Is nitrogen transfer between plants enhanced by contrasting nutrient-acquisition strategies?

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ABSTRACT

Nitrogen (N) transfer between plants has been found where at least one plant can fix N₂. In nutrient-poor soils, where plants with contrasting nutrient-acquisition strategies (without N₂ fixation) co-occur, it is unclear if N transfer exists and what promotes it. A novel multi-species microcosm pot experiment was conducted to quantify N transfer between arbuscular mycorrhizal (AM), ectomycorrhizal (EM), dual AM/EM, and non-mycorrhizal cluster-rooted plants in nutrient-poor soils with mycorrhizal mesh barriers. We foliar-fed plants with a K¹⁵NO₃ solution to quantify one-way N transfer from ‘donor’ to ‘receiver’ plants. We also quantified mycorrhizal colonisation and root intermingling. Transfer of N between plants with contrasting nutrient-acquisition strategies occurred at both low and high soil nutrient levels with or without root intermingling. The magnitude of N transfer was relatively high (representing 4 % of donor plant N) given the lack of N₂-fixation. Receiver plants forming ectomycorrhizas or cluster roots were more enriched, compared to AM-only plants. We demonstrate N transfer between plants of contrasting nutrient-acquisition strategies, and a preferential enrichment of cluster-rooted and EM plants compared to AM plants. Nutrient exchanges between plants are potentially important in promoting plant coexistence in nutrient-poor soils.

Key-words: Arbuscular mycorrhiza, biodiversity, cluster roots, ectomycorrhiza, isotope ¹⁵N, mycorrhizal networks, plant coexistence, ecological restoration.

INTRODUCTION

Belowground transfer of nitrogen (N) between plants has been reported frequently when one plant can fix N₂ (Arnebrant *et al.*, 1993, Ekblad & Huss-Danell, 1995, Haystead, Malajczuk & Grove, 1988, He *et al.*, 2009). Although the direction of transfer can vary, it typically occurs from a source, i.e. a N₂-fixing plant to a sink, a non-N₂ fixing plant (He *et al.*, 2009). In systems studied thus far, it is unclear if N transfer occurs between plants with contrasting nutrient-acquiring strategies (including a non-mycorrhizal) that do not fix N₂ in infertile soils. It has been suggested that N transfer between non-N₂-fixing AM plants may be important in infertile soils (Eissenstat, 1990). Plants with mycorrhizal associations are more likely to transfer nutrients than non-mycorrhizal plants (Moyer-Henry *et al.*, 2006), but plants with other nutrient-acquisition strategies such as non-mycorrhizal cluster-rooted plants, have never been tested (Lambers *et al.*, 2013, Lambers *et al.*, 2008). Plants that form cluster roots, particularly Proteaceae species, are highly effective in the acquisition of phosphorus (P) and produce prolific cluster roots in nutrient patches or at the soil surface where litter decomposes (Lambers *et al.*, 2012, Lamont, 1982, Shane & Lambers, 2005).

The study of N transfer between plants has more recently been directed towards agroecosystems under low external nutrient input (Hauggaard-Nielsen & Jensen, 2005, Shen & Chu, 2004, Wichern *et al.*, 2008). In natural ecosystems with nutrient-impooverished soils, where nutrients such as P and N can be co-limiting (e.g., on Australian coastal sand dunes; Laliberté *et al.*, 2012), N transfer has been suggested to be more prevalent (Jalonen, Nygren & Sierra, 2009). In such nutrient-poor soils, plants with contrasting nutrient-acquisition strategies co-occur, although it is uncertain how plants interact belowground and if nutrient transfers play an ecologically relevant role in promoting biodiverse plant communities. If N transfers occur, which nutrient-acquisition strategies promote transfer and what drives the transfer when there is no N₂-fixing plant providing a source of N? There is a need for studies using multiple species mixes to increase our understanding of processes that can promote plant coexistence,

75 maintenance/enhancement of biodiversity and plant community succession (Daehler &
76 Strong, 1996, Drake, Huxel & Hewitt, 1996, Høgh-Jensen, 2006).

77
78 Previous studies showed that N transfer between plants can occur through mycorrhizal
79 networks interconnecting plants or through release of N compounds from N₂-fixing plants
80 leading to uptake by non-N₂-fixing plants (Bethlenfalvay *et al.*, 1991, Frey & Schüepp,
81 1992, Lu *et al.*, 2013, Moyer-Henry *et al.*, 2006). However, there remains controversy
82 about whether transfer occurs preferentially via AM networks, EM networks, or
83 indirectly through the soil; perhaps due to the very small magnitude of transfers that have
84 been measured via direct pathways (Ikram, Jensen & Jakobsen, 1994, Johansen & Jensen,
85 1996). Root exudation can be a mechanism by which plants redistribute nutrients to
86 neighbours, but it is not clear how important it is quantitatively in the context of plants
87 forming mycorrhizal networks that act as dynamic transfer pathways (Haystead *et al.*,
88 1988, Jalonen *et al.*, 2009, Paynel, Murray & Cliquet, 2001). After root exudation, two
89 basic mechanisms may be transporting ions and simple amino acids to neighbouring
90 plants; the first is mass flow that moves ions along with the flow of water and is mainly
91 driven by plant transpiration and the second is diffusion of ions along a concentration
92 gradient without the flow of water. Determining the relative importance of these two
93 mechanisms compared to mycorrhizal-mediated movement is warranted.

94
95 Mycorrhizal fungi can promote transfer of N between plants (He *et al.*, 2006, He *et al.*,
96 2005, He *et al.*, 2004). Greater levels of colonisation have been linked to greater amounts
97 of transferred N (He *et al.*, 2005, Johansen & Jensen, 1996). However, it is unknown
98 which type of mycorrhiza provides a more effective pathway between plants (He *et al.*,
99 2009). Arbuscular mycorrhizal fungi (AMF) have a distinct nutritional ecology and are
100 more heavily dependent on host plants than EM fungi (EMF) are. The AMF have
101 mechanisms for N uptake such as extraradical hyphae regulated by N availability in the
102 soil (Govindarajulu *et al.*, 2005, Hodge, Helgason & Fitter, 2010), while the EMF are
103 efficient N scavengers and have a range of enzymes that degrade nitrogen-rich
104 compounds to access nitrogen from organic matter and various other sources of soil N
105 (Smith & Read, 2008).

