been shown to benefit from the association e.g. in terms of N and cereal grain yields (Fujita et al. 1992, Chalk 1998). Nitrogen of atmospheric origin constituted from 13% to 42% of total N in coffee (*Coffea arabica* L.) in several coffee – legume tree associations (Snoeck et al. 2000), up to 21% in *Setaria sphacelata* (Stapf) grass in association with legume trees *Gliricidia sepium* (Jacq.) Kunth ex Walp. and *Leucaena leucocephala* (Lam.) de Wit (Jayasundara et al. 1997), and from 31% to 35% of N in fodder grass *Dichanthium aristatum* (Poir) C.E. Hubb. intercropped with *G. sepium* (Sierra and Nygren 2006, Daudin and Sierra 2008).

Agroforestry refers to land use practices where woody perennials are intentionally integrated with crop or animal species. While agroforestry covers a wide variety of production systems, trees on agricultural land are common. According to a recent global study which used satellite data, half of agricultural land in Southeast Asia and Central America has more than 30% of tree cover (Zomer et al. 2009). Dinitrogen-fixing trees are widely used in agroforestry, and fast-growing fodder legumes such as *Leucaena leucocephala* and *Gliricidia sepium* are among the most popular agroforestry tree species. In densely populated rural areas agroforestry can contribute to cut-and-carry livestock systems where feed, crop residues or litter is collected from communal areas or other farms and carried to livestock confined on or close to the farms (LEAD 1999). Integrating fodder-

providing legume trees in the landscape with fodder crops can help source the needed feed effectively from a small area, and improve its nutritive quality (Niang et al. 1998). However, such production systems are vulnerable to the loss of soil fertility because of the high nutrient exports and because nutrients are not recycled back to the system in animal dung and urine as in the case of free-ranging livestock. Cut-and-carry systems illustrate the problem of competing demands for organic fertilisers for fodder, fuel, or sales for income, which often restrict fertiliser use (LEAD 1999).

Decomposing, nitrogen-rich leafs and twigs are usually thought to be the main mechanism of nutrient cycling from $N_2$-fixing trees (e.g. Kass et al. 1997, Beer et al. 1998, Giller 2003). In many agroforestry systems the trees are frequently pruned to provide green manure to the crops. Pruning also temporarily reduces competition with crops for light, water and nutrients, although it generally increases the competition for soil N; the trees absorb more N from the soil during regrowth because turnover of the $N_2$-fixing nodules impedes $N_2$ fixation (Nygren 1995, Nygren and Ramirez 1995). The quality of above-ground plant parts as N sources have been studied in numerous studies (Mafongoya et al. 1998, Palm et al. 2001). In a pot culture study maize (*Zea mays* L.) recovered 9-44% of N from the prunings of four legume tree species over 21 weeks (Cadisch et al. 1998). In field trials up to 80% of N in tree prunings was released during the annual cropping cycles, while the associated crops generally recovered less than 20% of it (Palm 1995).

Interestingly, substantial N transfer from $N_2$-fixing trees to associated crops was observed even in intensively managed cut-and-carry fodder production systems, where above-ground tree litter is practically eliminated by frequent harvests (Sierra et al. 2002, Dulormne et al. 2003). Similarly, maize N uptake in alley cropping with *Gliricidia sepium* was not reduced when mulch was removed from the system (Haggar et al. 1993). Mulch addition explained only partly the observed increase in soil N which developed over several years. These observations suggest that considerable N transfer happens via belowground processes. In fact, while most studies have concentrated on N release from above-ground biomass of legume trees, management of N release from belowground biomass maybe at least equally important for crop nutrition, since up to 50-60% of total plant N of frequently pruned agroforestry trees may occur in roots (Sanginga et al. 1995).

Nitrogen transfer from $N_2$-fixing tree species to associated plants via belowground processes has been observed under semi-controlled conditions (Virtanen and Saastamoinen 1936, Catchpoole and Blair 1990, He et al. 2004, Roggy et al. 2004, Sierra et al. 2007, Kurppa et al. 2010), but the methods of transfer are not well understood. Considerably more research on belowground N transfer has been conducted on herbaceous legumes, and it has led to the identification of potential N release and transfer pathways. Firstly, living roots of plants are known to exude nitrogenous compounds of low molecular weight, e.g. ammonium and amino acids, to the soil solution (Rovira 1956, Brophy and Heichel 1989, Paynel et al. 2001). Plants and their mycorrhizal fungal symbionts can directly absorb such compounds (Cliquet et al. 1997, Hodge et al. 2001, Lipson and Näsholm 2001). Secondly, N may be directly transferred from the N donor to the N recipient plant via mycelial networks of mycorrhizal fungi which connect the root systems of the plant species (common mycelial networks, CMN) (Brophy et al. 1987, Bethlenfalvay et al. 1991, Frey and Schüepp 1992).

Direct N transfer refers to situations where the N donor plant releases N in such form that the N recipient plant is able to absorb it directly without the complete mineralisation cycle by soil microbial organisms, and independently of C dynamics in the soil. It is conceptually different as N source from green manure, mulch, litter or residues, from which

N becomes again available for plant uptake only after mineralisation (Høgh-Jensen 2006). Direct N transfer may reduce the competition for N between the recipient plant species and soil microbial organisms (cf. Owen and Jones 2001, Jones et al. 2005), which makes it particularly interesting for crop nutrition.

Growing understanding about the complex biological interactions in agroecosystems underlines that optimal management of the tree component in agroforestry is not possible without considering both the facilitative and competitive relations of the trees and the crop species, but importantly also their symbionts such as $N_2$-fixing bacteria and mycorrhizal fungi, as well as soil microorganisms. Soil microorganisms on one hand decompose organic material and make its nutrients again available to plants, and on the other hand effectively compete with the plants and their fungal symbionts for the nutrients. If above-ground processes were the major pathway of N transfer from $N_2$-fixing trees to associated crops, as currently thought, choosing tree species with maximal production of N-rich leaf litter would suffice for the optimal N management in agroforestry systems. In contrast, if significant amounts of N are transferred via direct belowground processes, then what is crucial instead are good species combinations and management practices which consider N dynamics within and between plant species and their microbial symbionts. For example, N exudation might be related to balancing plant N demand with N supply from $N_2$ fixation after tree pruning (cf. Nygren 1995, Amtmann and Blatt 2009). Phosphorus was preferentially transferred between plants that shared the same type of mycorrhizal fungal symbionts (Eason et al. 1991), and significant differences were found in N transfer from legumes to non-legumes between mycorrhizal fungal isolates (Mårtensson et al. 1998).

So far, belowground matches and dynamics of intercropped species and their microbial symbionts have received little attention. Better understanding of the processes of belowground N transfer would help to develop new improved ways of managing agroforestry systems, contributing to a more efficient N transfer between trees and the associated crops and, thus, increasing productivity and sustainability of the systems and the nutritional quality of yielded products.

**Belowground pathways of N transfer**

*Common mycelial networks*

Carbon and nutrients have been observed to move between plants which share or are able to share the same mycorrhizal symbionts, even if the root systems of the plants are separated with a barrier impermeable to roots but permeable to the hyphae of mycorrhizal fungi (Bethlenfalvay et al. 1991, Simard et al. 1997, Mårtensson et al. 1998). Such transfer is accounted to common mycelial networks (CMN) which are formed between individual plants when the extraradical hyphae of their fungal symbionts fuse, i.e. form anastomosis. The complete fusion of cell walls allows cytoplasmic flow and transport of compounds between the fungi, and between their host plants (Giovannetti et al. 2006). Arbuscular mycorrhizal (AM) fungi, which colonise 80% of land plants, have a low host specificity (Sanders 2003, Smith and Read 2008), and they thus provide potential for the formation of CMN between plant species of different functional groups (Newman 1988). Anastomoses were observed in 34-90% of the cases where hyphae of the same isolates of AM fungi *Funneliformis caledonium* (ex *Glomus caledonium*) or *Rhizophagus intraradices* (ex *Glomus intraradices*) came into contact (Giovannetti et al. 1999), and in average 0.5

anastomoses mm$^{-1}$ of hyphae were observed in the mycelia of *Funneliformis. mosseae* (ex *Glomus mosseae*) spreading from plant roots (Giovannetti et al. 2001; see Schüßler and Walker 2010 for the revised taxonomy of AM fungi). Many studies show evidence of substantial N transfer from $N_2$-fixing legumes to non-legumes via CMN (Frey and Schüepp 1992, Martin et al. 1995, Johansen and Jensen 1996), while in other studies the transferred amounts were insignificant or too low to affect the nutritional status of the recipient plant (Hamel et al. 1991, Ikram et al. 1994). Controversy remains of whether N is transferred via CMN in amounts that are of ecological and physiological importance to the recipient plants.

Mycorrhizal symbiosis is important both for legumes and non-legumes for the acquisition of N and especially P from the soil. In contrast, reasons for why the legume host would release N to the fungal symbiont for transfer via CMN and the associated transfer mechanisms are not well understood. It has been demonstrated theoretically that N can move from the host to the fungus although the net flow would be towards the host (Smith and Smith 1990). Arbuscules and arbusculate coils are known as the sites where nutrients are transferred from the fungal symbiont to the plant and C to the opposite direction (Smith and Read 2008). Arbuscules are highly branched structures which are formed when AM fungal hyphae in the intercellular space of the root cortex penetrate individual cells (morphological type known as *Arum*-type), while in the case of arbusculate coils the fungus within plant root is virtually completely intracellular and forms extensive, non-terminal coils in cortical cells (*Paris*-type, after Gallaud 1905; reviewed in Smith and Smith 1997, Dickson et al. 2007). These interfaces might also involve bidirectional transfer of nutrients (cf. Smith and Smith 1990). In addition, intraradical mycorrhizal hyphae might be able to absorb N available in the host plant's cytoplasm similarly to biotrophic parasites (cf. Smith and Smith 1997, Harrison 1999), and then transfer it to associated plants.

It has been suggested that direct N transfer via CMN may be driven by sink-source processes between the donor and recipient plant species (Perry 1998). Nitrogen seems to flow from the more N-sufficient plant to the less N-sufficient one, e.g. from a $N_2$-fixer to a non-$N_2$-fixer, from N-fertilised (Bethlenfalvay et al. 1991) or defoliated N donor plants (Johansen and Jensen 1996), or towards physiologically younger plants (Frey and Schüepp 1992). In general, the role of mycorrhizal fungi in plant nutrient acquisition increases in N-deficient conditions (Johansen et al. 1994, Mäder et al. 2000). Common mycelial networks can be particularly important in plant nutrient acquisition in conditions where the relatively immobile ammonium is the main source of soil N (Johansen et al. 1992, Smith and Read 2008). This may be the case in ecosystems involving tropical grasses: some tropical grass species have been observed to release from their roots compounds which reduce the populations or activity of nitrifying bacteria (Ishikawa et al. 2003). In addition to arbuscules and arbusculate coils, vesicle colonisation could reveal useful information on the plant N status for interpreting N transfer. Vesicles, which occur in the intra- or intercellular space in the root cortex, are assumed to be storage organs for energy reserves within the fungus (Smith and Read 2008). Arbuscule:vesicle ratio has been suggested as an indicator of the relative cost or benefit of the fungus to the host plant (Braunberger et al. 1991, Titus and Leps 2000).

*Nitrogenous root exudates*

Root exudation is part of rhizodeposition, which is defined as the release of volatile non-particulate and particulate compounds from living plant roots (Wichern et al. 2008). Exudation of sugars and other carbonaceous compounds is increasingly understood as an

important process in regulating the interactions of plants and soil microbial organisms (Jones et al. 2004). Nevertheless, plant roots also exude many nitrogenous compounds such as ammonium, amino acids or even proteins (Sawatsky and Soper 1991), although they have generally received much less attention than sugars. The exuded nitrogenous compounds may contribute to N nutrition of neighbouring plants. Exuded N may be absorbed by the associated plants if the root systems or mycorrhizal fungal symbionts of the plants are in close contact, or after short-distance mass flow of compounds with soil solution to the vicinity of roots (Fig. 1).

Ammonium has been observed to be exuded from living plant roots in significant amounts, and it seems to be the predominant form of exudate N of legumes (Brophy and Heichel 1989, Paynel and Cliquet 2003). From 3 to 23 % of total N in a non-$N_2$-fixing ryegrass (*Lolium perenne* L.) was derived from the exudation of ammonium and to a lesser extent of amino acids by an associated $N_2$-fixing clover with hydroponics cultivation (*Trifolium repens* L.; Paynel et al. 2001). Exudation of amino acids from legume roots has been observed in hydroponic cultivation (Ofosu-Budu et al. 1990, Shepherd and Davies 1994) and in solid growth medium (Rovira 1956, Brophy and Heichel 1989, Paynel and Cliquet 2003), but with the exception of Rovira (1956) the quantities have generally been small.

The factors controlling exudation are not well known. Membrane permeability of the solute, concentration differences within root and in soil, and biotic and abiotic stress have been suggested as driving forces (Jones et al. 2004). Jones and Darrah (1994) observed that exudation of amino acids from maize roots occurred by passive diffusion, but that their recapture processes were active. Especially in legumes exudation may act as a regulation mechanism for controlling the concentration of amino acids in cytoplasm and balancing $N_2$ fixation with plant N requirements in the short term. Concentration of amino acids in cytoplasm regulates $NH_4^+$ and $NO_3^-$ transporters (Amtmann and Blatt 2009), and if the concentration increases too much, e.g. as a result of excess $N_2$ fixation, N transport within the root may slow down. Exudation of the excess N in such case could help to restore the N balance within roots. Defoliation of trees under a heavy fodder harvest regime may represent a stress factor which increases exudation. When N balance within a plant changes in pruning, flushes of N to the roots may be expected (Nygren 1995), and in herbaceous plants these have been associated with increased exudation (Eason and Newman 1990, Ayres et al. 2007). Shoot removal increases N transfer from a donor plant, both in mycorrhizal and non-mycorrhizal plants (Ikram et al. 1994, Johansen and Jensen 1996, Mårtensson et al. 1998), which may also imply increased exudation.

Relative competitive abilities between plant species in capturing exuded compounds from the soil solution may determine the net benefits of exudation for a species. Exudation could result in a net flux of N to the soil solution from plants which have low capacity for organic N uptake or low uptake rates (Lipson and Näsholm 2001). These plants would thus provide N to the associated plants that have a higher affinity for organic N. Legumes are generally less effective competitors for soil N than grasses (Ledgard and Steele 1992), and grasses may, thus, benefit from the exudates of legume species.

*Direct and indirect N transfer*

The underlying reasons for interplant N transfer are not well understood. Direct N transfer may be significant for plant nutrient acquisition especially in systems with high potential for nutrient immobilisation, because it can provide a shortcut to the nutrient mineralisation
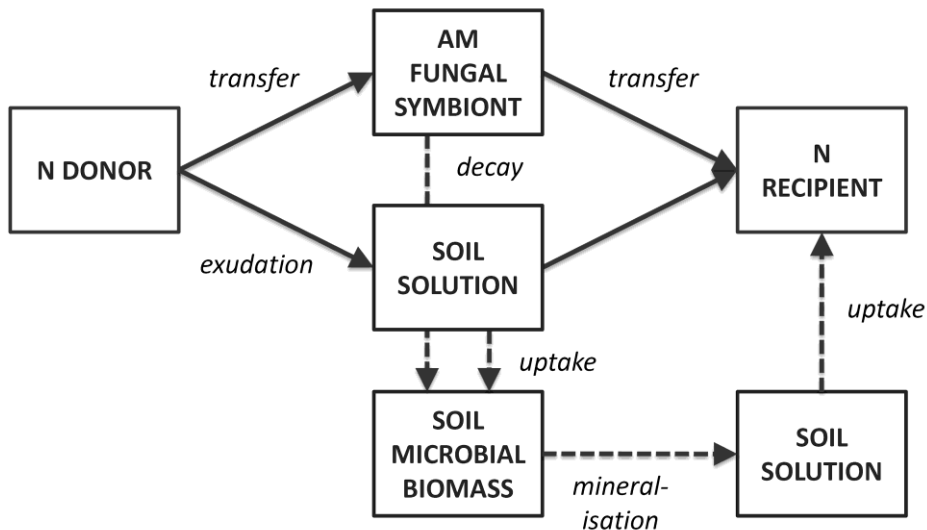
**Figure 1** Direct (solid line) and indirect (dashed line) N transfer pathways involving root exudation and common mycelial networks of arbuscular mycorrhizal (AM) fungi.

cycle and reduce competition with soil microbial organisms (cf. Owen and Jones 2001, Jones et al. 2005). Nitrogen transfer could contribute to species diversity and plant density in ecosystems, thus improving the overall resource use and increasing the stability and resilience of the systems. Such impacts have been associated to mycorrhizal fungal symbioses in general, since they are known to be able to alter plant competitive relationships and plant community composition as a consequence (Hart et al. 2003, van der Heijden et al. 2003).

It is important to note that N released by the N donor plant either via exudation or to AM fungal symbionts may also become subject to indirect N transfer, via microbial uptake or turnover of the mycelia which is known to be rapid (Staddon et al. 2003; Fig. 1). Suitable research methods to quantify whether N transfer via root exudation or mycorrhizal symbionts is direct or indirect are lacking, especially for CMN which are microscopic and fragile in nature. Consequently, the evidence of direct transfer of nutrients via these transfer pathways comes from indirect observations. For example, the amount of nutrients that plants are able to capture from the dying roots of associated plants has been shown to increase several-fold when the living and dying roots share or able to share the same mycorrhizal fungal symbionts (Hamel and Smith 1991). Such results also demonstrate the potential significance of direct N transfer.

In this study, *direct N transfer* is discussed theoretically, while making attempts to create experimental support to its role. *Nitrogen transfer*, in turn, is used to refer to both direct and indirect N transfer (via the mineralisation and immobilisation cycle in soil), as they are difficult to distinguish in experimental research. This use of the term is consistent with research literature where 'N transfer' commonly is used to refer to all N in the N recipient species originating from the N donor, without distinguishing between N mineralisation from organic residue and other N sources (Høgh-Jensen 2006).

**Isotope techniques**

Nitrogen has two stable isotopes, $^{14}N$ and $^{15}N$. Of these, $^{14}N$ is far more abundant in the atmosphere; $^{15}N$ accounts only for a very small but very stable ratio of 0,3663% of the atmospheric N. The bonds formed by the lighter $^{14}N$ are more easily broken (He et al. 2003), and most biological and physical processes in soil and plants, such as nitrification or transamination, discriminate against $^{15}N$. Thus, soils usually are enriched in $^{15}N$. The $N_2$-fixing enzyme nitrogenase is thought to have a minimal isotopic discrimination, and the proportion of $^{15}N$ in $N_2$-fixing plants is generally closer to the atmospheric $^{15}N$ ratio than in plants relying solely on N uptake from the soil (Högberg 1997). In the following, the $^{15}N:^{14}N$ isotopic ratio of plants and in the environment is generally referred to as *isotopic composition*.