The main aim of this study was to quantify N transfer between plants of different nutrient-acquisition strategies, all without N₂-fixation. Furthermore, we aimed to study these transfers under nutrient-poor and nutrient-rich conditions, where plants had the potential to form mycorrhizal networks with or without intermingling of their root systems. We hypothesised that, when roots could not intermingle, plants capable of forming both AM and EM networks would gain the most N from transfers since they could simultaneously transfer N with both types of networks. In the case when roots could intermingle, we expected plants with non-mycorrhizal cluster roots to gain more N than plants with AM and/or EM, based on the very high rootlet density of cluster roots (Shane & Lambers, 2005) and other studies that have shown higher transfer when plant roots are intermingled than when they are not (Jensen, 1996, Xiao, Li & Zhang, 2004). We also compared transfers between fertilised and non-fertilised soils, where we hypothesised that more transfer would occur in nutrient-limited soils driven by mycorrhizal colonisation and N sinks found in shoots of receiving plants.

MATERIALS AND METHODS

Experimental setup

Four Australian native plant species were grown together in novel microcosms in groups of three plants of different species, in 80 microcosm pots that allowed root and hyphal interactions or hyphal-only interactions (Fig. 1). The microcosm pots consisted of three fused PVC tubes, one (the donor compartment) wider than the other two, with the planes of fusion fully open or with a 50 µm mesh (Mesh treatment) that only permitted hyphae to grow between compartments and also restricted cluster roots (Fig. 1). We purposefully made the ‘donor compartment’ wider to allow neighbouring plants to interact and explore beyond their own compartment. Finally, half of the pots were fertilised (Fertilisation treatment) with a slow-release fertiliser for Australian native plants (Osmocote® fertiliser for Australian native plants N:17.9, P:0.8, K:7.3 with trace elements).

The species (*Melaleuca preissiana* Schauer (Myrtaceae), *Eucalyptus marginata* Sm. (Myrtaceae), *Verticordia nitens* (Lindl.) Endl. (Myrtaceae), and *Banksia menziesii* R.Br. (Proteaceae)) are native in the southwest of Western Australia (Western Australian Herbarium, 1998-). *Melaleuca* was selected since it forms dual AM/EM symbioses (Teste *et al.*, 2014), while *Eucalyptus* and *Verticordia* have been reported to be EM and AM, respectively (Brundrett, 2009). *Banksia* was used since it is non-mycorrhizal and uses cluster roots for nutrient acquisition (Lambers *et al.*, 2006). These four plant species were selected because they are widespread and common representatives of the southwest Australian flora, are long-lived and woody perennials with comparable growth rates as seedlings and do not differ in any critically confounding traits (Pate & Bell, 1999). Plants were grown from seed in commercial nurseries (Mucnea Tree Farm or Plant Rite) for eight months. Then, similar-size seedlings were transplanted in the microcosm pots filled with a mix of local Spearwood and Bassendean sand and Perlite (Teste *et al.*, 2014) and grown for seven months.

Donors consisted of all species except *Banksia*, while receivers comprised all four species (Tables 1 and S1). Half of the donor compartments had *Melaleuca* (40 pots) as ‘Donor’ and the remainder of the donor compartments had *Eucalyptus* (20 pots) or *Verticordia* (20 pots). Receiver species had five different arrangements (*Eucalyptus* & *Verticordia*; *Eucalyptus* & *Banksia*; *Verticordia* & *Banksia*; *Melaleuca* & *Verticordia*; *Melaleuca* & *Eucalyptus*). All treatments had four replicate pots for every treatment combination.

Mycorrhizas and root intermingling

Mycorrhizal colonisation of all plant species was quantified at the commencement (on a subset) and end of the experiment following a vinegar and ink clearing and staining protocol outlined in our companion study (Teste *et al.*, 2014). Root intermingling was quantified by severing roots proliferating outside their own compartment followed by a careful washing and extraction of the intact root system of each interacting plant. The

remaining root fragments were considered to make up the intermingling portion; see Teste *et al.* (2014) for more details.

¹⁵N foliar labelling

A leaf-feeding technique using ¹⁵N-nitrate to enrich donors and quantify N transfer was used (Fustec *et al.*, 2010, Lu *et al.*, 2013). The ¹⁵N pulse-labelling was undertaken over a three-week period at the end of the experiment; the end of the ‘chase’ period coincided with the start of the leachate and harvesting (see below). We ¹⁵N labelled only the ‘donor’ plants in the main compartment of the microcosm pots. Donor plants were labelled with two tubes attached to two separate branches using a 3 ml 0.31 M K¹⁵NO₃ solution where 99.8% of N was ¹⁵N. The tops of the tubes were sealed to avoid evaporation into the atmosphere.

¹⁵N analysis

After seven months growth in the glasshouse, seedlings were destructively harvested to measure shoot and root dry biomass, mycorrhizal colonisation, root intermingling, and shoot and root N concentrations (¹⁵N and ¹⁴N). Intermingling roots were not included in the ¹⁵N analyses. Ball-milled dried plant tissue was analysed for stable N isotope composition using an isotope ratio mass spectrometer (Sercon 20-22, Sercon Ltd, Crew, UK) in the West Australian Biogeochemistry Centre at The University of Western Australia. All results were initially expressed as ¹⁵N atom fraction in percent (% ¹⁵N) (Coplen, 2011) following multipoint normalisation (Skrzypek, 2013) based on three international standards (IAEA305A, IAEA305B, IAEA311) distributed by IAEA (International Atomic Energy Agency) and laboratory standards.

¹⁵N-enrichment calculations

With % ¹⁵N data, δ¹⁵N values were calculated from raw ¹⁵N atom fractions as outlined in Coplen (2011) with an air standard N₂ atmospheric gas (3.6764 x 10⁻³) for N. The

obtained $\delta^{15}\text{N}$ raw values were normalised using the standards and method listed above and were used to determine whether seedlings had an excess of ^{15}N above natural abundance levels. To convert $\delta^{15}\text{N}$ to mg of ^{14}N equivalent excess in plants, a modified version of the procedure outlined in Boutton (1991) was used. Sample tissue (shoots and roots separately) $\delta^{15}\text{N}$ values were converted to the absolute isotope ratio (R) and the molar fractional abundance (F):

$$R_{\text{sample}} = \left[\left(\frac{\delta^{15}\text{N}}{1000} \right) + 1 \right] \times R_{\text{standard}} \quad (\text{Eqn. 1})$$

$$F = \frac{R_{\text{sample}}}{R_{\text{sample}} + 1} \quad (\text{Eqn. 2})$$

Then we calculated the mass-based fractional abundance (MF):

$$MF = \frac{F \times 15}{[(F \times 15) + (1 - F) \times 14]} \quad (\text{Eqn. 3})$$

Natural abundance MF values were calculated for the whole plant (n=17 based on all species; *Banksia* (n=3), *Eucalyptus* (n=5), *Melaleuca* (n=6), *Verticordia* (n=3) or for each tissue type (shoot (n=11) and root (n=6) of plants not labelled), for appropriate and subsequent comparisons with enriched species or tissue types, and were subtracted from the sample MF values resulting in a change in MF (ΔMF). Then we calculated excess sample tissue ^{15}N (Excess ^{15}N in mg):

$$\text{Excess } ^{15}\text{N} = \text{tissue N concentration} \times \text{tissue mass} \times \Delta\text{MF} \quad (\text{Eqn. 4})$$

Lastly, we converted to excess ^{14}N equivalent (Excess ^{14}N in mg):

$$\text{Excess } ^{14}\text{N} = \text{excess } ^{15}\text{N} \times \left(\frac{14}{15} \right) \quad (\text{Eqn. 5})$$

We express enrichment of plants as ‘Excess ^{14}N equivalent’ to highlight that the N gains are shown in the common N form that the plant uses, thus making it biologically more relevant.

% N transfer

Nitrogen transfer was quantified as percent of N transferred (% N transfer) from labelled donors to both receiver plants with the equations (1) to (3) presented in He *et al.* (2009) and repeated here:

$$\text{atom}\%^{15}\text{N}_{\text{excess}_{\text{plant}}} = \text{atom}\%^{15}\text{N}_{\text{plant}_{\text{post labelling}}} - \text{atom}\%^{15}\text{N}_{\text{plant}_{\text{background}}} \quad (\text{Eqn. 6})$$

$$^{15}\text{N}_{\text{content}_{\text{plant}}} = \frac{\text{atom}\%^{15}\text{N}_{\text{excess}_{\text{plant}}} \times \text{total N}_{\text{plant}}}{\text{atom}\%^{15}\text{N}_{\text{excess}_{\text{labelled N}}}} \quad (\text{Eqn. 7})$$

$$\% \text{ N transfer} = \frac{^{15}\text{N}_{\text{content}_{\text{receiver}}} \times 100}{(^{15}\text{N}_{\text{content}_{\text{receivers}}} + ^{15}\text{N}_{\text{content}_{\text{donor}}})} \quad (\text{Eqn. 8})$$

In brief, % N transfer is based on the relative % ^{15}N enrichment in plants (donors and receivers) compared to background levels and the total amount of N found in plants at the end of the experiment (He *et al.*, 2009). We calculated % N transfer on a subset of 29 pots where we had % ^{15}N values for donor shoots and roots in conjunction with the two receiver shoots and roots (i.e. six values of % ^{15}N per pot). Absolute N transfer (i.e., net N transfer) could not be calculated since we did not perform a reciprocal labelling experiment; the values presented are therefore on the balance of N transfer into the plants and N release (if occurring).

Leachate analysis

To provide more evidence transfer of nutrients via diffusion and mass flow is more likely than movement via mycorrhizal networks, we collected leachates from pots prior to

harvesting the plants. Pots (all compartments inclusively) were watered to field capacity and then flushed with 2 l of deionised water, similar to a heavy rainfall event. Leachates from the bottom of the pots were collected and frozen. We freeze-dried leachates, then pulverised the remaining powder with a ball-mill and prepared a subsample (10 mg) to determine stable N isotope composition as above.

Data analysis

Statistical analyses were carried out using the R statistical computing and graphics program (R Core Team, 2013). First, the data were analysed using a fixed-effects model with a factorial treatment structure where the factors were mesh (2 levels: with or without), fertiliser (2 levels: with or without), and the ‘donor’ plant (3 levels: *Melaleuca*, *Eucalyptus* or *Verticordia*) with four replicate microcosms ($n = 4$). Equal variance and normality were assessed graphically in R using various plots of residuals (Logan, 2011). When assumptions were not met, we either used a simple transformation (e.g., log) or used generalised least squares to incorporate variance heterogeneity in the statistical model (Pinheiro & Bates, 2000, Zuur *et al.*, 2009). The pooled variance and experimental error degrees of freedom were used to calculate Tukey’s honestly significant difference (TukeyHSD) error bars, 95% confidence intervals (CIs), and effect-size estimates for statistical and ecological inferences (Di Stefano, Fidler & Cumming, 2005). The CIs were accompanied by TukeyHSD bars ($\alpha = 0.05$) and used as visual multiple-mean comparison tests in figures. Associated *P*-values were reported as supplementary evidence, yet TukeyHSD error bars and CIs provided sufficient information needed to detect statistical significance, conduct inferences, determine uncertainty, and suggest ecological importance of the effects (Altman *et al.*, 2000, Cumming, 2008). Finally, we conducted frequency analyses to compare proportion of enriched vs. not enriched with the treatments and regression analyses to suggest mechanisms for the N enrichment.