Isotope techniques are used to trace N throughout ecosystems, using the atmospheric $^{14}N:^{15}N$ ratio as a standard. The techniques allow minimal disturbance, great accuracy of detection, or both, depending on the choice between the $^{15}N$ natural abundance and $^{15}N$ enrichment methods, and the actual experimental design (Robinson 2001). Isotopic composition of a sample in the applications is usually expressed as atom-%, or as the deviation ($\delta^{15}N$) from the atmospheric standard, which can be calculated as

$$\delta^{15}N\text{‰} = \frac{atom\%^{15}N_{sa} - atom\%^{15}N_{at}}{atom\%^{15}N_{at}} x1000 \qquad (1)$$

where the subscript *sa* and *at* refer to the sample and the atmospheric $^{15}N$ atom-%, respectively.

In the $^{15}N$ natural abundance method, N isotopic composition of a sample is compared with that of the atmospheric standard, to determine the proportion of N originating from $N_2$ fixation (minimal deviation from atom-% $^{15}N_{at}$) and the soil sources (deviation as a result of $^{15}N$ discrimination in soil processes). Applying the method requires that the N isotopic composition of the available N sources and the discrimination against $^{15}N$ in N cycling processes are known. Soil processes discriminate against $^{15}N$ more than do plant N assimilation processes (Högberg 1997), and isotopic composition of N fixed from the atmosphere is assumed to change less in direct than indirect N transfer from $N_2$-fixing to non-$N_2$-fixing plants. Therefore, it is possible to distinguish using the $^{15}N$ natural abundance method whether the observed N transfer has occurred mainly via direct or indirect transfer processes (cf. Sierra and Nygren 2006). For reviews of the applications of the $^{15}N$ natural abundance method in N transfer studies, see He et al. (2003) and Unkovich et al. (2008).

The $^{15}N$ enrichment method involves applying a small amount of highly $^{15}N$ enriched substance in the studied system and tracing it thereafter. The high $^{15}N$ enrichment masks the effect of the natural discrimination processes and, therefore, practically eliminates errors related to unknown discrimination. The method can be especially useful for studying the belowground N cycling processes which are difficult to trace otherwise and where the discrimination against $^{15}N$ is not well known. Application of the method requires the assumption that the added $^{15}N$ and natural N are equally cycled in the system. Several methods for $^{15}N$ enrichment have been developed, for labelling either the growth medium or the N donor plant material directly with $^{15}N$. The most sophisticated methods for labelling legumes involve supplying them with $^{15}N$-enriched $N_2$. Because of the low $^{15}N$ concentration in nature, very small additions of $^{15}N$ to the donor plant are generally

sufficient to reliably track N cycling in the systems, and therefore the method does not interfere with N cycling or $N_2$ fixation. Soil labelling has proven to be more problematic, as it requires much higher amounts of additional $^{15}N$, and achieving a homogenous enrichment of the growth medium is difficult (Chalk and Ladha 1999). Labelling with $^{15}N$ is often conducted in pulses to distribute the label more evenly in the plant.

Quantification of N transfer in agroforestry systems using isotope techniques crucially depends on the determination of the isotopic composition of the N sources. Isotopic composition of plant organs or even compounds differ from each other and over time, both naturally and in $^{15}N$ enrichment studies. Discrimination against $^{15}N$ in biochemical processes results in variation of $^{15}N$ natural abundance between plant organs and their nitrogenous compounds (Högberg 1997). In $^{15}N$ enrichment studies assimilation of N from the soil, its allocation within the plant during growth, and in legumes also $N_2$ fixation, result in the dilution of the label at different rates for different organs (McNeill et al. 1997, Khan et al. 2002). Furthermore, recent studies suggest that the isotopic composition of N released from the decomposing roots of a legume tree after pruning differs from that of living roots just before pruning, both with $^{15}N$ natural abundance and $^{15}N$ enrichment methods (Sierra et al. 2007). Pruning or shoot removal of the N donor induced in the N recipient plants isotopic patterns which were inconsistent with common assumptions of $^{15}N$ tracer studies, making it impossible to reliably estimate N transfer (Sierra et al. 2007, Daudin and Sierra 2008, Sierra and Motisi 2012). Different decomposition rates of nitrogenous compounds of a plant could enhance the effects of isotopic variation among the compounds. Variation in decomposition rates is well-known from residue decomposition research (Henriksen and Breland 1999, Adair et al. 2008), yet not commonly considered in $^{15}N$ tracer studies. Together these two factors may result in considerable temporal variation in the isotopic composition of N released from organic inputs, e.g. roots decomposing after pruning. Such variation is subsequently reflected in the isotopic composition of the N recipient pools, e.g. soil compartments, soil microbial biomass or associated crops, and interferes with N transfer estimates.

**Research needs**

Substantial N transfer from $N_2$-fixing trees to associated crops in intensively managed cut-and-carry systems, where above-ground tree litter is practically eliminated by frequent harvests (Sierra et al. 2002, Dulormne et al. 2003; see also Haggar et al. 1993), suggests that belowground N transfer is important to the stability and productivity of agroforestry systems. However, the N transfer pathways and processes remain largely unknown. Dulormne et al. (2003), who estimated the N balance of a cut-and-carry fodder production system of the legume tree *Gliricidia sepium* and associated fodder grass *Dichanthium aristatum* over 11 years, found out that decomposition of tree litter, roots and nodules explained only about a third of the observed belowground N transfer from *G. sepium* to *D. aristatum* (Fig. 2). Similar results were obtained in an alley cropping system of *G. sepium* and maize (Haggar et al. 1993) and in annual legume-grass associations (Lory et al. 1992, Dubach and Russelle 1994). The results indicate that important N transfer pathways have been overlooked in research and management of agroforestry systems.

Nitrogen of atmospheric origin in the *D. aristatum* grass correlated with the root density of the *G. sepium* tree but not with soil N isotopic ratio, which suggests that the grass received N from the tree mainly through direct N transfer (Sierra and Nygren 2006). In
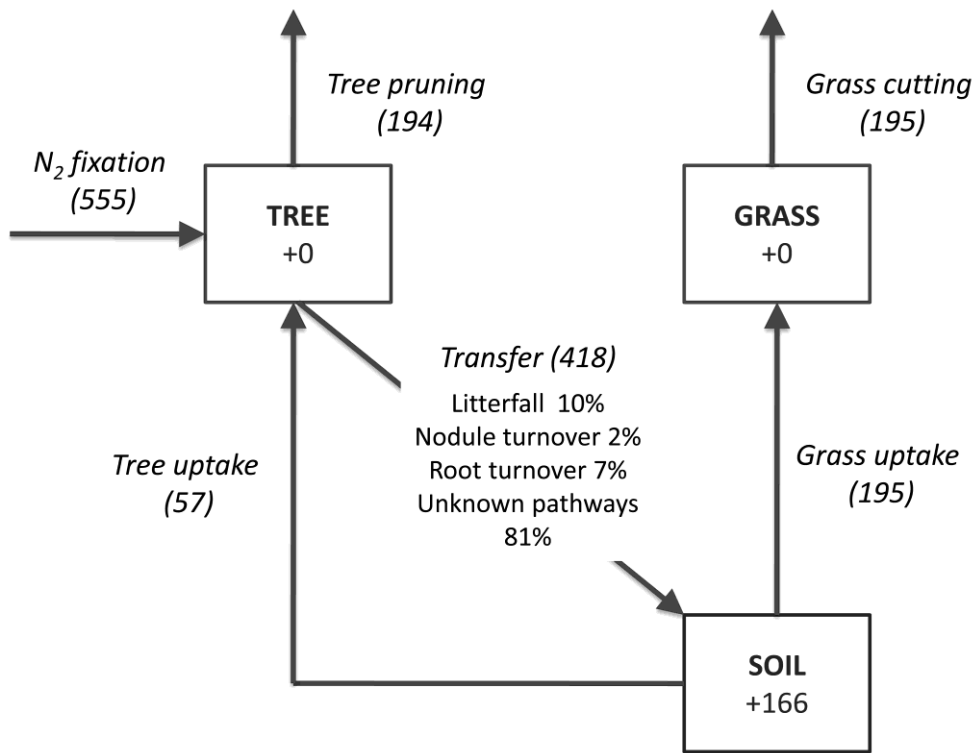
**Figure 2** Nitrogen balance of the agroforestry field site of *Gliricidia sepium* tree and *Dichanthium aristatum* grass. Values are in kg [N] ha$^{-1}$ yr$^{-1}$. See Dulormne et al. (2003) for an explanation of the calculation of the balance. Redrawn from Dulormne et al. (2003).

contrast to residue decomposition, CMN and root exudation enable such direct N transfer between plant species (Høgh-Jensen 2006). Previous evidence on direct N transfer via these pathways comes mainly from studies with herbaceous species (Bethlenfalvay et al. 1991, Frey and Schüepp 1992, Paynel and Cliquet 2003). Few studies have considered N transfer between trees and crops (Eason et al. 1991, Newman et al. 1994), although N$_2$-fixing trees provide interesting possibilities for more sustainable agricultural practices that are less dependent on external inputs. When the N donor is a woody perennial with an extensive root system, the characteristics of N transfer may differ greatly from those for herbaceous legumes. The existence and functioning of direct N transfer pathways remains little understood in associations of woody perennials and herbaceous plant species, functionally different yet commonly co-cultivated species,.

Furthermore, previous research on direct N transfer has concentrated either on quantifying total transfer without an attempt to distinguish the contribution of the involved transfer mechanisms, or, more recently, on the dynamics of N transfer via individual transfer pathways studied in isolation. Yet symbiotic N$_2$ fixation and the subsequent N cycling in ecosystems are complex processes. They probably are driven by source-sink relationships within a plant and possibly also in the ecosystem as a whole (Perry 1998, Nygren 2000, Moyer-Henry et al. 2006), and involve several components in the transfer

processes (Fig. 1). To my knowledge, no attempts have been made to understand the role of the N transfer pathways in interaction.

Due to the microscopic and fragile nature of the AM fungal hyphae, evidence for N transfer via CMN remains indirect. In studies which apply hyphal compartmentalisation it is technically not possible to distinguish N transfer via CMN from a situation where the fungal symbionts of the N recipient plant traverse the compartment wall and simply absorb N in the rhizosphere of the N donor (Fig. 1). Steps towards more conclusively establishing the role of CMN in N transfer include analysing the possibility of anastomoses between the AM fungal symbionts of the plant species as a precondition to the formation of CMN. Anastomoses have empirically been demonstrated only between fungal isolates which are genetically identical or belong to the same population (Giovannetti et al. 2006, Mikkelsen et al. 2008, Croll et al. 2009). Shared mycorrhizal symbionts would, thus, be necessary for direct N transfer between plant species. In addition, comparing the observed N transfer with the biological condition of the N donor and recipient plants may help to reveal source-sink relationships that can only be explained by CMN-mediated N transfer.

Both $^{15}$N natural abundance and $^{15}$N enrichment methods require the measurement, or a reasonable estimate, of the N isotopic composition of the actual N sources over time (e.g. root exudates or N compounds released from decomposing residue, instead of the whole root), in order to obtain reliable estimates of N transfer. However, because temporal changes in the isotopic composition of organic N sources are considered difficult to evaluate, it is often assumed that this composition remains stable over the length of an experiment. Although homogeneity of $^{15}$N labelling has improved through methodological development (Wichern et al. 2008), it remains difficult to achieve over time, especially at compound level (Khan et al. 2002). Tree pruning can also result in inconsistent isotopic ratios of the N recipient plants with both $^{15}$N tracing methods. Sierra et al. (2007) modelled N transfer via root exudation and root turnover from *G. sepium* to *D. aristatum* in pot culture, and obtained similar N transfer estimates with both $^{15}$N natural abundance and $^{15}$N enrichment methods over several months. However, after pruning of *G. sepium*, the model failed to predict the isotopic patterns of the N recipient *D. aristatum* with both $^{15}$N tracing methods assumably because roots recycled after tree pruning had a distinct N isotopic composition. Similarly, in their $^{15}$N natural abundance study, Daudin and Sierra (2008) could not estimate N transfer between *G. sepium* and *D. aristatum* in field conditions after tree pruning because of subsequent inconsistent isotopic signatures of *D. aristatum*. Temporal variation in the isotopic composition of N sources should be analysed as an alternative or a complementary method to aiming at, or simply assuming, homogenous isotopic composition in $^{15}$N tracer studies, in order to improve reliability of N transfer estimates.

## Objectives of the study

In this study I analyse the role of direct belowground pathways of N transfer in a tropical cut-and-carry agroforestry system comprising a $N_2$-fixing legume tree (N donor) and a herbaceous crop (N recipient). The model system used in the study consists of *Gliricidia sepium* tree and *Dichanthium aristatum* fodder grass. *Gliricidia sepium* is widely used as a fodder tree in cut-and carry systems in humid tropics, particularly in Central America and Southeast Asia (Suttie 2005). The study involves experimental research on the role of root exudates and common mycelial networks of arbuscular mycorrhizal fungi in N transfer, as

well as methodological research to evaluate and propose improvements to the use of the popular N isotope techniques for reliably estimating N transfer in agroforestry systems. The specific objectives of the study are as follows:

- Determine whether the legume tree and the fodder grass form symbiosis with same AM fungal strains in field conditions (I)
- Analyse how N transfer estimates obtained with $^{15}$N isotope techniques could be improved by considering the temporal and spatial variation in the isotopic composition of N sources (II, III)
- Quantify the role of common mycelial networks and root exudation in N transfer from the legume tree to the fodder grass (III, IV)

Key concepts and terms of the study are described in Table 1.

**Table 1** Key concepts and terms of the study.

| Concept or term | Explanation |
|---|---|
| Indirect N transfer | N released by the N donor plant is assimilated by soil microbial organisms and it becomes again available to associated plants only after mineralisation |
| Direct N transfer | N released by the N donor plant is assimilated by the N recipient plant as such, without immobilisation and mineralisation by soil microbial organisms |
| Root exudation | Excretion of non-particulate compounds from the roots of living plants; nitrogenous compounds in this study |
| Common mycelial networks (CMN) | Networks formed through the fusion of the extraradical hyphae of mycorrhizal fungal symbionts belonging to different host plants. The complete fusion of the hyphae allows cytoplasmic flow and transport of compounds between the fungi, and their host plants. |
| Isotopic composition of N | Ratio of the stable N isotopes $^{15}$N and $^{14}$N |
| $\delta^{15}$N | Deviation of the $^{15}$N ratio of a sample from the atmospheric standard (Eqn (1)) |
| %Ndft | Proportion of N derived from N transfer out of total N in the recipient plant (Eqn (2)) |
| $N_{tr}$ | Amount of N transferred (Eqn (3)) |
| %$N_{tr}$ | Proportion of N transferred out of total N in the N donor (Eqn (4)) |

# MATERIALS AND METHODS

## The model fodder production system

An experimental fodder production site (agroforestry field site in the following) of the legume tree *Gliricidia sepium* (Jacq.) Kunth ex Walp (Papilionoideae) and fodder grass *Dichanthium aristatum* (Poir) C.E. Hubbard (Poaceae) in Guadeloupe, French Antilles, was used as model system for this study. The site is located at the Godet Experimental Station of the French National Institute for Agronomic Research (INRA) (16°25' N, 61°30' W, 10 m a.s.l.). The climate in the area is warm and subhumid, the annual mean air temperature being 26 °C and annual mean rainfall 1300 mm. Dry season lasts from February to July, and 30% of the annual rainfall occurs during that time (weather station of INRA situated on the site). The soil on the site is Vertisol with 80% of clay rich in smectite, developed over coral reef limestone and with pH of 7.8. Average soil depth is 0.5 m. Cation exchange capacity of Vertisol is 52 cmol(+) kg$^{-1}$, with 75% of Ca$^{2+}$ saturation. Soil aggregates, therefore, are stable, which ensures a good aeration of the soil despite of the high clay concentration.

The agroforestry field site comprises two systems: (i) ***tree-grass plots*** where *D. aristatum* is grown between rows of *G. sepium*, and (ii) the ***adjacent grass plots*** of *D. aristatum*, where the grass is in contact with *G. sepium* roots but not with the tree canopies. In addition, (iii) a separate ***grass monocrop*** of *D. aristatum*, located approximately 200 m away from the agroforestry field site, was selected for comparison as a system where the grass has no contact with *G. sepium*.

The tree-grass plots were established in 1989 by planting cuttings of *G. sepium* in natural grassland of *D. aristatum*. The trees were planted in North-South aligned rows at a spacing of 0.3 m x 2 m. The tree-grass plots were 20 x 13 m in size and separated from each other by the grass plots of 15 m of width. At the time of this study the actual tree density of the tree-grass plots was about 12 000 ha$^{-1}$ because of mortality. The plots were not trenched, allowing contact of *G. sepium* roots with *D. aristatum* on the adjacent grass plots. The plots were managed since their establishment according to a cut-and-carry practice, with partial tree pruning every 2-6 months and grass cutting every 40-50 d. Grass on the grass plots and the grass monocrop was managed as on the tree-grass plot. All cut material was removed from the site.

Nitrogen fertilisers are not used on the agroforestry field site, and N$_2$ fixation by *G. sepium* is the sole N input to the tree-grass plots and the adjacent grass plots. Nitrogen derived from the atmosphere constitutes 60-87% of total N in the above-ground biomass of the tree, depending on the season (Nygren et al. 2000). Phosphorus and K fertilisers were applied five times since the establishment of the site at 100 kg [P] ha$^{-1}$ and 150 kg [K] ha$^{-1}$, last time in 2003. Available soil P is lower on the tree-grass plot than on the adjacent grass plot (11.5 mg kg$^{-1}$ vs. 18.1 mg kg$^{-1}$; Dulormne 2001), probably because of large biomass exports as tree prunings. Soil organic N content decreases with distance from the tree rows (Sierra and Nygren 2006; Table 2). Fine root densities of the two plant species correlate negatively, with the root density of *G. sepium* decreasing and that of *D. aristatum* increasing with distance from the tree rows (Sierra and Nygren 2006; Table 2). Biomass production and N accumulation by the grass are negatively affected by competition from the trees to approximately 3 m from the tree rows (Daudin and Sierra 2008), although tree roots are found throughout the adjacent grass plot.

**Table 2** Soil total N content and fine root densities of *Gliricidia sepium* and *Dichanthium aristatum* on the agroforestry field site (Sierra and Nygren 2006).

| Distance from the first G. sepium row | Soil total N (g kg$^{-1}$) | Fine root density (g dm$^{-3}$) | |
|---|---|---|---|
| | | G. sepium | D. aristatum |
| -1 m [a] | 3.05 | 0.43 | 1.52 |
| 1 m | 2.92 | 0.29 | 2.38 |
| 4 m | 2.81 | 0.22 | 3.14 |

[a] Between the first two tree rows

Organic C content of the soil on the agroforestry field site prior to the establishment of the tree-grass plots was approximately 21.7 g kg$^{-1}$, and total N content was 2.1 g kg$^{-1}$ (Dulormne et al. 2003). Soil organic C and N content on the tree-grass plots and the adjacent grass plot has considerably improved after the introduction of the trees. At the time of this study, soil organic C content was approximately 33.1 g kg$^{-1}$, organic N content 3.1 g kg$^{-1}$, and mineral N content varied from 10 to 20 mg kg$^{-1}$ depending on the season (Sierra et al. 2007). About 95-98% of the soil mineral N is in the form of $NH_4^+$ (Sierra et al. 2002). Soil organic C and N content on the *D. aristatum* monocrop remain similar to that on the agroforestry field site prior to tree planting.

A number of studies have been conducted on the agroforestry field site since its establishment, including modeling of evapotranspiration in *D. aristatum* and *G. sepium* (Tournebize et al. 1996), the effects of shading of the tree canopies on the growth of *D. aristatum* (Cruz 1997a, 1997b), effects of pruning regimes on $N_2$ fixation, nodule dynamics, biomass allocation and fodder production in *G. sepium* (Nygren and Cruz 1998, Nygren et al. 2000), dynamics of competition and complementarity between the two plant species for nutrients and water (Dulormne 2001, Dulormne et al. 2004), effects of *G. sepium* on soil N dynamics (Sierra et al. 2002), and N transfer in field conditions and the factors affecting it (Sierra and Nygren 2006, Daudin and Sierra 2008). This context importantly helps in interpreting the results of this study, to the detail that could not be achieved in individual studies. For more detailed descriptions of the site and the soil, see Daudin and Sierra (2008), Dulormne et al. (2003), and Sierra et al. (2002).

**Arbuscular mycorrhizal colonisation (I)**

*Sampling*

Root samples of *G. sepium* and *D. aristatum* were collected in August 2006 for identifying the AM fungal species and determining AM colonisation in the plant roots. Roots were sampled along four transects: on the tree-grass plot between the first two tree rows (T1), on the adjacent grass plot 1 m from the first tree row (T2) and approximately 3.5 m from the first tree row (T3), as well as on the *D. aristatum* monocrop (T4; *D. aristatum* roots only). On each transect six replications at 1 m spacing were collected for each plant species. Total number of samples was 18 for *G. sepium* and 24 for *D. aristatum* (Fig. 3).

In terms of the plant biomass and root density gradients of the two plant species (Table 2; Sierra and Nygren 2006, Daudin and Sierra 2008), *G. sepium* dominates on the tree-grass plot (T1) and *D. aristatum* on the adjacent grass plots at 3.5 m from the rows (T3), whereas radical competition is most even at 1 m away from the tree rows (T2). To
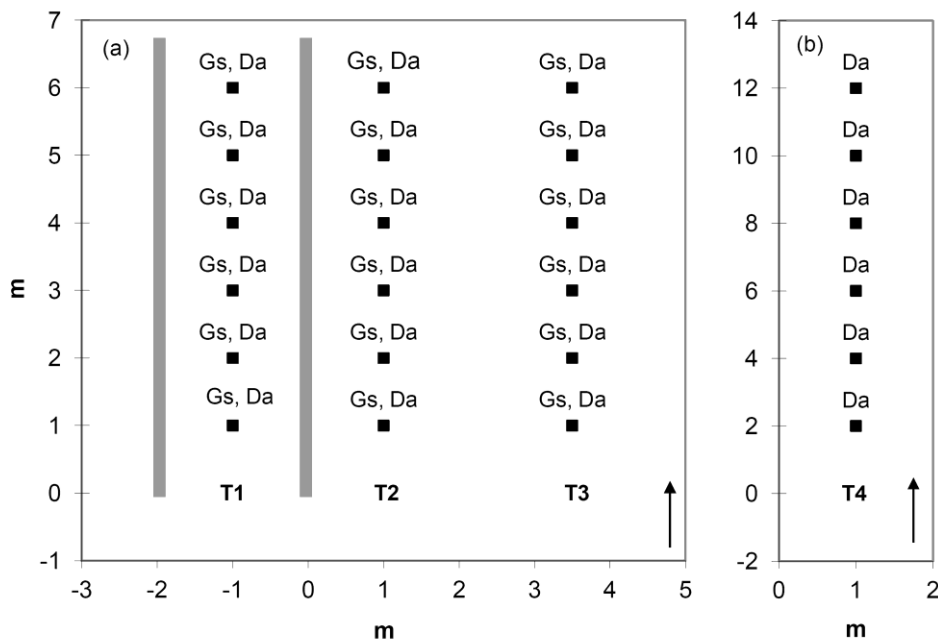
**Figure 3** Sampling procedure for mycorrhizal analyses on (a) the agroforestry field site, and (b) the *Dichanthium aristatum* monocrop. Grey bars indicate the location of the *Gliricidia sepium* tree rows. Abbreviations T1-T4 refer to the sampling transects, and *Gs* and *Da* to the root samples of *G. sepium* and *D. aristatum*, respectively.

evaluate the possibility of common AM fungal symbionts between *G. sepium* and *D. aristatum*, the samples were taken at points where roots of both plant species were present. As the density of tree roots decreased drastically with distance from the tree rows (cf. Sierra and Nygren 2006), tree roots could not always be located at the exact spot at 3.5 m from the rows. Search for the roots was started at the distance of 3.5 m and radially expanded until tree roots were located. Grass roots were then sampled at the same point. Average distance from the tree row for these samples was $3.6 \pm 0.1$ m. Roots of the two plant species were distinguished visually. The tree roots were nodulated, coarser and yellowish in colour. The grass roots were fine (<0.5-1 mm) and greyish in colour. Branching patterns of the roots of the two species were also different. On the *D. aristatum* monocrop roots were sampled from a N-S aligned transect, with six replications at 2 m spacing (T4; Fig. 3b). All root samples were collected at a depth of 0-10 cm.

Root samples were collected with the surrounding soil and stored at +6 °C until processed later on the same day. Roots were then washed clean of soil, and 6 x 10 mm fragments of fine roots per location and species were sampled close to the root tips for sequencing mycorrhizal fungal DNA. The samples were stored at -70 °C. Additional 12 x 10 mm fragments were sampled and conserved in 60% ethanol for determining mycorrhizal colonisation within the plant roots.

*Identification of mycorrhizal fungal strains from root samples*

DNA was extracted from the root samples by CTAB and proteinase K buffer (Timonen *et al.* 1997) after vigorous pulverisation in liquid nitrogen with pestle and mortar. Nested PCR with outer primers SSUmAf: 5'- TTG GTA ATC TTD TGA AAC TTY-3' and LSUmAr: 5'- GCT CTT ACT CAA AYC TAT CRA -3' and inner primers SSUmCf: 5'- TAT TGY TCT TNA ACG AGG AAT G -3' and LSUmBr: 5'- AA CAC TCG CAY AYA TGT TAG A -3' were modified from Krüger et al. (2009). The used primers are expected to have fairly good coverage of the most common families of Glomales (Krüger et al. 2009). The PCR was carried out as in Krüger et al. (2009) with the exception of using the annealing temperature of 58°C in the nested PCR. PCR reactions were run into 1% agarose-SYBR Green gel (Invitrogen^TM, Life Technologies, NY, USA). The correct-size products were cut cleanly from the gel under black light and frosen at -20°C overnight. The liquid from the gel pieces was centrifuged into eppendorf tubes, precipitated with ethanol and sequenced at Macrogen Inc., Seoul, Republic of Korea. The acquired sequences were manually checked using Geneious Pro 4.5.5 (Biomatters Ltd., Auckland, New Zealand).

Reference sequences were retrieved from the GenBank database and aligned with Geneious Pro 4.5.5. The phylogenetic analyses were performed by WinClada Ver. 1.00.08 (Nixon 2002) according to Timonen and Hurek (2006), with the following exceptions: maximum number of trees to keep [hold] 1000 000, number of replications [mult*N] 5000, and starting trees per replications [hold/] 20. A combined sequence of *Mortierella polycephala* sequences AB476414 and AF113464 was created to act as a fixed outgroup. The sequences retrieved from the examined roots were deposited to GenBank with the accession numbers FR873155-FR873165.

*Visual determination of mycorrhizal colonisation*

The sampled root fragments were stained following the method of Phillips and Hayman (1970). The roots were cleared at 2.5% KOH in 85 ˚C water bath for 30 min and rinsed with distilled water. They were then stained in a lactoglycerol mixture (lactic acid, glycerol, and water 1:1:1) with 0.05% w/v Trypan Blue, in 85 ˚C water bath for 25 min. The samples were immersed in lactoglycerol without the stain to remove excess colour, and conserved in a mixture of 50% of glycerol and 50% of water until mounting.

Mycorrhizal colonisation in the roots was visually determined applying the magnified intersections method (McGonigle et al. 1990). Briefly, the roots were mounted directly from the glycerol-water mixture on microscope slides, and examined with 400× magnification at 150 cross-sections per sample. The number of sections where the AM formations arbuscules (including arbusculate coils), vesicles or hyphae were observed was noted separately for each formation type. Dark or septate hyphae were not counted (Jumpponen and Trappe 1998). The results were expressed as the proportion of root length colonised by arbuscules, vesicles, and hyphae, and the proportion of uncolonised root, where no AM formations were encountered.

**Nitrogen transfer pathways (II-IV)**

*Study design (II-IV)*

The studies (II-IV) for analysing N transfer from *G. sepium* to *D. aristatum* via belowground pathways were conducted under greenhouse conditions at the Antillean Research Centre of INRA, Guadeloupe, Lesser Antilles (16°12'N, 61°39', 125 m a.s.l.), in 2006 and 2007. The climate in the area is warm and humid. At the time of conducting the experiments, from July to November both years, the monthly temperature maxima varied between 28 and 30 °C, and the minima between 21 and 23 °C. Air humidity was between 85 and 89%, and the global radiation between 15.8 and 22.3 MJ m$^{-2}$ d$^{-1}$. The greenhouse was well ventilated with the doors and windows open, and the growth conditions were similar to outside air, except for the exclusion of rain. Daily mean temperature in the greenhouse was in average 1.5 °C higher than outside.

Five treatments were designed to analyse the possible N transfer and uptake pathways. In each treatment the *G. sepium* trees were labelled with $^{15}$N, and the change in the N isotopic composition of *D. aristatum* was analysed. The treatments were as follows (Fig. 4):

1. Full belowground interaction (FI): *G. sepium* and *D. aristatum* grown in pots together, with their root systems fully mixed (8 pots) (III)
2. Mycelial networks (MY): *G. sepium* and *D. aristatum* grown in pots together, but their roots separated with a fine polyester mesh which only allowed fungal hyphae to pass (SEFAR PETEX 07-15/9; pore size 15 μm, open area 9%; SEFAR Inc., Heiden, Switzerland) (8 pots) (III).
3. Root exudation and exudate uptake in the case of full belowground interaction (EX$_{FI}$): N exudation by *G. sepium* was studied by growing the trees with hydroponics, and analysing N content of the culture solution. The results were used for estimating N exudation and exudate uptake by the recipient grass in treatment FI. (12 pots of *G. sepium*) (III).
4. Exudate uptake via mass flow (EX$_{MF}$): Alone-grown *D. aristatum* was irrigated with the culture solution collected from hydroponics with *G. sepium* trees (8 pots of *D. aristatum*, 11 pots of *G. sepium*), (IV). In the following, treatment EX without subscript refers to both EX$_{FI}$ and EX$_{MF}$.
5. Residue application (RA): Fine root and nodule residue of *G. sepium* trees was applied in pots of *D. aristatum* (4 pots of *G. sepium*, 8 pots of *D. aristatum*) (II). The results were used for measuring N uptake from decomposing residue by *D. aristatum*, and for parametrising a model on residue decomposition and N uptake.

Soil and plant material used in the studies originated from the *G. sepium* – *D. aristatum* agroforestry field site. Soil was collected from the topsoil layer of the site, and sieved to <1 cm aggregates for removing plant roots. *Gliricidia sepium* trees were established from cuttings in the study soil, and after their propagation transferred in pots of the same soil. For the treatments FI and MY, volume of the pots was 28 L. In treatment MY, the trees were planted in mesh bags of 14 L in the middle of the pots, in order to separate the root systems but allow contact via mycorrhizal fungal hyphae. Colonisation of previously uninfected maize (*Zea mays* L.) by AM fungi through the mesh was tested as part of the study and found extensive. Trees for the treatments EX and RA were planted in pots of 14 L. *Dichanthium aristatum* swards were transplanted from the agroforestry field site, around the trees in treatments FI and MY, and alone in pots of 14 L in treatments EX and RA. The
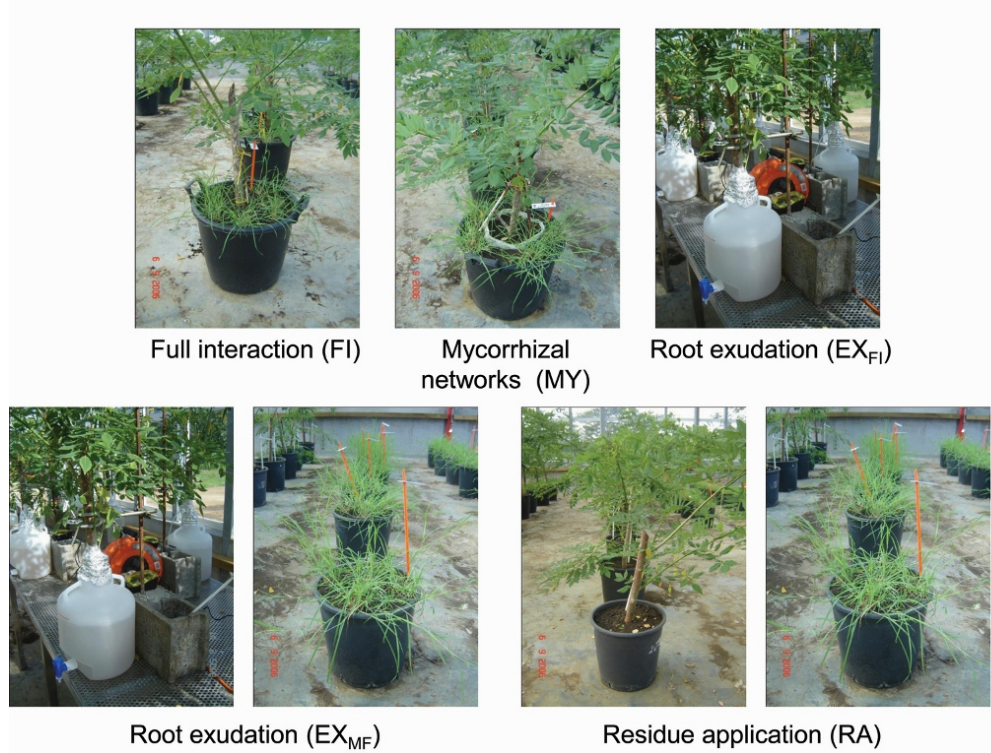
**Figure 4** Experimental design in the greenhouse studies (II-IV). In treatments FI and MY a plexiglass was placed between the grass and the tree branches before $^{15}$N labelling to eliminate tree litter from entering the pots. Photos are courtesy of Jorge Sierra.

pots were fertilised with 2 g of Triple Superphosphate and 2 g of $K_2SO_4$, corresponding to the fertiliser application rates on the agroforestry field site. The pots were irrigated daily during the experiment to maintain the soil water content close to field capacity.

After transplanting and establishment of the grass, the trees in all treatments were labelled using foliar feeding of 99% $^{15}$N-enriched $KNO_3$. Prior to labelling, the grass shoots were sampled for an analysis of $^{15}$N natural abundance, and then cut to a height of approximately 2 cm in order to homogenise the initial situation and to minimise the dilution of transferred $^{15}$N in the existent grass N pool. The cutting also corresponded to the standard management of the agroforestry field site. The $^{15}$N label was added on the tree leaves with a small paintbrush at 3 events with 2 day intervals, allowing time for absorption of the solution (Sierra et al. 2007), and applying in total 21 mg (FI, MY and $EX_{MF}$), and 30 mg ($EX_{FI}$ and RA) of $^{15}$N tree$^{-1}$. During labelling, the soil, and the grass in the shared pots, were covered with plastic in order to avoid contamination with $^{15}$N. The trees in treatments $EX_{MF}$ and RA were labelled 3 weeks earlier than the other trees, to synchronise the residue application and irrigation with exudates with $^{15}$N labelling in the other treatments.

All treatments were divided into three study periods, from 0 to 4, 4 to 7, and 7 to 10 weeks after tree $^{15}$N labelling in shared pots (FI, MY, $EX_{FI}$) or N source application to alone-grown grass ($EX_{MF}$, RA). Grass shoots were sampled for $^{15}$N at the end of each study period. Sampling was limited to these four events (including the initial sampling before $^{15}$N

labelling) in order to avoid excessive disturbance of grass growth. The biomass of the subsamples taken during the experiment represented approximately 5% of grass shoot biomass by the end of the experiment.

Trees were not pruned during the experiments, because the length of the experiments (10 weeks for observation of N transfer) was similar to pruning frequency under intensive management. On the agroforestry field site partial tree pruning is conducted every 2 to 6 months. Moreover, tree pruning results in turnover of fine roots and nodules which in previous studies has made it difficult to reliably assess N using isotopic techniques (Sierra et al. 2007, Daudin and Sierra 2008). The effects of turnover of $^{15}$N enriched root residue on the isotopic copmposition of the N recipient plant were studied as part of the experiment IV (section 2.3.3).