RESULTS

Based on the ^{15}N levels in unlabelled control plants (natural abundance data), 94 % of donors (100 % of shoots and 89 % of roots) were enriched in ^{15}N at time of harvest (Fig. 2, Table S1). We found significantly greater amounts of enrichment of donor roots in the unfertilised soil (0.595 % ^{15}N) compared with that of fertilised soil (0.402 % ^{15}N) (Fig. 2 and Fig. S1; difference = 0.193 [CI 0.057 to 0.328 % ^{15}N], T-test = 2.88, P -value = 0.0065). Similarly we found significantly greater amounts of enrichment of receiver roots in the unfertilised soil (0.409 % ^{15}N) compared with that of fertilised soil (0.378 % ^{15}N) (Fig. S1; difference = 0.031 [CI 0.008 to 0.053 % ^{15}N], T-test = 2.66, P -value = 0.0094). Furthermore, 77 % of receivers (92 % of shoots and 47 % of roots) were enriched and had an excess ^{14}N equivalent well above zero (Fig. 2). As predicted, fertilisation increased shoot and root biomass of all plant species in both donor and receiver roles (Table 1). Shoot and root N concentrations also followed the expected increase with fertilisation (Table 1). Based on a subset of 29 pots that included all % ^{15}N values for donor and receiver tissues, we calculated that 3.9 % [CI 2.4 to 5.4 %] of N from donor plant N was transferred. Nitrogen transfer in nutrient-poor soils was 2.9 % [CI 1.6 to 4.2 %] and not statistically different from the 4.4 % [CI 2.3 to 6.5 %] measured in fertilised soils (T-test = -1.25, P -value = 0.2130).

Receivers grown in fertilised soil compared to unfertilised soil had greater amounts of excess ^{14}N equivalent (Fig. 3). Overall the mesh treatment did not affect the enrichment found in receivers (Fig. 3). Using frequency analysis, we found that a significantly greater proportion of receivers were enriched in microcosm pots that were not fertilised (92 %) compared to fertilised pots (72 %) (Table 2).

Overall enrichment in receivers showed that *Banksia*, *Eucalyptus*, and *Melaleuca* had greater excess ^{14}N equivalent compared to *Verticordia* (Fig. 4a). We found the same trend, using the treatment combination (fertilisation with mesh) that promoted the greatest amount of enrichment and a similar trend, although not statistically significant (Table S2) with the treatment combination (not fertilised with mesh) that promoted the greatest frequency of enrichment (Figs 4b & 4c). These trends were similar when pots

had no mesh (Fig. S4). Finally, the identity of the donor species did not influence the amount of enrichment in receivers (data not shown).

Receiver ^{14}N enrichment in *Eucalyptus* and *Melaleuca* was positively related to receiver root biomass gain with the mesh treatment (Fig. S5). There was an interaction between the fertilisation treatment and the root biomass gain for the overall receiver ^{14}N enrichment response (data not shown), so we suspect nutrient availability was confounding. Similarly, overall we found a positive relationship between receiver root ^{14}N enrichment and receiver root N concentration only for *Eucalyptus* and *Melaleuca* (Fig. S2). More importantly, the possibility that low ^{14}N enrichment levels in *Verticordia* were due to the small root size of this species was not supported: there was no significant relationship between root size and ^{14}N enrichment (Fig. S3). Finally, we also noted positive relationships for *Eucalyptus* and *Melaleuca* between receivers shoot enrichment and EMF colonisation levels with mesh (Fig. 5). Transfer was not related to overall mycorrhizal colonisation or root intermingling (data not shown although see Teste *et al.* (2014) for root intermingling data).

Mycorrhizal colonisation levels in receiver plants were similar in fertilised and unfertilised soil (data not shown, T-test = 0.20, P -value = 0.8399). *Melaleuca preissiana* seedlings were evenly colonised by both AMF and EMF (Table 3, T-test = -8.1, P -value < 0.0001). The *Eucalyptus* seedlings had some AMF colonisation, but EMF was considerably more abundant (Table 3). Finally *Verticordia* was only colonised by AMF, whereas *Banksia* was non-mycorrhizal, as expected (Table 3).

The ^{15}N composition of a subsample of pot leachates revealed that most ^{15}N tracer remained in plant tissue at time of harvest (Fig. 6). However, one leachate sample originating from an unfertilised pot with no mesh hosting *Melaleuca* as donor and *Banksia* and *Verticordia* as receiver plants was enriched (Fig. 6).

DISCUSSION

Nutrient-acquisition strategies and N transfer

Transfer of N, in the absence of N₂ fixation, occurred between plants with contrasting nutrient-acquisition strategies, including non- mycorrhizal plants. We estimated that overall, 4 % of N from donor plant N was transferred to receivers. Transfer was found in both nutrient-poor and fertilised soils, where plants forming cluster roots and dual mycorrhizal strategies were more enriched. Overall, *Banksia*, *Eucalyptus*, and *Melaleuca* received the greatest amount of transferred N. Being non-mycorrhizal, *Banksia*'s ability to receive N from neighbouring plants may be related to its efficient nutrient-mining cluster roots (Shane & Lambers, 2005). *Melaleuca*'s ability to receive N is likely facilitated by its symbioses with both AMF and EMF. *Verticordia* that formed associations only with AMF, was the least enriched, therefore it acted as a poor receiver of transferred N, while *Eucalyptus* had modest amounts of enrichment and was dominated by EMF. These findings provide evidence that AMF and their networks did not appear proficient at transferring N. Although plant size would be expected to influence N transfer, it did not relate to enrichment levels in any of the regression analyses.

Transfer pathways

The observed patterns of N transfer between plants suggest that different pathways were operating. We provide evidence for N transfer predominantly facilitated via EMF and mass flow or diffusion. We cannot discern if a direct ectomycorrhizal network pathway was more important than an 'ectomycorrhizal-mediated' pathway (Simard & Durall, 2004, Teste *et al.*, 2010); in either case, it appears that ectomycorrhizal hyphae played a more dominant role than AMF.

Diffusion of N in soil after root exudation, root hair and EMF hyphae senescence (Fusseder, 1987, McElgunn & Harrison, 1969), followed by mass flow or diffusion, is a transfer pathway that likely occurred, since non-mycorrhizal *Banksia* plants were enriched. Short-term glasshouse experiments with relatively slow-growing woody plants

are expected to only have root-hair decomposition, root exudation, and direct transfer via mycorrhizal networks as nutrient-transfer pathways. Decomposing roots have been shown a source of N for AMF forming an active network and allowing the transfer of N and P between plants (Johansen & Jensen, 1996), but this mechanism is likely underestimated in experiments of short duration using ^{15}N as a nutrient tracer.