*Root exudation and exudate uptake by grass (III, IV)*

Root exudation of *G. sepium* in treatment $EX_{FI}$ was measured during 4 days at the midpoint of each study period. The results were assumed representative of average daily net exudation in treatment FI during that period. At each occasion, the roots of 4 *G. sepium* trees were gently washed from the soil and rinsed with distilled water. Thereafter, the trees were transferred to aerated hydroponic tanks. Distilled water was chosen as culture solution in order to minimise the content of non-evaporable compounds other than of tree origin in the solution. This facilitated the collection of the exudate residue and ameliorated the quality of the samples for mass spectrometry. The hydroponic tanks were covered with a plastic sealed around the tree stems in order to avoid air-borne contamination of the culture solution. After 4 days, the water in the tanks was collected and filtrated with Whatman No. 1 filter paper for removing root fragments and dead cells, since the aim was to study the role of readily absorbable N compounds exuded by the tree. Measured exudation corresponded to net exudation over 4 days. Nitrogen content, $^{15}$N enrichment and C:N ratios of exudates were analysed as described by Sierra et al. (2007). In short, exudates were slowly concentrated in glass beakers at 50 ºC for several days, until only the solid residue was left. In order to prevent $NH_3$ volatilisation, the pH of the solution was maintained near 4 during evaporation. The solid residue was then collected, weighed, and ground to <0.2 mm. After homogenisation, a 8.0-µg-subsample was taken for isotopic analysis.

Hydroponics cultivation in treatment $EX_{MF}$ was conducted as continuous over 10 weeks with 11 *G. sepium* trees. Foliar fertilisation of P, K and micronutrients was applied daily by spraying. Application of macronutrients was based on the growth requirements of *G. sepium* (Cruz 2001) and on estimated tree growth, and micronutrient concentrations were estimated based on a legume growth medium (Somasegaran and Hoben 1985). During the following 10 weeks, water in the hydroponics tanks was changed weekly, and exudation therefore corresponded to weekly net exudation. Exudates of 3 trees (*analysis trees* in the following) were collected, filtered and prepared for the analysis of N content and $^{15}$N enrichment as described above. Exudates of the other 8 trees were used for irrigating alone-grown *D. aristatum* grass. Isotopic composition of N in irrigation water was estimated from the isotopic composition of the roots of each tree, and the observed linear relationship between the isotopic composition of roots and exudates in the 3 analysis trees. Grass shoots were sampled for the analysis of exudate N uptake as described in section 2.3.1.

*Residue decomposition and N uptake by grass (IV)*

The experiment consisted of 4 potted *G. sepium* trees for providing the decomposing root and nodule residue, and 8 pots of *D. aristatum* grass for studying the uptake of $^{15}$N mineralised from residue. Residue application took place three weeks after tree $^{15}$N-labelling. Grass was first cut as in the other treatments. The trees were harvested for collecting fine roots (<2 mm of diameter) and nodules which were not detached from the roots. Twelve holes were then carefully drilled in the soil of the grass pots, and fresh tree roots and nodules, mixed with a small amount of soil, were applied in the holes. The residue was not mixed homogeneously within the soil in the grass pots in order to avoid destructing the grass. Each pot received 3.2 ± 0.6 g of residue, which corresponded to approximately half of the fine root and nodule mass of the trees. Fine root biomass of *G. sepium* has been observed to decrease by 49-60% after pruning (Schroth and Zech 1995). The remaining residue material was weighed, oven-dried at 70 °C for 72 h, and ground to <0.2 mm for isotopic analysis and for determining the relation of residue fresh and dry weight. Grass shoots were sampled for analysis of N uptake from the residue as described in section 2.3.1.

*Analyses of mycorrhizal colonisation, plant biomass and N isotopic composition*

At the end of the experiments at week 10, all plants in treatments FI and MY, and *D. aristatum* in treatments $EX_{MF}$ and RA were harvested by biomass compartment (leaves, branches, stem, and roots of the trees; shoot, stubble, and roots of the grass). The trees were found to be effectively nodulated. It was also verified that the soil had remained well aggregated in the pots, and had not acquired a massive structure that would have restricted its aeration. In order to determine mycorrhizal colonisation within plant roots, 12 x 1 cm fragments of fine roots per pot and per species were sampled, prepared and analysed as described in section 2.2.3. All other plant material, sampled during and at the end of the experiment, was weighed, oven-dried at 70 °C for 72 h, and ground to <0.2 mm for isotopic analysis. Sample N contents and their isotopic ratios were determined at the Stable Isotope Facility of the University of California-Davis, USA, using an element analyser (PDZ Europa ANCA-GSL) interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20; Sercon Ltd., Cheshire, UK). Carbon and N content of exudates for calculating their C:N ratio were determined with an element analyser at the Antillean Research Centre of INRA.

*Calculations of N transfer*

Since the $^{15}$N-labelled plants and exudates displayed relatively low values of $^{15}$N atom-% excess, the enrichment of the samples with $^{15}$N was expressed as deviation from the atmospheric standard, $\delta^{15}$N (‰; Eqn (1)) rather than as $^{15}$N atom-% excess. The higher the deviation in the N recipient grass, the more N it would have obtained through the active N transfer pathways in each treatment.

Proportion of N derived from transfer (%Ndft) out of grass total N was estimated separately for each study period from the $^{15}$N enrichment of the samples by using the 2-pool isotope mixing model:

$$\% Ndft_t = \frac{\delta^{15}N_{D0} - \delta^{15}N_{Dt}}{\delta^{15}N_{S0} - \delta^{15}N_{Gt}} \times 100 \qquad (2)$$

where the subscripts *D, S* and *G* refer to *D. aristatum*, the soil, and to N derived from *G. sepium*, respectively, and *0* and *t* refer to the values at the beginning of the experiment and by the end of each study period, respectively. Value of $\delta^{15}N_{S0}$ was set identical to that of $\delta^{15}N_{D0}$ in all treatments. In treatments $EX_{MF}$ and RA the soil was the only source of N for the grass prior to the irrigation with exudates and root application, while in treatments FI and MY grass probably obtained N from *G. sepium* also before $^{15}N$ labelling. In both cases the difference between $\delta^{15}N_D$ and $\delta^{15}N_G$ or $\delta^{15}N_S$ was considered negligible when compared with the difference between $\delta^{15}N_D$ and $\delta^{15}N_G$ after $^{15}N$ labelling.

Because of an uneven partitioning of the $^{15}N$ label within the tree, isotopic composition of tree roots and exudates may differ (Sierra et al. 2007), which in turn affects the N transfer calculations. Isotopic composition of roots may be assumed to represent root N content as a whole, including both mobile N and N bound in radical structures. It is, therefore, applicable to cases where N transfer occurs mainly via root residue (cf. Wichern et al. 2008). In turn, it has been suggested that exuded N is mainly recently fixed N (Ta et al. 1986), representing root mobile N. To account for the variation of isotopic composition of transferred N, %Ndft in experiments FI and MY was estimated using the $\delta^{15}N$ values of both the roots and exudates of *G. sepium* as $\delta^{15}N$ of the N source (Eqn (2)). This method gave the probable upper and lower boundaries for the isotopic composition of transferred N. The $\delta^{15}N$ values of *G. sepium* roots and exudates were obtained from treatment $EX_{FI}$ where they were measured at the midpoint of each study period. In order to account for the variation of N content of the N source between the study periods, $\delta^{15}N$ of roots and exudates of each study period were weighed with their respective N content prior to the calculations. Nitrogen transfer was calculated as cumulative by the end of each study period.

Total amount of N transferred to the grass ($N_{tr}$) was calculated as

$$N_{tr} = \%Ndft_t \times N_{Dt} \tag{3}$$

where $N_D$ denotes grass N content, and the subscript *t* refers to the end of the 10-week experiment. During the experiment, only samples of grass shoots were taken, and prior to the final harvest at week 10, N transfer was, therefore, calculated only as proportional and not in mass terms. Proportion of N transferred to grass out of tree total N (%$N_{tr}$) was, in turn, obtained from

$$\%N_{tr} = N_{tr} / N_{Gt} \times 100 \tag{4}$$

where $N_G$ denotes total N content of *G. sepium* (FI, MY) or total N content of exudates or root residue (EX, RA) at the end of the 10-week experiment.


## Nitrogen isotopic composition during residue decomposition and its effect on N transfer estimates (II)

*Study approach*

A dynamic model was developed for (i) quantifying how temporal variation in the N isotopic composition of decomposing residue affects N transfer estimates, and (ii) evaluating how this variation could be taken into account by considering both the

differential isotopic composition and the differential decomposition kinetics of residue compounds. Isotopic composition of a N-recipient plant was used as integrator of the effects of isotopic heterogeneity on the various N pools in the soil-plant system.

The model was parameterised for two pot culture studies: (a) the experiment RA, where root and nodule residue was harvested from $^{15}$N-labelled *G. sepium* trees and applied in pots of *D. aristatum*, and (b) for the study of Sierra et al. (2007), where *G. sepium* labelled with $^{15}$N through foliar feeding was grown in shared pots with *D. aristatum*. Three months after $^{15}$N labelling the trees were pruned to induce root turnover, and the evolution of the N isotopic composition of *D. aristatum* was then studied for 48 weeks. For this study we used the data for the first 24 weeks, after which the N source from the decomposing residue appeared exhausted. *S*hoots of *D. aristatum* were harvested every 4-8 weeks during the experiment (Sierra et al. 2007). Model parameterisation for the two datasets helped to evaluate model performance and the effects of $^{15}$N labelling on the results in two different experimental designs.

*Model structure*

Residue decomposition was modelled according to the STICS residue decomposition model (Nicolardot et al. 2001). A single fraction for the residue, which decomposes with first order kinetics at a rate determined by its C:N ratio, is considered in the STICS model. It has reasonably well explained overall C and N mineralisation from plant residues in tropical environments (Raphael et al. 2012), including for roots of *G. sepium* (Sierra and Motisi 2012).

The model developed in this study applies a modified decomposition component with regard to STICS, in order to allow simulation of heterogeneity of residue and its isotopic composition, and the implications to soil and recipient plant in the system. Residue is divided into fractions, and $\delta^{15}$N, N content, and C:N ratio can be specified individually for each of these (Fig. 5). Uptake of N and its partitioning within the recipient plant was modelled according to a box model (Sierra and Nygren 2006) which has explained well biomass accumulation and $\delta^{15}$N in *D. aristatum* shoots (Sierra and Nygren 2006, Sierra et al. 2007). The final model consisted of seven types of N pools, namely residue ($N_R$), microbial biomass involved in decomposition ($N_B$), humified organic matter ($N_H$), soil inorganic N originating from residue ($N_{SR}$), soil native inorganic N ($N_{SN}$), and plant roots ($N_{RO}$) and shoot ($N_S$). All pools were divided to $^{14}$N and $^{15}$N according to their initial $\delta^{15}$N values. Isotopic ratios of the N flows were defined by those of the source pool.

All residue fractions and the associated microbial biomass pools were set to either deplete or contribute to the same soil inorganic N pool, depending on the C:N ratios of each residue fraction (Nicolardot et al. 2001). The model enables the simulation of differential N uptake from the $N_{SR}$ and $N_{SN}$ pools by the recipient plant, by adjusting a N source factor, *Sf*. If Sf > 0, then N is first absorbed from the $N_{SR}$ pool and only secondarily from $N_{SN}$, if the supply from $N_{SR}$ is insufficient. If Sf < 0, N is first absorbed from the $N_{SN}$ pool. Because $N_{SN}$ represents net N mineralisation from soil organic matter, it takes into account N immobilisation during mineralisation. However, further N immobilisation from $N_{SN}$ may occur when N demand for growth of the microbial biomass decomposing residues is greater than N supplied from $N_{SR}$ (Fig. 1; Nicolardot et al. 2001).

Mineralisation of soil native organic N ($m_{SO}$) was simulated as a function of soil temperature according to the equation proposed for the same soil and site by Sierra et al.
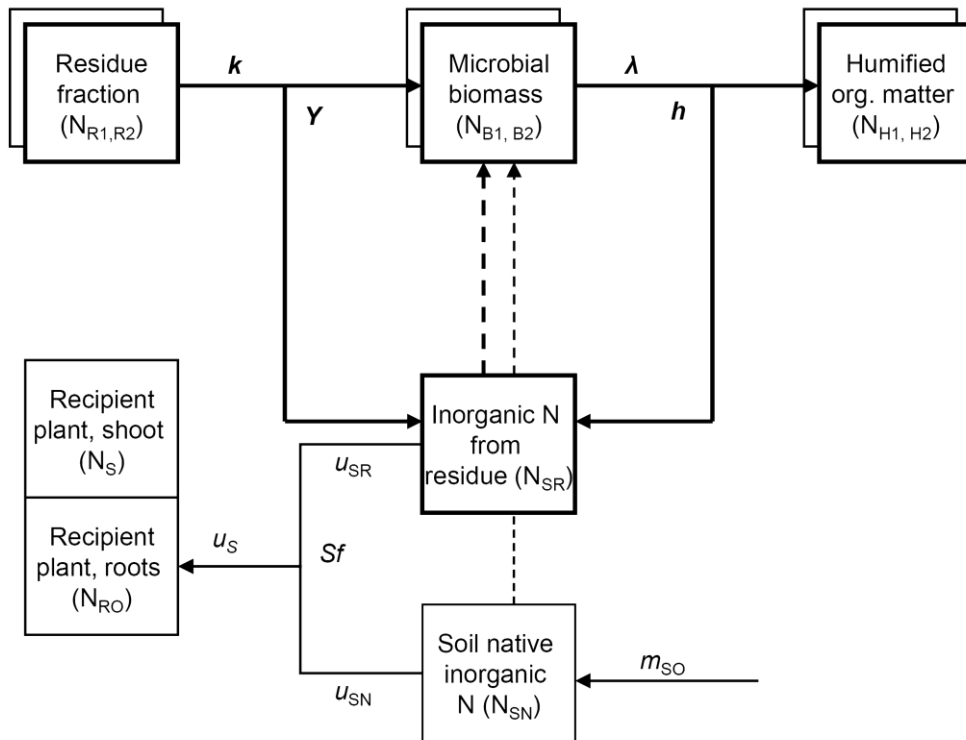
**Figure 5** Model of residue decomposition and subsequent uptake of residue N by a recipient plant. Each pool is divided into $^{14}$N and $^{15}$N. Subscripts *R1* and *R2*, *B1* and *B2*, and *H1* and *H2* refer to the pools related to the two residue fractions in the residue, microbial biomass and humified organic matter, respectively. Parameters *k* and $\lambda$ are decomposition rate constants for residue and microbial biomass, respectively, *Y* is assimilation yield of C from residue by microbial biomass, and *h* is humification rate of microbial biomass. Parameter *u* is N uptake rate by the recipient plant, and its subscripts *SR* and *SN* refer to the uptake rates from the two soil inorganic pools (Table 2). Parameter *Sf* is N source factor which determines the relative N uptake rates from the two soil N pools. Parameter $m_{SO}$ is mineralisation rate for soil organic N. Pools and processes indicated with bold lines are simulated according to Nicolardot et al. (2001).

(2002). Ambient temperature and soil humidity affect mineralisation of both residue and microbial N pools (Nicolardot et al. 2001) and soil native organic N. Mineralisation of humified organic matter originating from the residue was considered negligible as N source for the recipient plant, in comparison to the decomposing residue and soil inorganic N (Brisson et al. 1998). Denitrification and volatilisation were excluded from the model because of the very low proportion of $NO_3^-$ in the studied soil, previous observations of negligible volatilisation in unfertilised Vertisols (Courtaillac et al. 1998), and frequent irrigation which was assumed to further reduce the concentration of $NH_4$ in the soil.

Daily uptake of soil inorganic N by the recipient plant was modelled as linear over the time span of the experiments and considering that grass shoots were regularly harvested in

the experiment of Sierra et al. (2007). Linear N uptake was based on the results of Sierra et al. (2007) and previous observations for the N recipient grass (Cruz 1997a).

*Model parameterisation and simulations*

We assumed that the residue consisted of two fractions, labile and stable. The following constraints were imposed for the labile fraction: (i) The minimum C:N ratio was set to 3.0, and the maximum N content to 30% of total residue N. These are in the range of values measured for water-soluble N in plant roots (Bending et al. 1998, Chavez et al. 2004, Jensen et al. 2005). (ii) The maximum $\delta^{15}N$ was set to 600 for the RA experiment, and to 495 for the data of Sierra et al. (2007). With the maximum N content and respective $\delta^{15}N$ of the labile residue fraction, $\delta^{15}N$ of the stable fraction would equal to 1 in each dataset.

   Input parameters for total N content, C:N ratio and $\delta^{15}N$ of the residue in the RA experiment were the values measured for fine roots and nodules. For the dataset of Sierra et al. (2007) the input parameters corresponded to the amount of N recycled after pruning, and total C:N ratio and $\delta^{15}N$ of roots (including nodules).

   The model was run for a period of 70 days for the RA experiment and 168 days for the data of Sierra et al. (2007). Fixed parameter values used in the simulations are given in Table 3. Temperature in the simulations was daily mean temperature in the greenhouse during the experimental studies, and soil humidity was set equal to field capacity as the grass pots were irrigated daily.

   Three simulation steps were performed for each dataset to evaluate the assumptions about residue characteristics. The steps considered (i) one single residue fraction, i.e. homogenous residue, (ii) two residue fractions with equal isotopic composition, and (iii) two residue fractions with different isotopic composition. The aim was to test the hypotheses that simulating the observed trends of the isotopic composition of the N recipient plants requires more than one residue fraction, and that the fractions differ in their isotopic composition. Each simulation step was run with two options for the N source factor: N uptake proportionally equal to the size of each inorganic N pool (Sf = 0), and optimising the factor in the simulation. In simulation step 3 for the RA experiment, the N content of the labile fraction was fixed to 29.0, the value obtained in all previous simulations, in order to limit the number of optimised parameters.

      Agreement with simulations and experimental observations was evaluated using the coefficient of variation of the root mean square error (Mayer and Butler 1993):

$$CV(RMSE) = \frac{1}{\bar{y}}\sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n}} \qquad (5)$$

and values <0.05 were defined to represent satisfactory model performance.

   After finding the parameter values which allowed satisfactory simulation of the experimental observations, a fourth simulation step was performed to study the impacts of isotopic heterogeneity of the decomposing residue on the N uptake estimates by the N recipient plant. Nitrogen uptake from the residue was estimated according to Eqn (2), using two options for $\delta^{15}N$ of N derived from the residue: (i) total $\delta^{15}N$ of the residue at the time of residue application, which corresponds to the assumption of a homogenous residue over time (uptake denoted %Ndft$_R$), and (ii) simulated $\delta^{15}N$ of N derived from residue (%Ndft$_S$), which can vary over time.

The model was built using the Simile software, version 4.9 (Simulistics Ltd., Edinburgh, UK). Optimal fit was searched by using the PEST software (Model-Independent Parameter Estimation and Uncertainty Analysis, version 11.8; Watermark Numerical Computing, Australia). For a detailed description of the software and the optimisation method, see the PEST manual (PEST 2004).

**Table 3** Parameters of the residue decomposition model for the residue application study (RA, II) and the dataset of Sierra et al. (2007) (abbreviated S07).

| Parameter | Code | Value | | Unit | Source | |
|---|---|---|---|---|---|---|
| | | RA | S07 | | RA | S07 |
| *Values specific to each dataset* | | | | | | |
| Initial value of soil native inorganic N | $N_{SN0}$ | 21 | 17 | mg kg$^{-1}$ | II | S07 |
| Initial $\delta^{15}N$ of soil native inorganic N | $\delta^{15}N_{SN0}$ | 4.3 | 7.2 | ‰ | II | S07 |
| Initial shoot $\delta^{15}N$ | $\delta^{15}N_{SH0}$ | 4.3 | 19.8 | ‰ | II | S07 |
| N content of root residue [a] | $N_R$ | 97 | 1332 | mg pot$^{-1}$ | II | S07 |
| C:N ratio of root residue [a] | R | 14.5 | 30.2 | | II | S07 |
| $\delta^{15}N$ of root residue [a] | $\delta^{15}N_R$ | 180 | 318 | ‰ | II | S07 |
| Total N uptake rate | u | 3.2 | 3.0 | mg d$^{-1}$ | II, estimation | S07 |
| *Common values* | | | | | | |
| C:N ratio of the newly formed humified organic matter | $R_h$ | 10.5 | | | Sierra and Nygren 2005 | |
| C:N ratio of microbial biomass | $R_b$ | $R_b = 16.1 - 123 / R$, $R_b = 7.8$ when $R < 14.8$ | | | Nicolardot et al. (2001) | |
| Decomposition rate constant of residue | k | $0.07 + 1.94 / R$ | | nday$^{-1}$ [b] | Nicolardot et al. (2001) | |
| Decomposition rate constant of microbial biomass | $\lambda$ | 0.0110 | | nday$^{-1}$ [b] | Nicolardot et al. (2001) | |
| Assimilation yield of C from residue by microbial biomass | Y | 0.62 | | | Nicolardot et al. (2001) | |
| Humification rate of microbial biomass | h | $1 - 0.326 R / (11.2 + R)$ | | | Sierra and Motisi (2012) | |

[a] Corresponds to fine roots and nodules for RA, and all roots and nodules for S07.

[b] nday refers to a 'normalised day' (25 °C and optimum water content). In this study, we used 25 °C as the reference temperature, instead of 15 °C in Nicolardot et al. (2001).

**Statistical analyses**

Mycorrhizal colonisation on the agroforestry field site was analysed as factorial design, with the host plant species and the distance from tree rows as the factors. Results of mycorrhizal colonisation in the root fragments, expressed as %, were normalised by arcsine transformation for both the field experiment and the pot culture studies. The frequency distribution of the arcsine-transformed data from the field experiment did not differ statistically significantly from the normal distribution for the studied variables, except for arbuscular colonisation and arbuscule:vesicle ratio in *G. sepium* (Kolmogorov-Smirnov test). Variances did not differ statistically significantly between the species for any of the studied variables. Treatment means were, thus, compared by using Tukey's HSD (Honestly Significant Difference). In the pot culture studies, the frequency distribution of the arcsine transformed data did not differ statistically significantly from the normal distribution for any studied variables. Comparison of mycorrhizal colonisation between the plant species was, thus, conducted with Student's t-test. Treatment means for each species were compared using Student's t-test (*G. sepium*) and analysis of variance followed by the separation of means using Tukey's HSD (*D. aristatum*).

The differences between $\delta^{15}N$ of grass shoot subsamples and grass total or shoot biomass at week 10 in the pot culture studies were tested with Student's t-test, in order to estimate the sampling error on the %Ndft estimates during the experiment. Differences in plant biomass, N content and $^{15}N$ enrichment between the treatments were tested with Student's t-test (*G. sepium*) or analysis of variance followed by the separation of means using Tukey's HSD (*D. aristatum*).

Correlation between grass shoot $\delta^{15}N$ and N concentration over time in the RA experiment was calculated as described by Hamlett et al. (2004), taking into account that the variable values were obtained as repeated measures of individual plants and were linked over time. Correlations of N transfer variables with tree and grass final N content and N concentration in treatments FI and MY were tested with Pearson's correlation analysis and expressed as Pearson's correlation coefficients.

Values of $P < 0.05$ were interpreted as indicating statistically significant differences in all statistical tests. Results were analysed with SAS statistical analysis software, version 9.1 (SAS Institute Inc., Cary, NC, USA).

# RESULTS

**Arbuscular mycorrhizal colonisation on the agroforestry field site (I)**

Five different types of AM fungal sequences were found from the analysed root fragments from the agroforestry field site and the *D. aristatum* monocrop (Fig. 6). Most of the sequences clustered into two different subgroups of *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler comb. nov. (ex *Glomus intraradices*; Schüßler and Walker 2010). One subgroup included sequences from both *G. sepium* and *D. aristatum*, and all the sequences from *D. aristatum* were from samples taken on the tree-grass plot (T1; *R. intraradices* group II in Fig. 6). The other subgroup included sequences from *D. aristatum* from both the adjacent grass plot and the grass monocrop (T2, T4; *R. intraradices* I in Fig. 6). One *Paraglomus* sequence was detected from *D. aristatum*

*Mortierella polycephala* AB476414, AF113464

100

*Paraglomus laccatum* AM295494

84

T3, host *Dichantium aristatum*, FR873156

100

*Paraglomus occultum* DQ273827

*Paraglomus occultum* FJ461883

Paraglomeraceae

100

T3, host *Gliricidia sepium*, FR873165 *

81

100

*Gigaspora margarita* FN547547

*Scutellospora gilmorei* FN547598

Gigasporaceae
Scutellosporaceae

100

*Acaulospora laevis* FN547507

*Entrophospora kentinensis* FM876829

100

*Diversispora spurca* FN547637

*Glomus versiforme* FN547666

Acaulosporaceae
Diversisporaceae

100

*Funneliformis coronatum* FM876794

*Funneliformis caledonium* FN547494

Glomeraceae

97

T2, host *Gliricidia sepium*, FR873155 *

100

80

T2, host *Dichantium aristatum,* FR873162

66

*Rhizophagus intraradices* FM865602

61

T4, host *Dichantium aristatum*, FR873157

80

T4, host *Dichantium aristatum*, FR873158

T4, host *Dichantium aristatum*, FR873163

*R. intraradices I*

66

100

*Rhizophagus intraradices* FM865576

*Rhizophagus intraradices* FM865577

78

T1, host *Dichantium aristatum*, FR873160

T1, host *Dichantium aristatum*, FR873161

76

T1, host *Dichantium aristatum*, FR873159

*Rhizophagus intraradices* AM980857

T3, host *Gliricidia sepium,* FR873164

*R. intraradices II*

**Figure 6**

**Figure 7** Structures of arbuscular mycorrhizal fungi in root samples from the agroforestry field site: (a) arbuscules and (b) vesicles in *Gliricidia sepium* roots, (c) arbuscules, (d) arbusculate coils, and (e) vesicles in *Dichanthium aristatum* roots. Bar: 10 μm (a, c, d) or 50 μm (b, e).

**Figure 6** (facing page) Phylogenetic tree (partial small subunit, internal transcribed spacer, partial large subunit rDNA sequences) of arbuscular mycorrhizal fungi on the agroforestry field site, with *Mortierella polycephala* as outgroup. The strict consensus tree was created from five equally parsimonious trees (2978 steps, Ci 60, Ri 63). Jackknife values over 50, which indicate strong support for the branches, are marked above the branches. Asterisks (*) indicate the two samples from *G. sepium* whose identity remained unclear as they did not cluster with any sequences available from the current databases.

roots. Identity of two sequences retrieved from *G. sepium* on the adjacent grass plot (T2, T3), could not be determined. They clearly belonged to Glomeromycota as indicated by the phylogenetic tree (Fig. 6), but they did not neatly cluster with any sequences available in the current databases.

Arbuscules were observed in all root samples collected from the agroforestry field site and the *D. aristatum* monocrop, except for two *G. sepium* samples (Fig. 7). Average arbuscular colonisation was $7.7 \pm 0.9\%$ of root length for *G. sepium* and $13.1 \pm 0.9\%$ for *D. aristatum*. In *D. aristatum* both Arum- and Paris type arbuscules, and hyphal coils, were observed, while in *G. sepium* only Arum type arbuscules were observed.*Gliricidia sepium* had significantly more vesicles and hyphae but less arbuscules and a lower arbuscule:vesicle ratio than *D. aristatum* (Fig. 8). Arbuscular colonisation of *G. sepium* was higher on the tree plot (T1) than on the adjacent grass plot 3.5 m away from the trees (T3).
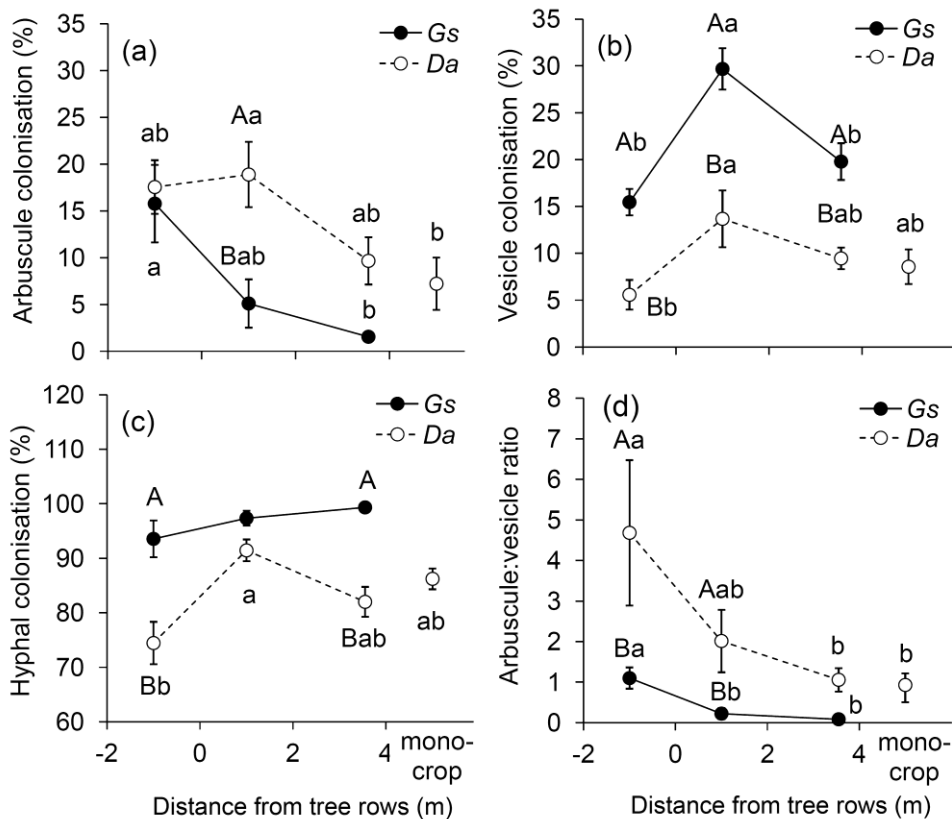


**Figure 8** Variation in arbuscular mycorrhizal colonisation on the agroforestry field site by host plant species and distance: percentage of root length colonised by (a) arbuscules, (b) vesicles, (c) hyphae, and (d) arbuscule:vesicle ratio. Gs = *Gliricidia sepium* and Da = *Dichanthium aristatum*. Sampling points from left to right correspond to the sampling transects T1-T4. Capital letters indicate statistically significant differences between species at the given distance, and small letters indicate within-species differences by distance ($P < 0.05$). Vertical bars indicate standard error (n = 6).

Vesicle colonisation for both species was highest on the adjacent grass plot at 1 m from the tree rows (T2). Arbuscule:vesicle ratio decreased with distance from the tree rows for both species (Fig. 8d). Arbuscular colonisation on the *D. aristatum* monocrop (T4) was lower than on the grass plot (T2), and arbuscule:vesicle ratio lower than on the tree-grass plot (T1).

**Variation of isotopic composition of N sources and its effects on N transfer estimates (II, III)**

Isotopic composition of N varied significantly within *G. sepium* at the end of the experiments, 14 weeks after foliar $^{15}$N labelling. Notably lower $^{15}$N enrichment was measured in exudates than in roots or above-ground plant parts (III, Table 4). Isotopic composition of roots and root exudates correlated positively and statistically significantly (r = 0.82, n = 12) over the 10-week experiment. However, $^{15}$N enrichment of exudates was always lower than that of roots. Enrichment of both exudates and roots decreased statistically significantly from the first to the last study period (III; Table 5).

Shoot $\delta^{15}$N of the N recipient grass in treatment RA increased rapidly after residue application at the beginning of the experiment, and decreased thereafter during the last two study periods (Fig. 9). Model simulations with a single residue fraction resulted in low $\delta^{15}$N values for the N recipient plant in both experimental datasets (Sf > 0, II; Fig. 9a,b). Neither the trends nor the ranges of plant shoot $\delta^{15}$N corresponded to the observations. When the residues were divided into two fractions with different C:N but equal $\delta^{15}$N, a decreasing trend of shoot $\delta^{15}$N after an initial peak was obtained for both datasets, as also observed in the experimental studies. However, the simulations deviated too much from the observations for satisfactory results (Fig. 9c,d; Table 6). Best agreement with th observations for both datasets was reached when $\delta^{15}$N of the two residue fractions were allowed to differ (Sf > 0, Fig. 9e,f; Table 6). Characteristics of the residue fractions in those simulations are given in Table 5. Values of $\delta^{15}$N for the N recipient plant in both experimental datasets could only be satisfactorily simulated when proportionally more N was absorbed from the inorganic N pool originating from residue, in comparison to soil native inorganic N (Sf > 0; Table 6).

Values of $\delta^{15}$N of the grass shoot subsamples used for estimating N transfer did not differ statistically significantly from $\delta^{15}$N of total shoot biomass or total plant biomass at the end of the experiments in any treatments (data not shown).

**Table 4** Variation of N isotopic composition in *Gliricidia sepium* trees labelled with $^{15}$N via foliar feeding. For each treatment, small letters indicate significant differences between plant parts (analysis of variance and Tukey's HSD, *P* <0.05).

| Variable | Treatment | | | |
|---|---|---|---|---|
| | EX | | RA | |
| Leaf $\delta^{15}$N (‰) | 1141 ± 113 | a | 1851 ± 72 | a |
| Root $\delta^{15}$N (‰) | 370 ± 38 | b | 347 ± 21 | c |
| Fine root $\delta^{15}$N (‰) | - | | 180 ± 15 | d |
| Coarse root $\delta^{15}$N (‰) | - | | 499 ± 38 | b |
| Exudate $\delta^{15}$N (‰) | 90 ± 12 | c | - | |

**Table 5** Properties of *Gliricidia sepium* trees (N donor) in treatment $EX_{FI}$ (root exudation) during the experiment. Values are mean ± standard error (n = 4). For each variable, small letters indicate significant differences between the study periods (analysis of variance and Tukey's HSD, $P < 0.05$).

| Variable | Study period (wk after $^{15}$N labelling) | | | | | |
|---|---|---|---|---|---|---|
| | 1 (2) | | 2 (5.5) | | 3 (8.5) | |
| Total dry mass (g tree$^{-1}$) | 210 ± 17 | c | 311 ± 27 | b | 452 ± 25 | a |
| Total N content (mg tree$^{-1}$) | 2305 ± 37 | c | 4279 ± 78 | b | 6304 ± 439 | a |
| Root N content (mg tree$^{-1}$) | 268 ± 25 | c | 592 ± 20 | b | 921 ± 36 | a |
| Root N concentration (%) | 3.8 ± 0.1 | a | 3.5 ± 0.1 | b | 2.7 ± 0.0 | c |
| Total $\delta^{15}$N (‰) [a] | 2026 ± 65 | a | 1037 ± 14 | b | 715 ± 38 | c |
| Root $\delta^{15}$N (‰) | 793 ± 37 | a | 351 ± 17 | b | 370 ± 38 | b |
| Exudate $\delta^{15}$N (‰) | 179 ± 34 | a | 105 ± 13 | ab | 90 ± 12 | b |
| Exudate C:N ratio | 3.8 ±0.5 | | 4.7 ±0.3 | | 3.9 ± 0.2 | |

[a] Calculated from the proportion of plant total $^{15}$N out of total N content

Nitrogen transfer estimates in treatments FI and MY were approximately 3.8 times higher when the isotopic composition of transferred N was set equal to that of the exudates rather than the roots (III; cf. Eqn (2); Table 7). Despite of the variation in the isotopic composition of N released from the decomposing residue in treatment RA, the N transfer estimates calculated assuming homogenous residue (%Ndft$_R$) differed only slightly from simulated N transfer (%Ndft$_S$), except for the first week after residue application (II; Fig. 10a). For the dataset of Sierra et al. (2007), however, the N transfer estimates calculated assuming homogenous residue were 40-51% lower than simulated N transfer during the first 10 days, and 36-39% lower during the rest of the experiment, with the difference slowly decreasing over time (Fig. 10b).

**Nitrogen transfer pathways (II-IV)**

*Nitrogen transfer in full root interaction and via mycelial networks (III)*

The N donor *G. sepium* trees in treatments FI and MY did not differ statistically significantly for dry mass, N content or isotopic composition of any biomass compartment, nor for leaf or stem N concentration. Nitrogen concentration in tree roots was significantly higher in treatment MY than FI (Table 8). Arbuscules were observed in all root samples in both treatments FI and MY. Arbuscular colonisation in *D. aristatum* was significantly higher in treatment MY than in treatment FI (Table 9).