Some studies have shown that N transfer occurred mainly by root exudation and to a lesser extent via mycorrhizal networks (Haystead *et al.*, 1988, He *et al.*, 2005, Jalonen *et al.*, 2009), while others have shown the importance of a mycorrhizal network pathway in N transfer between plants (Arnebrant *et al.*, 1993, He, Critchley & Bledsoe, 2003, Johansen & Jensen, 1996). Our study refines the relative importance of transfer via mycorrhizal networks versus other pathways, since we used a strictly-non-mycorrhizal receiver plant species and a strictly-AM species in conjunction with a mesh treatment. Transfer appeared greater in ectomycorrhizal networks compared to arbuscular mycorrhizal networks. In the case of pots without mesh, where roots could intermingle, we did not find more N enrichment in plants with the cluster-root strategy than in mycorrhizal plants, thus not supporting our hypothesis on root intermingling effects.

Our findings make an important contribution to answering the ‘old question’ of direct and/or indirect transfer pathways (Høgh-Jensen, 2006). All mycorrhizal donor and receiver plants were well colonised by AMF, EMF or both. Since we found high enrichment in non-mycorrhizal *Banksia* and ectomycorrhiza-forming species when plants were separated by a mesh barrier, diffusion, mass flow and ectomycorrhizal networks were more important than AM networks. The results of the ^{15}N composition of the leachates should be interpreted cautiously. However, since enriched leachate was found originating from a pot that harboured *Melaleuca* as donor and *Banksia* and *Verticordia* as receiver plants, where only AM networks could form, there is evidence that AM networks are perhaps not functioning extensively in N transfer between plants.

Source-sink relationships

The type and mycorrhizal colonisation level of the three mycorrhizal donor plant species (*Eucalyptus*, *Melaleuca*, *Verticordia*) did not influence the enrichment levels in the receiver plants; there was no evidence that any donor species acted as a greater source of N. Donor shoots were highly enriched with ^{15}N , while most donor roots (89 %) were enriched. Donors took up ^{15}N efficiently during the foliar-feeding pulse period since the most enriched donor had 19.3 mg of ^{15}N , close to the maximum theoretical limit of 27.5 mg of ^{15}N . Some of the ^{15}N was then transferred to their roots, and eventually into soil, EMF- and AMF-extraradical hyphae, since unlabelled neighbouring receiver plants ended up considerably enriched in ^{15}N . Previous studies have used plant assemblies that enhanced N transfer by choosing plants that represented a clear source and sink for N (He *et al.*, 2003, He *et al.*, 2009). Typically, the donor plant was a N_2 -fixing species that acted as a source when growing beside a non- N_2 -fixing plant species (He *et al.*, 2009). Here, we demonstrate N transfer without a N_2 -fixing species and without any obvious N gradient between donors and receiver plants. There are less than a ‘hand full’ of reports of N transfer from non- N_2 -fixing plants to N_2 -fixing plants or between two non- N_2 -fixing plants, but amounts were low compared to our findings (Ek, Andersson & Söderström, 1996, Li *et al.*, 2009, Moyer-Henry *et al.*, 2006, Shen & Chu, 2004). We propose that for species that evolved in nutrient-poor soils, in particular when P is the key limiting nutrient, sharing of N is a consequence of plant interactions, but the magnitude appears partly driven by a plant’s nutrient-acquisition strategy. We also propose that we are the first to provide some evidence for what can best be referred to as maladaptation in the studied plants. These plants have invested heavily in traits to use P efficiently; they operate at very low ribosomal RNA levels and, as a consequence, at low protein levels (Sulpice *et al.*, 2014), without a strong evolutionary pressure to retain N.

The transfer that we report appeared driven by source-sink relationships, where growth demands tended to increase the amount transferred. We noted that more ^{15}N stayed in donors in the nutrient-poor soils compared to the donors growing in the fertilised soil. This suggests that transfer of N was equally driven by N source strength in both situations (i.e., donor N concentration relative to receiver N concentration). In other studies, donor source strength alone was the main driver of N transfer (Ek *et al.*, 1996, Ekblad & Huss-

Danell, 1995, Teste *et al.*, 2009). Interestingly, the level of EM colonisation was related to an increase in the N enrichment in receivers, contrary to other systems (Johansen & Jensen, 1996, Kurppa, Leblanc & Nygren, 2010). These positive relationships were found stronger for receiver species capable of forming ectomycorrhizal networks. A greater density of extraradical EMF hyphae in soil might be more effective at gathering N and allow direct transfer of N between plants compared to an AM network that is typically less extensive (Leake *et al.*, 2004, Simard *et al.*, 1997). Fundamentally, EMF form more complex transport structures (e.g., rhizomorphs) for long-distance exploration of N patches or formation of interplant networks (Agerer, 2001). Rhizomorphs can produce rapid and efficient N transfer, because the inner medulla of the rhizomorph has hollow vessel hyphae up to 15 μm in diameter with few septal pores (Brownlee *et al.*, 1983, Duddridge, Malibari & Read, 1980). Furthermore, AM fungi do not appear to have the enzymatic capabilities to break down organic N lost from donor plants (Hodge *et al.*, 2010), thus reducing the relative importance of AM networks compared to EMF networks in N transfer. Finally, cluster roots are expected to efficiently capture N via interception of mass flow and organic N sources and appeared equally effective to gain N without mycorrhizas.

N transfer in nutrient-poor soils

We estimated that 2.9 % of N was transferred between plants in nutrient-poor soils, and 4.4 % in fertilised soils. Furthermore, positive relationships between receiver enrichment and indicators driving the N transfer were maintained between fertilised and unfertilised pots. This suggests that sink strength for N transfer was not influenced by soil fertility. However, the soil fertility did appear to influence the source strength of donors and this may have partly regulated N transfer. Receiver net root biomass gains may have confounded the effects of soil fertility. Nevertheless, our findings point to the nutritional needs of faster-growing receivers and the level of EMF colonisation as the main drivers for the amount of N transfer, regardless of the soil fertility. Due to the present relatively high levels of N transfer, our results also agree with those of Eissenstat (1990), who suggested N transfer of this magnitude likely impacts plant nutrition in nutrient-poor soils.