Proportion of grass N obtained from transfer (%Ndft) increased continuously in treatment FI, being $3.7 \pm 0.5$ % and $14.0 \pm 2.0$ % at the end of the experiment when calculated with $\delta^{15}$N of roots and exudates, respectively (Fig 11). Nitrogen transfer in treatment MY at the end of the first study period corresponded to 32% of that observed in treatment FI. However, in contrast to treatment FI, %Ndft in treatment MY levelled out after the first study period (Fig. 11). At the end of the experiment, %Ndft was $0.7 \pm 0.1$ % and $2.5 \pm 0.4$ % of grass total N when calculated with $\delta^{15}$N of roots and exudates, respectively, and corresponded to 18% of that observed in treatment FI.
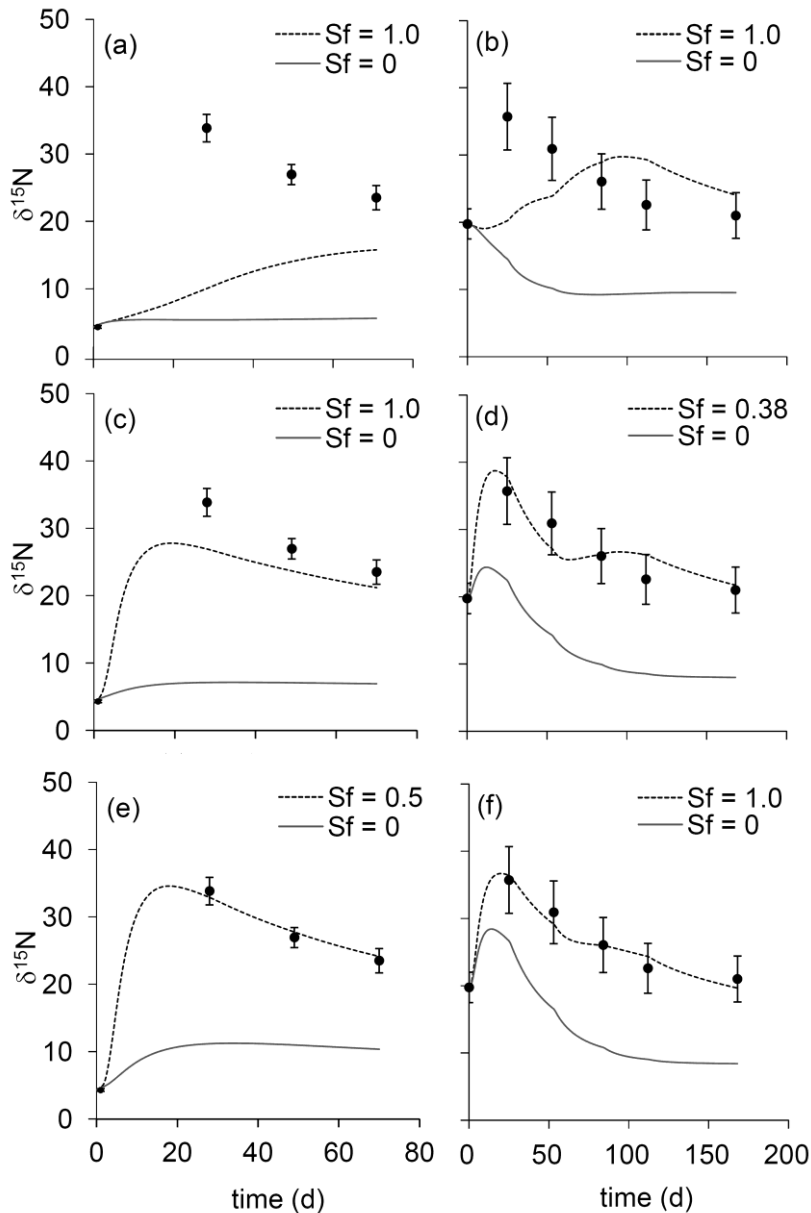
**Figure 9** Simulated change of plant $\delta^{15}N$ after the input of $^{15}N$-labelled decomposing residue in the residue application treatment (RA; a, c, e) and the dataset of Sierra et al. (2007) ( b, d, f), when the residue is considered to consist of (a,b) a single fraction with uniform C:N and $\delta^{15}N$, (c,d) two fractions with different C:N but uniform $\delta^{15}N$, and (e,f) two fractions with different C:N and $\delta^{15}N$. Simulations were conducted with proportionally equal N uptake from soil inorganic N sources (Sf = 0) and by optimising the Nsource factor in the simulations. Values of Sf > 0 refer to a situation where the plant takes up proportionally more N originating from the residue than soil native N. Circles indicate the measured values in pot culture. Vertical bars indicate the standard error of mean for the observations (n = 8 and n = 4 for the RA treatment and the dataset of Sierra et al. 2007, respectively).

Grass N content at the end of the experiment was statistically significantly higher in treatment FI than in treatment MY (Table 8). Ratio of N concentration in grass roots to N concentration in tree roots (*relative N concentration of grass*) correlated positively with the amount of N transferred ($N_{tr}$) and %Ndft in treatment FI, and N transfer thus increased N concentration in the grass roots in comparison with the tree roots (Table 10). No correlations were found between AM colonisation and N transfer. Interestingly, in treatment MY, %Ndft, $N_{tr}$, and %$N_{tr}$ all correlated negatively with N concentration of grass roots (Table 10). In other words, the smaller the N concentration of grass roots, the more N was transferred from the tree to the grass. In addition, $N_{tr}$ correlated positively and statistically significantly with arbuscular colonisation in tree roots, but not with tree total or root N content. Arbuscular colonisation, thus, increased N transfer from the tree independently of tree N content. No correlations were found between AM colonisation in grass roots and N transfer.

**Table 6** Simulation steps, optimal parameter values and performance of the residue decomposition model. Values indicated in bold were obtained through model optimisation; other values were fixed in the respective simulation steps. Residue parameter values are for labile fraction, except for the final simulation step for which values are given also for the stable fraction. For CV (RMSE), asterisk (*) indicates statistically significant fit between observed and simulated $\delta^{15}$N values of the N recipient plant (< 0.05).

| Step | Fraction | Characteristics | | | N source factor (Sf) [a] | CV (RMSE) |
|---|---|---|---|---|---|---|
| | | C:N | N (mg) | $\delta^{15}$N (‰) | | |
| *Residue application experiment (RA)* | | | | | | |
| (i) Single residue | labile | 14.5 | 97 | 180 | 0.0 | 0.820 |
| fraction | labile | 14.5 | 97 | 180 | **1.0** | 0.579 |
| (ii) Two fractions with | labile | **3.0** | **29** | 180 | 0.0 | 0.764 |
| equal δ$^{15}$N | labile | **3.0** | **29** | 180 | **1.0** | 0.164 |
| (iii) Two fractions with | labile | **3.0** | **29** | **600** | 0.0 | 0.630 |
| different δ$^{15}$N | labile | **3.3** | 29 | **273** | **0.50** | 0.029* |
| | stable | **19.3** | 68 | **140** | | |
| *Sierra et al. (2007)* | | | | | | |
| (i) Single residue | labile | 30.2 | 1332 | 318 | 0.0 | 0.553 |
| fraction | labile | 30.2 | 1332 | 318 | **1.0** | 0.454 |
| (ii) Two fractions with | labile | **3.0** | **400** | 318 | 0.0 | 0.538 |
| equal δ$^{15}$N | labile | **3.4** | **41** | 318 | **0.38** | 0.093 |
| (iii) Two fractions with | labile | **3.0** | 400 | **495** | 0.0 | 0.482 |
| different δ$^{15}$N | labile | **3.3** | **66** | **167** | **1.0** | 0.047* |
| | stable | **31.6** | **1266** | **330** | | |

[a] 0 corresponds to proportionally equal uptake from the two soil inorganic N pools, and 1 to preferential uptake from N mineralised from residue over soil native N (II).

In treatment FI grass total and root N content correlated negatively and significantly with those of the tree (r = -0.88 and -0.77 for total and root N content, respectively; n = 8). In contrast, in treatment MY the correlations between grass and tree root N content were positive (r = 0.80, n = 6). In treatment FI %Ndft correlated negatively and statistically significantly with arbuscular colonisation in tree roots, whereas in treatment MY arbuscular colonisation in tree roots facilitated N transfer (Table 10). Vesicle colonisation was significantly higher in treatment FI than MY for both plant species (Table 9).

The analyses excluded two repetitions of treatment MY where the tree roots had penetrated the mesh bags and mixed with grass roots (n = 6).

**Table 7** Nitrogen transfer from *Gliricidia sepium* trees to *Dichanthium aristatum* grass during the 10-week pot culture study. Values are mean ± standard error. For each variable, values followed by different letters differ significantly between the treatments (Student's test, *P* <0.05).

| Variable | $\delta^{15}N$ of transferred N | Treatment | | | |
|---|---|---|---|---|---|
| | | FI | | MY | |
| $N_{tr}$ [a] | root $\delta^{15}N$ | 14.1 ± 2.8 | a | 2.0 ± 0.3 | b |
| (mg pot$^{-1}$ 10 wk$^{-1}$) | exudate $\delta^{15}N$ | 53.5 ± 10.5 | a | 7.6 ± 1.1 | b |
| %Ndft [b] | root $\delta^{15}N$ | 3.7 ± 0.5 | a | 0.7 ± 0.1 | b |
| | exudate $\delta^{15}N$ | 14.0 ± 2.0 | a | 2.5 ± 0.4 | b |
| %$N_{tr}$ [c] | root $\delta^{15}N$ | 0.17 ± 0.05 | a | 0.02 ± 0.00 | b |
| | exudate $\delta^{15}N$ | 0.66 ± 0.17 | a | 0.08 ± 0.02 | b |

FI = full interaction (n = 8), MY = mycelial networks (n = 6).
[a] N transfer (Eqn (3))
[b] Proportion of grass N derived from transfer (Eqn (2))
[c] N transferred out of tree total N (Eqn (4))
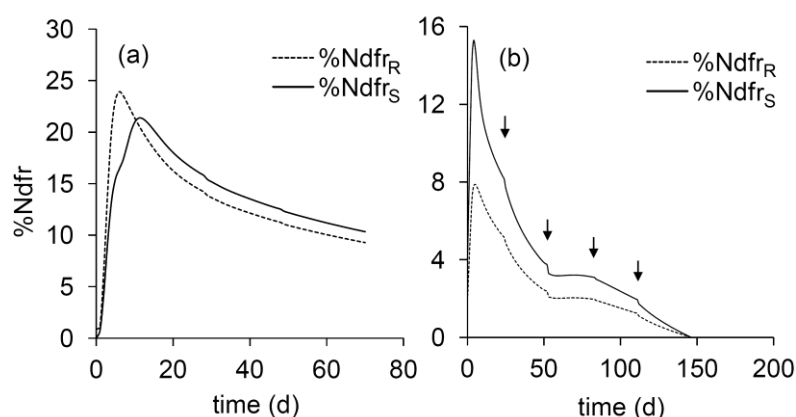


**Figure 10** Effect of $\delta^{15}N$ of residue on N uptake estimates (proportion of plant N derived from transfer) in (a) the residue application treatment (RA), and (b) for the dataset of Sierra et al. (2007). %Ndfr$_R$: calculated with total initial $\delta^{15}N$ of residue, and %Ndfr$_S$: calculated with simulated $\delta^{15}N$ of N released from residue. Arrows indicate shoot harvesting (b).

42

**Table 8** Biomass properties of *Gliricidia sepium* trees (*Gs*, N donor) and *Dichanthium aristatum* grass (*Da*, N recipient) at the end of the pot culture study. Values are mean ± standard error. For each variable, small letters indicate significant differences between the treatments (Student's t-test for *G. sepium* and Tukey's HSD for *D. aristatum*, $P$ <0.05).

| Variable | Sp. | FI | MY | EX$_{MF}$ | RA |
|---|---|---|---|---|---|
| Total dry mass | *Gs* | 573 ± 40 | 573 ± 27 | - | - |
| (g pot$^{-1}$) | *Da* | 76.3 ± 7.1 a | 62.3 ± 2.5 ab | 47.2 ± 1.3 c | 51.7 ± 1.1 bc |
| Total N content [a] | *Gs* | 8915 ± 719 | 9543 ± 432 | - | - |
| (mg pot$^{-1}$) | *Da* | 436 ± 31 a | 366 ± 12 b | 268 ± 5 c | 264 ± 5 c |
| Shoot N content | *Gs* | - | - | - | - |
| (mg pot$^{-1}$) | *Da* | 236 ± 18 a | 195 ± 7 ab | 156 ± 3 bc | 135 ± 8 c |
| Root N content | *Gs* | 1820 ± 141 | 2175 ± 130 | - | - |
| (mg pot$^{-1}$) | *Da* | 96 ± 9 a | 64 ± 7 b | 62 ±4 b | 70 ± 3 b |
| Shoot N conc. (%) | *Gs* | - | - | - | - |
| | *Da* | 0.56 ± 0.04 b | 0.52 ± 0.02 b | 0.69 ±0.01 a | 0.57 ± 0.03 b |
| Root N conc. (%) | *Gs* | 2.0 ± 0.1 b | 2.3 ± 0.0 a | - | - |
| | *Da* | 0.51 ± 0.02 b | 0.64 ± 0.02 a | 0.46 ±0.02 bc | 0.42 ± 0.01 c |

FI = full interaction (n = 8), MY = mycelial networks (n = 6), EX$_{MF}$ = exudation via mass flow (n = 8), and RA = residue application (n = 8).

[a] Including stubble N content for *D. aristatum.*

**Table 9** Mycorrhizal colonisation in the roots of *Gliricidia sepium* tree (*Gs*) and *Dichanthium aristatum* grass (*Da*) at the end of the pot culture study. Values are mean ± standard error. For statistical tests, the data were normalised by arcsine transformation. For each variable, values followed by different small letters differ significantly between the species of the same treatment, and values followed by different capital letters differ significantly between the treatments for a species (Tukey's HSD for the comparison of treatments for *D. aristatum*, and Student's t-test for the other comparisons; $P$ <0.05).

| Mycorrhizal colonisation (% of root length) | Sp. | FI | MY | EX$_{MF}$ | RA |
|---|---|---|---|---|---|
| Arbuscular | *Gs* | 26.0 ± 4.0 A | 28.0 ± 4.9 A | - | - |
| colonisation | *Da* | 6.2 ± 2.9 Bb | 13.6 ± 1.6 Ba | 14.4 ± 1.9 a | 11.4 ± 1.8 ab |
| Vesicle | *Gs* | 36.5 ± 4.4 Aa | 20.5 ± 2.0 Ab | - | - |
| colonisation | *Da* | 21.8 ± 2.2 Ba | 11.0 ± 2.3 Bb | 3.3 ± 1.0 c | 8.6 ± 1.0 b |
| Hyphal | *Gs* | 89.4 ± 2.3 A | 77.4 ± 5.1 | - | - |
| colonisation | *Da* | 73.9 ± 3.6 Ba | 75.0 ± 3.0 a | 51.8 ± 3.9 b | 74.2 ± 2.2 a |
| Uncolonised | *Gs* | 10.6 ± 2.3 B | 21.3 ± 4.9 | - | - |
| | *Da* | 26.0 ± 3.6 Ab | 24.8 ± 3.1 b | 48.3 ± 3.9 b | 26.3 ± 2.2 a |

FI = full interaction (n = 8), MY = mycelial networks (n = 6), EX$_{MF}$ = exudation via mass flow (n = 8), and RA = residue application (n = 8).

**Table 10** Correlations of properties of *Gliricidia sepium* trees (N donor) and *Dichanthium aristatum* grass (N recipient) with N transfer variables at the end of the pot culture study. Asterisks (*) indicate significant correlations (Pearson's correlation analysis, *P* <0.05).

| Variable | %Ndft [a] | | $N_{tr}$ [b] | | %$N_{tr}$ [c] | |
|---|---|---|---|---|---|---|
| Treatment | FI | MY | FI | MY | FI | MY |
| N concentration (%) of grass roots | 0.71 | -0.88* | 0.73 | -0.83* | 0.53 | -0.85* |
| Relative N concentration of grass [d] | 0.89* | -0.79 | 0.93* | -0.73 | 0.82* | -0.59 |
| Arbuscular colonisation in tree roots | -0.90* | 0.76 | -0.72 | 0.81* | -0.41 | 0.86* |

FI = full interaction (n = 8), and MY = mycelial networks (n = 6).

[a] Proportion of grass N derived from transfer (Eqn (2))

[b] N transfer (mg 10 $wk^{-1}$; Eqn (3))

[c] N transferred out of tree total N (Eqn (4))

[d] the ratio of grass root N concentration to tree root N concentration

*Root exudation and exudate N uptake (III, IV)*

Total amount of N exuded from *G. sepium* roots over the 10-week experiment ($EX_{FI}$) was estimated at $245 \pm 20$ mg [N] $tree^{-1}$ (III). Exudation of nitrogenous compounds increased continuously from the first to the last study period, from $2.9 \pm 0.2$ to $4.3 \pm 0.5$ mg [N] $d^{-1}$. Average daily N exudation varied from 0.07% to 0.12% of tree total N, decreasing significantly with tree growth towards the end of the experiment. The C:N ratio of exudates varied from 3.3 to 5.3, with no observable trend between the study periods (Table 5). Amount of N in exudates correlated positively and significantly with tree total, root and stem N content (r = 0.73, 0.69, and 0.71, respectively; n = 12).

Total root exudation of *G. sepium* would have been enough to supply 63% of grass total N in treatment FI by the end of the experiment (III). Average N transfer calculated with exudate $\delta^{15}$N in treatment FI was 0.76 mg [N] $d^{-1}$, totalling 54 mg $pot^{-1}$ over the experiment. The results suggest that during the 10-week experiment, the grass absorbed up to 22% of N exuded by *G. sepium* when the roots of the two plant species were mixed together in the pots (III). In contrast, when the alone-grown grass was irrigated with exudates in treatment $EX_{MF}$, it recovered only $5.6 \pm 1.2$% of the estimated N in exudates (IV). Proportionally exudate uptake from mass flow continuously increased over the experiment (data not shown).

*Nitrogen uptake from decomposing residue (II)*

Grass shoot N concentration (data not shown) peaked simultaneously with shoot $\delta^{15}$N (Fig. 9) and correlated statistically significantly with it (r = 0.38, *P* <0.001; n = 32). According to the simulations, 10.3% of N in the N recipient plant originated from the residue at the end of the RA experiment. This corresponded to 25 mg of N and 26% of initial residue N.
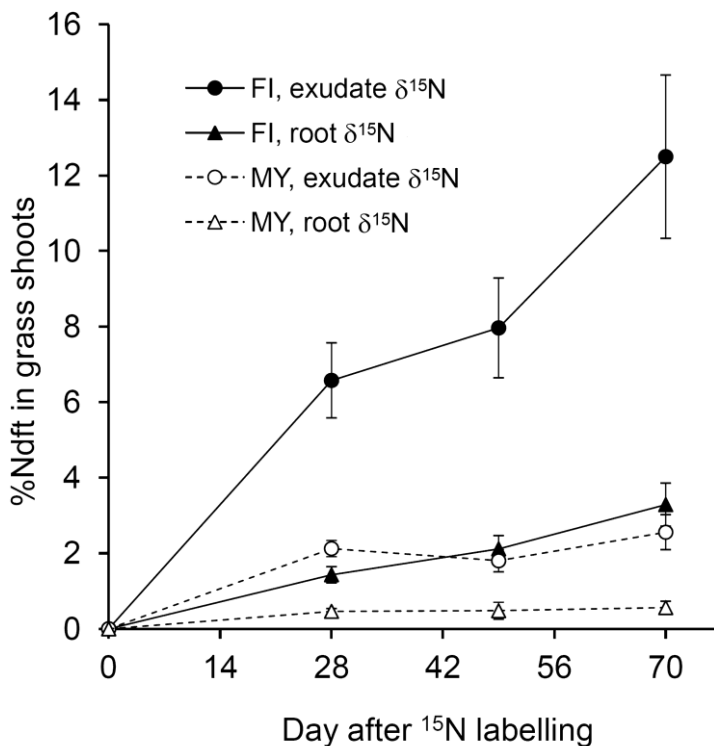
**Figure 11** Proportion of N derived from transfer (%Ndft) in shoots of *Dichanthium aristatum* grass during the experiment, calculated with exudate and root $\delta^{15}N$ as $\delta^{15}N$ of the N source (Eqn (2)). Treatments are FI = full interaction (n = 8) and MY = mycelial networks (n = 6). Vertical bars indicate the standard error of mean.

## DISCUSSION

### Arbuscular mycorrhizal fungal symbionts on the agroforestry field site (I)

*Gliricidia sepium* and *Dichanthium aristatum* were observed to form symbiosis with the same subgroup of *Rhizophagus intraradices* on the agroforestry field site. The result indicates favourable conditions to the formation of anastomoses between the mycorrhizal fungal symbionts of the two plant species, and thereby for N transfer via CMN (Croll et al. 2009; Mikkelsen et al. 2008). Comparison of the fungal strains identified from the agroforestry field site and the grass monocrop indicated that on the agroforestry field site the two plant species had developed symbiosis with common fungal symbionts of Glomeromycota (*R. intraradices* group II) which differed from those observed on the grass monocrop (*R. intraradices* group I). It is known that mycorrhizal infection may spread from one plant to another of different species (Ingleby et al. 2007; Ikram et al. 1994). *Rhizophagus intraradices* has been observed in a wide variety of ecosystems from tropical

and temperate forests to grasslands, where they were commonly the dominant taxon, as well as in some arable lands, indicating that they are relatively tolerant to anthropogenic disturbance (Öpik et al. 2006).

Fungal strains from *G. sepium* included two distinct strains that may represent novel types of fungal symbionts under Glomeromycota, as they did not closely resemble any strains previously recorded in the phylum. To my knowledge, mycorrhizal fungal species have not previously been identified with DNA sequencing techniques from field samples of *G. sepium* or *D. aristatum* roots. Overall, very little molecular research has been conducted on mycorrhizal symbionts of tropical tree species in field conditions (but see Husband et al. 2002, Wubet et al. 2004, Shepherd et al. 2007). The results of this study support the view that diversity of Glomeromycota may yet be largely underestimated (Redecker and Raab 2006).

## Methodological considerations in quantifying belowground N transfer

*Quantifying N transfer with isotope techniques (II, III)*

Nitrogen transfer from the legume tree to the recipient grass via root exudation and CMN was calculated using two estimates for the $\delta^{15}N$ of the transferred N – $\delta^{15}N$ of the roots and of the exudates – in order to account for the uneven partitioning of $^{15}N$ within the tree biomass and obtain an estimate for the probable range of N transfer (III). Results of N transfer differed almost 4-fold depending on the estimate used, which demonstrates the importance of evaluating the isotopic composition of the *actual* N sources for reliably quantifying N transfer with isotope labelling techniques. The lower $\delta^{15}N$ of root exudates in comparison to other biomass compartments, including roots, were coherent with previous observations (Sierra et al. 2007), and in line with the suggestion that exuded N is mainly recently fixed N (Ta et al. 1986) although also the exudates were clearly enriched with $^{15}N$ (III). Range of C:N measured for root exudates was similar to the estimated C:N of labile N recycled from decomposing tree roots (II). The results support the view that transferred N was mainly mobile N in the belowground parts of *G. sepium*, which had clearly lower $\delta^{15}N$ than roots as a whole. This, in turn, implies that actual N transfer was closer to the upper range of the estimates given in this study, 14% of grass total N when the root systems of the N donor and N recipient plants were fully mixed.

Methods for the separation of root exudates from root debris for determining N transfer via exudation are limited. Microlysimeter techniques applied in some other studies (Cliquet et al. 1997, Paynel et al. 2001) for herbaceous plants were not feasible in this study because of the high water requirements of the N donor trees, and hydroponic cultivation was conducted instead. Because of the short time with hydroponics, potential differences in tree root development versus natural soil or nutrient availability with hydroponics were not of concern in this study. It is important to note that N transfer calculations via exudation were not based on measuring exudate N content, but instead exudate $\delta^{15}N$. If we assume a relatively homogenous labelling of *mobile N* within roots at a given time, potential changes in the extent of exudation should not affect exudate $\delta^{15}N$ and therefore not the transfer estimates either.

Direct measuring of $\delta^{15}N$ of N transferred via CMN is not possible because of the microscopic and fragile nature of mycelial connections, and the difficulty of separating the hyphae from the very clayey soil used in this study. Nevertheless, N transferred to

mycorrhizal hyphae from the donor plant roots can be assumed to be mobile N, similarly to exuded N. For ectomycorrhizal fungi there is evidence that the fungal symbiont obtains N from a $N_2$-fixing host in the form of amino acids (Arnebrant et al. 1993). Therefore, the actual N transfer via CMN probably also was closer to the higher transfer estimate obtained with exudate $\delta^{15}N$, 2.5% of grass total N.

Using simulation methods, it was demonstrated that the labile and stable fractions of organic residue of $^{15}N$-labelled plants were differently enriched with $^{15}N$, independent of the timing of $^{15}N$ labelling (II). The results are in line with the observations of Sierra et al. (2007) who showed that isotopic composition of N released from decomposing roots may not correspond to that measured from the living roots just before pruning. Assuming homogenous isotopic composition of organic residue when the residue fractions actually had different isotopic compositions impaired N recycling estimates, depending on the relative sizes of the labile and stable fractions (II). Nitrogen recycling estimates may not be largely affected by isotopic heterogeneity when only relatively high-quality residues (e.g. fine roots of legume trees) are concerned. These residues may decompose entirely over a short time and N originating from the residue fractions become effectively mixed in the system. In contrast, substantial errors in N recycling estimates may be obtained for lower-quality residues with large stable fractions, if isotopic composition of released N is assumed homogenous within the residue and constant over time. In such cases, either over- or underestimates for N recycling may be obtained, depending on the relative $^{15}N$ enrichment of the residue fractions. Relative $^{15}N$ enrichment of the fractions can be assumed to change over time, as initially metabolically active or stored $^{15}N$ is converted to structural components during biomass growth. Measuring $\delta^{15}N$ and N content of labile and stable residue fractions can be a useful first step in evaluating whether isotopic heterogeneity is likely to result in too small or large estimates for N recycling from organic sources in the concerned system.

The observed correlation between shoot N concentration and $\delta^{15}N$ of the N recipient grass after the incorporation of $^{15}N$-labelled residue in the RA experiment indicates that grass obtained proportionally more N from decomposing residue than from soil native N pools. This was supported by the results of the modeling study, where proportionally higher N uptake of N mineralised from residue compared to soil native N was necessary for obtaining a satisfactory agreement with the experimental observations. Residue application appeared, therefore, to enhance grass N nutrition instead of merely substituting soil native N as N source (cf. Jenkinson et al. 1985). The differences in N uptake between the two inorganic N sources could be explained by heterogenous distribution of the residue in the soil, increased soil porosity around decomposing residue which could have facilitated N absorption (Jingguo and Bakken 1997, Hinsinger et al. 2007), or restricted solute flow in the clayey soil (Cabidoche and Guillaume 1998, IV). For the dataset of Sierra et al. (2007), differences in the competitive abilities of *G. sepium* and *D. aristatum* (cf. Ledgard and Steele 1992) could also have contributed. The differences in N uptake between the soil N pools do not, however, affect the results about residue fractionation and distinctive $^{15}N$ enrichment of the labile and recalcitrant residue fractions.

Method of $^{15}N$ labelling may importantly affect the relative $^{15}N$ enrichment of plant organs and N compounds (Wichern et al. 2008). An early start of $^{15}N$ labelling would help to enrich plant structural components more evenly, but may in turn disturb the system more through repeated interventions and the need of higher $^{15}N$ inputs. Analysis of the temporal isotopic variation of the N sources and reasons affecting it, as illustrated in this study (II), could provide an alternative or complementary method to aiming at, or simply assuming,

homogenous isotopic composition in $^{15}$N tracer studies. If no information of the variation of $\delta^{15}$N within the N donor plant in $^{15}$N enrichment studies is available, caution should be exerted in estimating N recycling from the $\delta^{15}$N values of the recipient pools in the short term. This is especially the case after discrete events in time such as pruning or residue application, or under pronounced seasonality, if there are large inputs of residue from senescing biomass over a short time period. Previous research indicates that these considerations are important also in studies applying the $^{15}$N natural abundance method (Sierra et al. 2007, Daudin and Sierra 2008), and the results presented here suggest that residue fractionation merits research in explaining isotopic patterns related to N recycling in $^{15}$N natural abundance studies.

*Competition in pot culture studies*

In a limited growth space, root competition between the assumed N donor and recipient plants may alter the dynamics of mycorrhizal colonisation and N transfer (Ingleby et al. 2007, Kurppa et al. 2010). In the experimental setup of this study, the relative importance of N transfer via CMN with regard to total N transfer was evaluated by comparing N transfer observed in treatments MY and FI. If N transfer in treatment FI was considerably reduced because of root competition, the role of CMN in N transfer obtained from the comparisons may in turn be overemphasised.

Both *G. sepium* and *D. aristatum* seem to have experienced more competition for soil N in treatment FI than in treatment MY, because both plant species had lower root N concentration in that treatment than in treatment MY. Vesicle colonisation, which has been associated with increased carbon investments from the host plant to the fungal symbionts under nutrient-limiting conditions (Cooke et al. 1993, Treseder and Allen 2002), was higher in both plant species in treatment FI than in the other treatments. This increase was in accordance with the observations on the agroforestry field site where the highest vesicle colonisation in both plant species was observed at 1 m from the tree rows (T2), the location of the most even competition (I). In general, legumes are considered less effective competitors for soil nutrients than grasses (Ledgard and Steele 1992). Legumes are often strongly dependent on mycorrhizal fungi for nutrient acquisition, as demonstrated by their growth response to mycorrhizal inoculation (Habte and Turk 1991, Okon et al 1996), and also by the more frequent mycorrhizal formations in the roots of the legume tree versus the grass in pot culture in this study (III). Furthermore, the mycorrhizal dependency of legumes has been observed to increase when they are intercropped with grasses, compared to a legume monocrop (Scheublin et al. 2007).

Root competition may result in enhanced mycelial transport of nutrients towards the larger legume trees, and, thereby, restrict the possibilities of interplant N transfer in limited growth space (Trannin et al. 2000, Ingleby et al. 2007). The observation that N transfer in treatment FI decreased with increasing arbuscular colonisation in *G. sepium* roots is consistent with this view. The fact that the biomass and N content of *G. sepium* were, nevertheless, similar in treatments FI and MY indicates that either the enhanced mycorrhizal N supply or enhanced $N_2$ fixation (van Noordwijk and Dommergues 1990) compensated for the eventual reduction of soil N availability for the tree in treatment FI, and N availability did not become growth-limiting for either plant species. While the total and root N content of the grass were higher in treatment FI than MY, this N gain was small when compared with the total N content of the tree. Therefore, it seems that while competition may have limited N transfer via CMN in treatment FI, the effect on total N

transfer was small. The potential effects of competition on N transfer dynamics should be taken into consideration in pot culture studies where growth space and nutrient availability are limited, especially in experiments lasting for several months. Changes in mycorrhizal colonisation can be helpful indicators for changes in competitive relations between plants and the subsequent impacts on N transfer among treatments in pot culture studies.

**Nitrogen transfer pathways (III-IV)**

When the root systems of *G. sepium* and *D. aristatum* were fully mixed in the pots, *D. aristatum* obtained up to 14% of its N from *G. sepium* via belowground pathways over 10 weeks (III). The results were in line with the evidence from an earlier pot culture study with the same soil and plant species as in this study, where *D. aristatum* gained 10-15% of its N from *G. sepium* over 12-13 weeks (Sierra et al. 2007). Nitrogen transfer of the same order of magnitude has earlier been measured in pot culture studies with herbaceous legumes: barley *(Hordeum vulgare* L.) gained 19% of its N from an associated field pea *(Pisum sativum* L.) over 10 weeks (Jensen 1996), and timothy *(Phleum pratense* L.) approximately 10% of its N from alfalfa *(Medicago sativa* L.; Ta et al. 1989).

*Common mycelial networks (III)*

The positive correlation of grass and tree root N content in treatment MY suggests a beneficial effect of the tree presence to grass N nutrition also when the root systems of the plants were separated by a thin mesh that only allowed mycorrhizal hyphae to penetrate. Nitrogen transfer in that treatment could only have occurred via mass flow through the mesh, which however was considered unimportant in the studied very clayey soil (Cabidoche and Guillaume 1998; IV), or via CMN. The extensive AM colonisation in the roots of both *G. sepium* and *D. aristatum* suggests a good basis for N transfer via CMN. The fact that the plant species were observed to form symbiosis with the same subgroup of *G. intraradices* on the agroforestry field site (I) also supports the formation of CMN.

Biologically, N transfer via CMN in this study was small with respect to the total N content of the N recipient grass. The results were at the lower end of the range of N transfer observed in other pot culture studies applying hyphal compartmentalisation. Values of %Ndft higher than 10% have repeatedly been measured from recipient plants associated with herbaceous legumes (Haystead et al. 1988, Bethlenfalvay et al. 1991, Frey and Schüepp 1992, Mårtensson et al. 1998). At minimum, N transfer amounted to less than 2% of the recipient plant N (Johansen and Jensen 1996; Ikram et al. 1994). The deviation in the results may be explained by differences in species' physiology or plant nutritional status (Simard et al. 2003), or the methods of root separation. Small pore size of the separating mesh with regard to other studies (Johansen and Jensen 1996; III), or a low proportion of pores per total mesh area may limit N transfer.

However, N transfer in treatment MY equalled to 18% of total N transfer in the case of fully mixed root systems of the two plant species, which can be considered proportionally significant. Moreover, the fact that %Ndft in treatment MY saturated after the first few weeks, while in treatment FI it continuously increased until the end of the experiment, could be explained by the saturation of mesh penetration by mycelial hyphae resulting in a bottleneck to N transfer across the mesh. At the end of the first study period, N transfer in treatment MY was 32% of that observed in treatment FI, but the proportion reduced to 18%

by the end of the experiment. Had the proportion remained the same throughout the experiment, N transfer in treatment MY at the end of the experiment could have exceeded 5% of grass total N. Mycelial connections between the root systems of the N donor and recipient plants in full root interaction are likely both more frequent and shorter in distance than in the case of root compartmentalisation. Considerably more N transfer via CMN could, therefore, be expected in systems involving fully mixed root systems of the N donor and recipient plants.

The experiment MY yielded novel quantitative observations that the amount of N transferred ($N_{tr}$) increased with tree arbuscular colonisation in treatment MY, and that N transferred out of the tree total N (%$N_{tr}$) increased with decreasing grass root N concentration. These results suggest that in this study, N transfer at least partly occurred directly via CMN. If the mycorrhizal symbionts of the grass had only taken up N excreted by *G. sepium* in the rhizosphere of the tree, such correlations between the AM colonisation of the N donor and N transfer would not have been observed. The negative correlation of grass root N concentration with N transfer in treatment MY suggests that source-sink relationships exist between the N donor and recipient plants in N transfer mediated by CMN, as anticipated in many studies (Smith and Smith 1990, Bethlenfalvay et al. 1991, Simard et al. 2003). Previously, N transfer via CMN has been linked to donor stress (Haystead et al. 1988, Bethlenfalvay et al. 1991, Johansen and Jensen 1996). The results of this study indicate also a sink effect exercised by the recipient plant towards the N donor in normal physiological condition, suggesting that such effect may be of relevance in a wider range of systems and conditions.

*Root exudation (III, IV)*

The estimated root exudation in treatment $EX_{FI}$ would have been enough to supply all transferred N measured in full root interaction (III). It can be assumed that root recycling of trees in active growth phase and non-N-deficient conditions was limited (cf. Burton et al. 2000, Gill and Jackson 2000). Thus, the comparison of N transfer results of the treatments suggests that the observed N transfer in full belowground interaction occurred mainly via root exudation. Exudates appeared an important N source also in comparison with N recycled from decomposing roots. Assumed root and nodule recycling after tree pruning (50% of fine roots, IV) would have explained approximately only half of the N transfer observed in full root interaction without pruning (II). Nitrogen transfer via CMN could have accounted for approximately 18% of transfer observed in full interaction (FI), or possibly more because of the restriction of mycelial connections by the mesh interface (III).

Exudate C:N ratios were remarkably low. It is generally held that majority of root exudates are sugars and other C compounds (Grayston et al. 1996, Jones et al. 2004). The results of this study, however, suggest that legume root exudates potentially are a good source of N to associated crops, either when directly absorbed or after a rapid recycling via soil microbial organisms. Very low C:N ratios of 4.7 to 6.8 were previously measured also for the root exudates of the $N_2$-fixing legume tree *Robinia pseudoacacia* L. (Uselman et al. 1999).

Mass flow of exuded compounds does not seem an important pathway of N transfer in the studied soil (Vertisol) even in the spatial scale of $10^{-1}$ m (IV). Diffusion of $NH_4^+$ in soil generally is slow, only of the magnitude of 0.2 mm $d^{-1}$ (Owen and Jones 2001). Pools of $NH_4^+$ in soils are generally relatively large but very dynamic, and their average turnover time is only a day. Amino acids in soil solution are immobilised within hours (Jones et al.

2005). Mass flow of exuded compounds may be further restricted by the tendency of microbes to form sheaths around plant roots and, thus, effectively filter N in soil solution (Owen and Jones 2001).