Of note, we found a greater frequency of N enrichment in receivers that occurred under nutrient-poor compared to fertilised conditions. In N-limited pastures, pioneer plant communities, or ecosystems with impoverished soils, N transfer is potentially more important (Haystead *et al.*, 1988, Høgh-Jensen, 2006, Jalonen *et al.*, 2009). It has been proposed that transfer of N between plants is ecologically relevant if the mineralisation of N can be partly by-passed by reducing competition for N with soil microorganisms (Jalonen *et al.*, 2009). Our study only partly supports this hypothesis: while there was a 20 % higher occurrence of enrichment in nutrient-poor soils, there still was enrichment in 72 % of the fertilised receivers.

Concluding remarks

We demonstrate that transfer of N between plants with contrasting nutrient-acquisition strategies occurs, in the absence of N₂-fixation, at both low and high soil nutrient levels, without the need for root contact (intermingling). The magnitude of N transfer was relatively high (4 % of donor plant N) given the lack of any N₂-fixing donors. Most N-enriched were plants forming cluster roots and plants capable of forming dual AMF-EMF associations, compared with plants only forming symbioses with AMF. Efficient N scavenging via ectomycorrhizas, likely through ectomycorrhizal networks, and cluster roots, facilitated more of the N transfer than AM networks did.

Future tracer studies using ¹⁵N and ³³P could measure diffusion and transpirationally-induced mass flow using microdialysis methods (Oyewole, Inselsbacher & Nasholm, 2013) in conjunction with the use of non-mycorrhizal plants, hyphal barriers (Teste *et al.*, 2006), and leachates as we did, to better quantify transfer of nutrients via mass flow and diffusion. Also, mycorrhizal networks may indeed have other ecologically important roles, such as nutrient retention (van der Heijden, 2010, van der Heijden & Horton, 2009) that have not been appreciated in plant community ecology and in the context of ecosystem services. Although we did not assess if there was any direct benefit of one-way N transfer to receiver plants, our findings suggest that any transfer of nutrients in

nutrient-poor soils has the potential to increase growth of neighbours, plant fitness, and ultimately ecosystem productivity.

The relatively high levels of transfer in the absence of N₂-fixing plants and compared to other studies (Teste *et al.*, 2009) may well be indicative of plant communities limited by P without any proficient mechanisms for retention of N, which is not a key limiting nutrient (Hayes *et al.*, 2014). This study suggests that plant communities evolved and adapted to soils very low in P may have partly lost their ability to retain other important nutrients such as N, an example of maladaptation in plants; it would be interesting to follow up and contrast with a study measuring P transfer. We hypothesise that in plant communities where N and P are co-limiting, liberal movement of N between plants could equalise competitive effects between species. Therefore, we propose that exchanges of nutrients between plants in nutrient-poor soils can play important ecological roles in promoting plant coexistence and sustaining biodiversity. Finally, nutrient exchanges of the type found may represent a mechanism for aggregating more plant species into an area than predicted under competition theory. Indeed, the extraordinarily nutrient impoverished yet biodiverse southwest Australian flora where this study was undertaken may rely on nutrient exchanges as one of the means that helps maintain biodiverse yet ecologically resilient plant communities. For restoration of such ecosystems, this study indicates that it may be advantageous to design restored soil profiles that are low in nutrients to ensure plant co-existence is promoted.

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777 **Tables and figures**

778

779 **Tables**

780

781 **Table 1** Characteristics of the plant species used as donors (D) and receivers (R) at the end of the
782 experiment. Values are means with 95 % confidence intervals.

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Characteristic	Species	Role	Treatment	
			Not fertilised	Fertilised
Shoot dry mass (g)	<i>Melaleuca preissiana</i>	D	4.9 (3.5 to 6.2)	29.5 (24.9 to 34.1)
		R	3.5 (2.7 to 4.3)	26.0 (18.1 to 34.0)
	<i>Eucalyptus marginata</i>	D	9.0 (3.8 to 14.1)	39.8 (26.4 to 53.3)
		R	4.8 (4.1 to 5.4)	24.8 (19.5 to 30.0)
	<i>Verticordia nitens</i>	D	4.0 (1.6 to 6.3)	8.4 (4.6 to 12.3)
		R	3.4 (2.8 to 4.1)	7.9 (6.6 to 9.2)
	<i>Banksia menziesii</i>	R	8.3 (7.0 to 9.6)	28.4 (24.9 to 31.8)
Root dry mass (g)	<i>Melaleuca preissiana</i>	D	2.8 (2.3 to 3.4)	10.3 (7.7 to 13.0)
		R	2.2 (1.6 to 2.7)	9.1 (5.0 to 13.2)
	<i>Eucalyptus marginata</i>	D	6.4 (3.5 to 9.3)	12.2 (7.1 to 17.3)
		R	3.6 (3.0 to 4.1)	10.4 (8.4 to 12.4)
	<i>Verticordia nitens</i>	D	2.9 (1.7 to 4.1)	5.2 (3.4 to 6.9)
		R	1.7 (1.3 to 2.1)	3.5 (2.6 to 4.4)
	<i>Banksia menziesii</i>	R	11.5 (9.3 to 13.8)	17.6 (11.5 to 23.7)
Shoot N concentration (mg g ⁻¹)	<i>Melaleuca preissiana</i>	D	9.3 (8.3 to 10.4)	14.2 (8.3 to 10.4)
		R	11.5 (9.9 to 13.1)	17.9 (16.0 to 19.7)
	<i>Eucalyptus marginata</i>	D	11.9 (10.2 to 13.6)	15.1 (13.2 to 16.9)
		R	8.1 (7.2 to 9.0)	12.7 (11.6 to 13.9)
	<i>Verticordia nitens</i>	D	11.0 (8.8 to 13.1)	15.8 (13.6 to 18.1)
		R	11.9 (10.6 to 13.3)	15.8 (14.4 to 17.3)