The proportion of N transfer in the recipient grass in treatment $EX_{MF}$ increased over the course of the experiment, although N accumulation rate in grass biomass was much faster than exudate N addition rate (IV). This observation could indicate increasing importance of exudates to grass N nutrition over time, and could be explained by remineralisation of the initially immobilised exudate N. However, grass absorption of N exuded by *G. sepium* even in full belowground interaction (FI) was relatively low, 22% of all exudate N if it was assumed that all observed N transfer occurred via root exudation (III). The results of $\%N_{tr}$ were of the same order of magnitude as in a previous study with the same soil and plant species, where grass absorption of exudate N over 58 weeks was estimated at 1.1 mg [N] $d^{-1}$ or 29% of all N exuded by the tree (Sierra et al. 2007). The comparison of the results suggests that the majority of exuded N may remain in soil even over several months, e.g. immobilised in soil microbial biomass (cf. Mayer et al. 2003) or, in the case of $NH_4^+$, fixed on the surface of the soil clay particles (Paul and Clark 1996). In total 95-98% of inorganic N in the soil used for the experimental studies is in the form of $NH_4^+$ (Sierra and Nygren 2006). *Dichanthium aristatum* (J. Sierra, unpubl.), as some other tropical grasses (Ishikawa et al. 2003), has been observed to release compounds which reduce the populations or activity of nitrifying bacteria which could explain the proportion of $NH_4^+$. Negligible volatilisation of $NH_3$ has been observed in a non-N-fertilised Vertisol similar to that used in the experimental studies (Courtaillac et al. 1998), and frequent irrigation was assumed to further reduce concentration of $NH_4$ in the soil. Therefore, loss of $^{15}N$ from the system via denitrification and volatilisation can be assumed negligible. In conclusion, root exudation by the N donor plant may benefit the N recipient mostly indirectly, e.g. by facilitating decomposition of residues and soil organic matter.

Rhizodeposition of particular organic compounds, e.g. root border cells and sloughed epidermal root cells, root hairs, microroots and root fragments (Wichern et al. 2008, Fustec et al. 2010) was not considered in this study which focused on direct N transfer, and root exudates were filtered prior to analysis to exclude such particles. Rhizodeposition of particulate compounds has been observed to constitute in average 64% of belowground N in herbaceous legumes (Wichern et al. 2008, Fustec et al. 2010), and 9-12% of rhizodeposits of three legume species was recovered in subsequent crops (Mayer et al. 2003). It can be assumed that rhizodeposition of particulate compounds contributed to the observed N transfer in pot culture in this study, while root exudation played the major role, since estimated root exudation in treatment $EX_{FI}$ would have been enough to supply all transferred N in treatment FI (III).

In general, N transfer or uptake via belowground transfer pathways in this study was on the lower side of the values measured in previous studies. Variation in the observations may result e.g. from residue type and method of application, biological or environmental conditions facilitating or restricting decomposition, capacity of the soil to sequester organic inputs, synchrony with the nutrient needs of the recipient plant, methods for estimating N uptake, and the length of the observation period (Hesterman et al. 1987, Jensen 1994, Palm 1995, Chintu and Zaharah 2003, Sierra and Motisi 2012).

# IMPLICATIONS OF THE FINDINGS TO AGROFORESTRY RESEARCH AND PRACTICES

## Nitrogen transfer in agroforestry systems

This study was motivated by the observation that recycling of organic matter could not explain most of the observed N inputs from *Gliricidia sepium* to the system on the cut-and-carry fodder production site of *G. sepium* and *Dichanthium aristatum* (Dulormne et al. 2003). The findings of this study now indicate that root exudation and N transfer via common mycelial networks are important N transfer pathways which can potentially explain most of the belowground N transfer on the agroforestry field site and could considerably contribute to N transfer in agroforestry systems in general, also in cases where above-ground N inputs are practically eliminated through frequent biomass harvests (Fig. 12). The results are in line with previous observations from the agroforestry field site. Concentration of N fixed from the atmosphere in *D. aristatum* correlated with fine root density of *G. sepium* but not with soil isotopic ratios, indicating that most of the N transfer occurred via direct N transfer pathways (Sierra and Nygren 2006). Direct N transfer can improve plant nutrient acquisition especially in conditions where the relatively immobile ammonium is the main source of soil N (Johansen et al. 1992, Smith and Read 2008). Role of root exudation and CMN in the provision of N inputs to the soil (Dulormne et al. 2003) is probably yet more important than their subsequent contribution to N transfer to the recipient plant which was quantified in this study.

In field studies N transfer generally increases over cropping seasons, and in established systems it often exceeds that measured in pot culture studies (van Kessel et al. 1994, Jayasundara et al. 1997, Høgh-Jensen and Schjoerring 2000). On the agroforestry field site of *G. sepium* and *D. aristatum*, symbiotically fixed N constituted 31-35% of grass total N 12-15 years after site establishment (Sierra and Nygren 2006, Daudin and Sierra 2008). Pulses of exuded compounds have been observed from the roots of herbaceous legumes after shoot harvest (Eason and Newman 1990). Tree pruning which is practiced regularly on the field site could induce similar pulses from *G. sepium* roots (cf. Nygren 1995) and partly explain the scale difference in N transfer between short-term pot cultures and field measurements in established systems. Moreover, extraradical hyphae of AM fungi extend slowly (Camel et al. 1991, Ingleby et al. 2007), and the development of the networks over time may contribute to higher N transfer in the field over time.

Although N transfer via CMN was biologically small in this study, the results indicate that proportionally it may be an important transfer pathway (III). The role of CMN in this study may also have been underestimated as a result of root compartmentalisation. Observations from the agroforestry field site lend support to the results of the pot culture studies about the potential of interplant N transfer through CMN. Arbuscular colonisation in *G. sepium* roots, which correlated positively with N transfer via CMN (II), was highest under the tree rows. Same mycorrhizal fungal strains observed in the roots of both plant species indicated the possibility of anastomoses between their fungal symbionts (I). Interestingly, in *D. aristatum* proximity to the legume trees appeared to stimulate AM symbiosis, as arbuscular colonisation and the arbuscule:vesicle ratio in the grass roots were higher on the agroforestry field site than on the *D. aristatum* monocrop. Intercropping with a legume has been observed to stimulate AM colonisation also in the *Lolium perenne* grass (Barea et al. 1989).
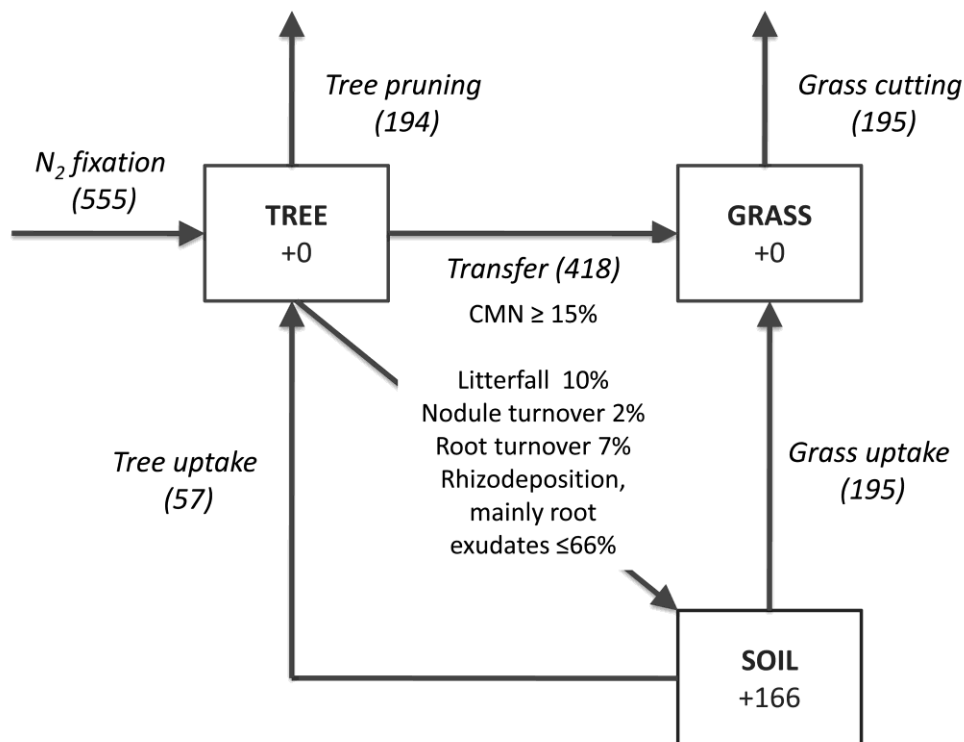
**Figure 12** Nitrogen balance of the agroforestry field site of *Gliricidia sepium* tree and *Dichanthium aristatum* grass, including indicative estimates of previously unknown N transfer pathways from this study (cf. Fig. 2). Values are in kg [N] ha$^{-1}$ yr$^{-1}$. Nitrogen transfer via common mycelial networks (CMN) and root exudation are given proportional to total N transfer, as opposed to the proportions given in the text which excluded litter and biomass turnover. See Dulormne et al. (2003) for detailed explanation of calculation of the balance.

Mass flow of exuded compounds was considered unimportant in the studied soil (IV). Similarly, the extent over which CMN may effectively contribute to N transfer is short, of the magnitude of a few centimeters only (Newman 1988, Hodge 2000). The apparent direct N transfer observed in earlier pot culture (Sierra et al. 2007) and field studies (Sierra and Nygren 2006; Daudin and Sierra 2008) seems to require an intimate contact between the donor and recipient plants – either of roots directly or mediated by CMN (He et al. 2003).

Root exudation and N transfer via CMN are assumed to be mechanistic processes which depend e.g. on membrane permeability of the solute and concentration differences between the N donor plant and soil solution (Jones et al. 2004), or between the N donor and N recipient plants (Perry 1998, III). Both N transfer processes have been observed in a number of different types of plants, including herbaceous legumes (e.g. Brophy et al. 1987, Bethlenfalvay et al. 1991, Frey and Schüepp 1992, Paynel et al. 2001) and N$_2$-fixing tree species. Exudation has been established in legume trees *Robinia pseudoacacia* (Fabaceae) (Uselman et al. 1999) and *Inga edulis* Mart (Mimosoideae) (H. Leblanc and P. Nygren unpubl.), and CMN transfer in actinorhizal species *Alnus glutinosa* (L.) Gaertn.

(Betulaceae) (Arnebrandt et al. 1993) and *Casuarina cunninghamiana* Miq. (Casuarinaceae) (He et al. 2004). The extent of N transfer via root exudation and CMN transfer between *Gliricidia sepium* and *D. aristatum*, physiologically distinct plant species, indicates that these processes can potentially transfer N also in many other species associations.

**Future prospects**

Despite of its potential as N transfer pathway, little is known about the exudation of nitrogenous compounds from the roots of living plants. To my knowledge, no comprehensive reviews exist about the extent of N exudation and the factors driving and affecting it (as distinguished from overall rhizodeposition; Wichern et al. 2008, Fustec et al. 2010). Synthesising previous observations about N exudation and the existing knowledge about plant physiological processes which may explain it would be a useful first step towards improved understanding on the role of N exudation in facilitating plant nutrition and for motivating further research.

Identity of AM fungal symbionts is known to affect transfer of nutrients between plant species (Eason et al. 1991, Mårtensson et al. 1998). Research on N transfer for different combinations of legume tree host species and mycorrhizal fungal isolates could lead to identification of combinations of plant and fungal species which result in particularly high N transfer. Diversity of AM symbionts is assumed higher in legume than non-legume species (Shepherd et al. 2007), and analysing the differences between functional plant groups can help to identify AM fungal species which have the highest potential for CMN. Plant species may also be infected by multiple mycorrhizal fungal symbionts at a time (Shepherd et al. 2007), and effects of such multiple infection on N transfer could reveal options for optimising N transfer. Some mycorrhizal fungal species are more sensitive to anthropogenic disturbance than others (Öpik et al. 2006), and optimal fungal symbionts in terms of N transfer for a given host plant species may depend on the intensiveness of management of the host plant in the system.

Environmental conditions and management practices of agroforestry systems can affect processes related to N transfer both directly and through their effects on nutrient demand and availability (Boddington and Dodd 2000, Titus and Leps 2000). For example, results of this study indicate that N status of the N recipient plant correlated with N transfer via CMN (III). Tree pruning is known to induce changes in C and nutrient allocation within the tree and with regard to its microbial symbionts, directing resources towards foliar regrowth (García et al. 2001) and reducing C supply to $N_2$-fixing nodules (Nygren 1995, Nygren and Cruz 1998). Similar effects of pruning on mycorrhizal fungal symbionts can be assumed, while in previous studies both negative, positive and no significant impacts on AM colonisation were reported depending on the host plant species (Ingleby et al. 2007, Martin et al. 2010). Tree pruning is also assumed to induce exudation (Nygren 1995). Limited N transfer via mass flow observed in this study (IV) indicates that spatial arrangement of the N donor and recipient plants in agroforestry systems importantly affects N transfer. Future research on the effects of the system design and management practices on N exudation and CMN formation – e.g. intensity and timing of pruning of the N donor, harvesting of the N recipient species, spacing and relative densities of the N donor and N recipient species, or fertilisation or nutrient deficiencies – can contribute to defining good management practices which favour N transfer and to identifying conditions under which sustainability of the

systems may be compromised. Furthermore, the complexity of the interactions between the legume tree, its rhizobial and mycorrhizal fungal symbionts, and associated crops indicate that modeling approaches linked to carefully designed pot culture and field experiments would be useful in improving understanding about the dynamics of N transfer in agroforestry systems.

Future studies which apply isotope techniques to quantify N transfer should strive to assess the isotopic composition of the actual N sources in order to obtain reliable N transfer estimates. Isotopic composition of mobile and structural N can differ both in $^{15}$N enrichment (Sierra et al. 2007; II, III) and $^{15}$N natural abundance studies (Sierra et al. 2007, Daudin and Sierra 2008), especially after management interventions such as tree pruning. Analysing the extent and explaining factors of such variation would help to improve the reliability of N transfer estimates obtained with either isotope technique.

The consequences of soil fertility depletion in the tropics hit hardest the already resource-poor farmers for whom agriculture is the main source of livelihood and who have limited economic or physical access to fertilisers and land for compensating the loss in productivity. Farmers' knowledge about soil quality and processes varies widely, especially according to how long they have resided in the area, but many farmers are knowledgeable about their environment and adapt farming practices and species choices to changing conditions over time through experimentation and expertise (Hiemstra et al. 1992, Thapa et al. 1995). Studying the effects of farmers' management practices on root exudation, mycorrhizal symbioses and belowground N transfer would help to identify good practices for facilitating N transfer, indicators for sustained transfer, and thresholds where risks to soil fertility loss increase disproportionally. Research could help identify conditions and practices under which belowground N transfer can adequately compensate for exports of above-ground biomass as N source, and thereby enable harvesting of tree prunings, litter or residues without compromising the sustainability of the production systems. Overall, research should increasingly focus on indigenous legume tree species, their microbial symbionts, management practices in intercropping systems, and their belowground N transfer potential. Farmers use dozens of indigenous tree and shrub species for fodder alone, many of them leguminous (Roothaert and Franzel 2001), and consider indigenous tree species more compatible with their cropping systems than exotic species (Mekoya et al. 2008), possibly because of the often extensive local knowledge on the ecology and biology of indigenous species.

Even when improved farming technologies are aimed at poor farmers, the technologies are often more frequently adopted by wealthier farmers (Tripp 2006). Economic, social, political and cultural incentives may all be important in encouraging soil conservation, and community-based approaches and action research can be particularly useful in studying and testing sustainability of farming practices and feasibility of proposed improvements (Stocking 2003). Adoption of *Faidherbia albida* (Del.) A. Chev, *Gliricidia sepium* and other $N_2$-fixing legume trees for mulch production by hundreds of thousands of African farmers illustrates that mainstreaming of soil conservation practices among smallholder farmers is possible (Garrity et al. 2010). Could knowledge about the role and optimal management of belowground N transfer in tropical farming systems ultimately become as widespread?

# CONCLUDING REMARKS

This study was motivated by the observation that recycling of organic matter, commonly assumed as the major N transfer pathway between plant species in agroforestry, could not explain most of the observed N inputs from the $N_2$-fixing legume tree *Gliricidia sepium* to the system in a cut-and-carry fodder production system of *G. sepium* and the *Dichanthium aristatum* fodder grass (Dulormne et al. 2003). To analyse the role of belowground pathways in N transfer from the legume tree to the associated grass in the system (referred to as agroforestry field site), three research objectives were formulated.

The first objective was to determine whether *G. sepium* and *D. aristatum* form symbiosis with the same arbuscular mycorrhizal fungal strains in field conditions, as a precondition for the formation of common mycelial networks (CMN) between the plants. The two plant species were found to form symbiosis with the same subgroup of *Rhizophagus intraradices* on the agroforestry field site, which indicates good potential for CMN and, consequently, direct N transfer between the plant species.

The second objective was to analyse how N transfer estimates obtained with N isotope techniques could be improved by considering the temporal and spatial variation in the isotopic composition of the N sources. Applying both experimental and simulation methods it was demonstrated that transfer estimates can considerably vary according to the assumed isotopic composition of the transferred N, which itself can differ between plant metabolic and structural compounds and change over time. Homogeneity of $^{15}N$ enrichment within the plant could be improved by $^{15}N$ labelling techniques, yet homogenous labelling of different N compounds over time is likely difficult to achieve. Efforts to accurately measure or estimate the isotopic composition of the *actual* N sources for transfer (e.g. root exudates instead of root biomass as a whole) may provide an alternative or complementary approach to aiming at, or simply assuming, homogenous isotopic composition of the N donor plant over time.

The third objective was to quantify the role of CMN and root exudation in N transfer from the legume tree to the fodder grass. Exudation of nitrogenous compounds by the legume tree was identified as an important N transfer pathway which may have explained most of the observed belowground transfer in the study. Although N transfer via CMN in the short-term pot culture study was biologically small, it formed a notable proportion, 18%, of total belowground transfer. The potential of CMN in supporting crop nutrition was further highlighted by the observation that N transfer via CMN was related to the nutrient status of the N recipient plant. Root exudation and CMN can importantly contribute to N transfer in agroforestry systems also in cases where above-ground N inputs are practically eliminated through frequent biomass harvests. Recognition of the significance of belowground N transfer could result in new improved design and management options for agroforestry systems, and, consequently, in increased productivity and sustainability of the systems, especially where external farming inputs are not easily accessible.

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