	<i>Banksia menziesii</i>	R	6.6 (6.0 to 7.2)	12.4 (11.5 to 13.2)
Root N concentration (mg g ⁻¹)	<i>Melaleuca preissiana</i>	D	6.1 (5.5 to 6.7)	13.0 (12.0 to 14.0)
		R	7.6 (6.6 to 8.6)	14.9 (13.7 to 16.0)
	<i>Eucalyptus marginata</i>	D	6.4 (5.6 to 7.1)	13.6 (12.0 to 15.3)
		R	4.4 (4.1 to 4.8)	10.1 (8.9 to 11.3)
	<i>Verticordia nitens</i>	D	6.5 (5.5 to 7.4)	11.5 (10.1 to 12.9)
		R	5.9 (5.4 to 6.4)	10.6 (9.0 to 12.3)
	<i>Banksia menziesii</i>	R	4.0 (3.4 to 4.7)	12.8 (11.1 to 14.5)

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786 **Table 2** Frequency analyses of receiver excess ¹⁴N equivalent with the fertilisation and mesh
787 treatments. Receivers were categorised as enriched based on 95 % confidence intervals for control
788 plants (natural abundance) data. Pearson’s Chi-squared test (df = 1) confirmed with Log likelihood
789 ratio test (G-test) of independence with ‘Williams’ correction.

Treatment	Not enriched	Enriched	χ^2 test	<i>P</i>	G-test	<i>P</i>
Not fertilised	12	136	19.82	<0.0001	20.45	<0.0001
Fertilised	40	102				
Mesh	31	118	1.72	0.1896	1.71	0.1905
No mesh	21	120				

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792 **Table 3** Overall mycorrhizal colonisation of the donor and receiver roots at the end of the experiment.
793 Values are means with 95 % confidence intervals (CI).

Plant species	Role	Colonisation (%)	
		Arbuscular	Ectomycorrhizal
<i>Melaleuca preissiana</i>	Donor	14.6 (9.8 to 19.3)	25.3 (17.2 to 33.4)
	Receiver	12.9 (6.9 to 18.9)	32.5 (23.5 to 41.5)
<i>Eucalyptus marginata</i>	Donor	6.7 (0.6 to 12.8)	24.4 (12.9 to 35.9)
	Receiver	5.6 (3.7 to 7.5)	36.6 (29.1 to 44.1)
<i>Verticordia nitens</i>	Donor	26.7 (14.7 to 38.6)	0 (0 to 0)
	Receiver	27.5 (21.6 to 33.4)	0 (0 to 0)
<i>Banksia menziesii</i>	Receiver	0 (0 to 0)	0 (0 to 0)

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801 **Fig. 1**
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Fig. 2

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Fig. 4

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Fig. 5

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Fig. 6

826 **Figure legends**

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828 **Figure 1** Microcosm pots (35 cm deep) viewed from above and from the side, showing the three fused
829 compartments, separated with or without mesh. Plants grown in the larger compartment (diameter = 12
830 cm) were labelled with a ^{15}N solution (see Methods) and were one of three species (Mp: *Melaleuca*
831 *preissiana*, Em: *Eucalyptus marginata*, Vn: *Verticordia nitens*). Species growing in smaller fused
832 compartments (diameter = 9 cm) were also one of these three species (but not the donor species) or
833 *Banksia menziesii* (Bm).

834

835 **Figure 2** Nitrogen enrichment of donor (as % ^{15}N) and receiver (as excess ^{14}N equivalent) plants.
836 Enrichment was based on 95 % CIs around unlabelled control plants (natural abundance) data. Natural
837 abundance values were not significantly different across tissue type and species (Table S1). Receiver
838 species excess ^{14}N raw data are presented to show the enrichment overall and to easily compare to the
839 ‘natural abundance’ (background) levels as marked by the dotted line. The associated bar graph shown
840 in Figure 3 is presented to show the effects of the main treatments on receiver ^{14}N equivalent. Mp:
841 *Melaleuca preissiana*, Em: *Eucalyptus marginata*, Vn: *Verticordia nitens*, and Bm: *Banksia menziesii*.

842

843 **Figure 3** Amount of N enrichment in receiver plants subject to the mesh and fertilisation treatments.
844 Values are back-transformed log means with 95% confidence intervals (dotted lines) with Tukey’s
845 HSD error bars (solid). Statistically different means can be assessed visually; they exist when two
846 Tukey’s HSD error bars do not overlap. Only the effect of fertilisation was statistically significant (P -
847 value < 0.0001; see Table S2 for the analysis of variance table).

848

849 **Figure 4.** N enrichment levels between receiver plant species. a) Overall, b) Planned comparisons of N
850 enrichment levels between receiver plant species for the fertilisation and with mesh treatment
851 combination, c) Planned comparisons of N enrichment levels between receiver plant species for the
852 unfertilised and with mesh treatment combination. Values are back-transformed log means with 95%
853 confidence intervals (dotted lines) with Tukey’s HSD error bars (solid). Statistically different means
854 can be assessed visually; they exist when two Tukey’s HSD error bars do not overlap. Analysis of
855 variance tables and Tukey multiple comparison tests and associated P -values can be found in Table S2.

856

857 **Figure 5** Regression analyses of a) *Eucalyptus* and b) *Melaleuca* receiver species' enriched shoot
858 excess ^{14}N -equivalent with mean EMF colonisation of the two receiver plants. Linear regression lines
859 (Em: $Y = 0 + 0.47X$, $R^2 = 0.22$, $P = 0.02$; Mp: $Y = 0 + 0.95X$, $R^2 = 0.41$, $P = 0.01$) were fitted with
860 95% confidence intervals (gray ribbon). Mp: *Melaleuca preissiana*, Em: *Eucalyptus marginata*.

861

862 **Figure 6** Nitrogen enrichment of leachates (as % ^{15}N) derived at the end of the experiment (prior to
863 destructively harvesting plants) from the microcosm pots with *Melaleuca preissiana* as focal donor
864 plant. Enriched leachates are seen above the 95% confidence band. The mean (solid line) and 95 %
865 CIs (dotted lines) were based on unlabelled control pots (natural abundance) data. Em: *Eucalyptus*
866 *marginata*, Vn: *Verticordia nitens*, and Bm: *Banksia menziesii*.

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868

869 **SUPPORTING INFORMATION**

870

871 **Table S1** Donor and unlabelled control plant % ¹⁵N at the end of the experiment. Values are means
872 with range in square brackets or 95 % confidence intervals in parentheses. Unlabelled control plant
873 tissue type did not differ significantly (T-test = 1.23, df = 6, *P*-value = 0.2673).

874

Plant species	% ¹⁵ N	
	Donor shoot	Control
<i>Banksia menziesii</i>	not a donor	0.3694 (0.3666 to 0.3722)
<i>Eucalyptus marginata</i>	4.583 [0.958 to 20.483]	0.3658 (0.3636 to 0.3680)
<i>Melaleuca preissiana</i>	1.857 [0.443 to 5.639]	0.3668 (0.3648 to 0.3687)
<i>Verticordia nitens</i>	4.942 [1.033 to 11.684]	0.3691 (0.3663 to 0.3719)

875

876

877 **Table S2** Analysis of variance (ANOVA) tables for the responses graphically represented in Fig. 3 &
878 Fig. 4. Output of the full statistical model including the intercept is shown and associated Tukey HSD
879 tests for Fig. 4. Mp: *Melaleuca preissiana*, Em: *Eucalyptus marginata*, Vn: *Verticordia nitens*, and
880 Bm: *Banksia menziesii*.

881
882 ANOVA Table (Type III tests) for Fig. 3
883 Response: log(receiver excess ¹⁴N equivalent (mg))

Source of variation	Sum of squares	df	F-value	P-value
(Intercept)	2485.52	1	616.6	< 2.2e-16
Mesh	12.85	1	3.1877	0.0754
Fertilisation	72.59	1	18.0069	3.1e-05
Mesh:Fertilisation	6.77	1	1.6802	0.1961
Residuals	999.69	248		

884
885 ANOVA Table (Type III tests) for Fig. 4 a) Overall all mesh treatments
886 Response: log(excess ¹⁴N equivalent (mg))

Source of variation	Sum of squares	df	F-value	P-value
(Intercept)	2918.49	1	640.9738	< 2.2e-16
Species	59.81	3	4.3783	0.005784
Residuals	555.49	122		

887 Tukey multiple comparisons of “Species” means

Comparison	Mean difference	Adj. P-value
Em-Bm	-0.3049190	0.9553929
Mp-Bm	-0.1505587	0.9955892
Vn-Bm	-1.6461887	0.0315902
Mp-Em	0.1543602	0.9918839
Vn-Em	-1.3412697	0.0263580
Vn-Mp	-1.4956300	0.0331260

895
896 ANOVA Table (Type III tests) for Fig. 4 b) Fertilised with mesh
897 Response: log(excess ¹⁴N equivalent (mg))

Source of variation	Sum of squares	df	F-value	P-value
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(Intercept)	773.20	1	152.75	< 2.2e-16
Species	87.56	3	5.7657	0.001818
Residuals	253.09	50		

898 Tukey multiple comparisons of “Species” means

899	<u>Comparison</u>	<u>Mean difference</u>	<u>Adj. <i>P</i>-value</u>
900	Em-Bm	-0.2988776	0.9895610
901	Mp-Bm	0.1691291	0.9985634
902	Vn-Bm	-2.7406831	0.0281323
903	Mp-Em	0.4680067	0.9534113
904	Vn-Em	-2.4418055	0.0107517
905	Vn-Mp	-2.9098122	0.0091045

906

907 ANOVA Table (Type III tests)

908 Response: log(excess ¹⁴N equivalent (mg)) Fig. 4 c) Not fertilised with mesh

Source of variation	Sum of squares	df	F-value	<i>P</i> -value
(Intercept)	2211.28	1	771.19	< 2.2e-16
Species	7.08	3	0.8235	0.4854
Residuals	194.98	68		

909

910

Figure S1 Raw nitrogen enrichment (as % ^{15}N) for donor and receiver plants. Enrichment was based on 95 % CIs around unlabelled control plants (natural abundance) data. Natural abundance values were not significantly different across tissue type and species (Table S1). Mp: *Melaleuca preissiana*, Em: *Eucalyptus marginata*, Vn: *Verticordia nitens*, and Bm: *Banksia menziesii*.

919

920 **Figure S2** Regression analysis of receiver's enriched root excess ^{14}N -equivalent with receiver's root N
921 concentrations. Linear regression lines (overall: $Y = -0.001 + 0.001X$, $R^2 = 0.07$, $P = 0.001$) were
922 fitted with 95% confidence intervals (grey ribbon).

923

925

926 **Figure S3** Regression analyses of the four receiver species' enriched whole plant excess ¹⁴N-
927 equivalent with their dry root biomass values. Linear regression lines (overall: $Y = 0.003 + 0.0007X$,
928 $R^2 = 0.06$, $P < 0.001$) were fitted with 95% confidence intervals (gray ribbon). Mp: *Melaleuca*
929 *preissiana*, Em: *Eucalyptus marginata*, Vn: *Verticordia nitens*, and Bm: *Banksia menziesii*.

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934 **Figure S4** N enrichment levels between receiver plant species. a) Planned comparisons of N
935 enrichment levels between receiver plant species for the fertilisation and without mesh treatment
936 combination, b) Planned comparisons of N enrichment levels between receiver plant species for the
937 unfertilised and without mesh treatment combination. Values are back-transformed log means with
938 95% confidence intervals (dotted lines) with Tukey's HSD error bars (solid). Statistically different
939 means can be assessed visually; they exist when two Tukey's HSD error bars do not overlap.

940

Figure S5 Regression analyses of the four receiver species' enriched whole plant excess ^{14}N -equivalent with their net root biomass gain (final root biomass minus initial root biomass at start of experiment). Similar relationships were found for net shoot biomass gain (data not shown). Linear regression lines (overall: $Y = 0.0005 + 0.0006X$, $R^2 = 0.05$, $P < 0.001$) were fitted with 95% confidence intervals (gray ribbon). Mp: *Melaleuca preissiana*, Em: *Eucalyptus marginata*, Vn: *Verticordia nitens*, and Bm: *Banksia menziesii*.