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Assessing nitrogen fixation in mixed- and single-species plantations of *Eucalyptus globulus* and *Acacia mearnsii*

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Summary Mixtures of *Eucalyptus globulus* Labill. and *Acacia mearnsii* de Wildeman are twice as productive as *E. globulus* monocultures growing on the same site in East Gippsland, Victoria, Australia, possibly because of increased nitrogen (N) availability owing to N_2 fixation by *A. mearnsii*. To investigate whether N_2 fixation by *A. mearnsii* could account for the mixed-species growth responses, we assessed N_2 fixation by the accretion method and the ^{15}N natural abundance method. Nitrogen gained by *E. globulus* and *A. mearnsii* mixtures and monocultures was calculated by the accretion method with plant and soil samples collected 10 years after plantation establishment. Nitrogen in biomass and soil confirmed that *A. mearnsii* influenced N dynamics. Assuming that the differences in soil, forest floor litter and biomass N of plots containing *A. mearnsii* compared with *E. globulus* monocultures were due to N_2 fixation, the 10-year annual mean rates of N_2 fixation were 38 and 86 kg ha^{-1} year^{-1} in 1:1 mixtures and *A. mearnsii* monocultures, respectively. Nitrogen fixation by *A. mearnsii* could not be quantified on the basis of the natural abundance of ^{15}N because such factors as mycorrhization type and fractionation of N isotopes during N cycling within the plant confounded the effect of the N source on the N isotopic signature of plants. This study shows that *A. mearnsii* fixed significant quantities of N_2 when mixed with *E. globulus*. A decline in $\delta^{15}N$ values of *E. globulus* and *A. mearnsii* with time, from 2 to 10 years, is further evidence that N_2 was fixed and cycled through the stands. The increased aboveground biomass production of *E. globulus* trees in mixtures when compared with monocultures can be attributed to increases in N availability.

Keywords: accretion, mycorrhizae, ^{15}N natural abundance.

Introduction

Growth of non-N_2-fixing trees can increase when grown in mixture with an N_2-fixing species. Nitrogen input rates from symbiotic N_2 fixation in tree systems have been estimated to be as high as 200 kg ha^{-1} year^{-1}, or about 10% to nearly 100% of the total N used by the host plant (Binkley 1992, Binkley and Giardina 1997, Khanna 1998, Fisher and Binkley 2000, May and Attiwill 2003). Plants that are incapable of fixing N_2 can use N acquired by associated N_2-fixing plants when dead plant tissues and microbial cells decompose and cycle through the ecosystem (Van Kessel et al. 1994, Stock et al. 1995, Fisher and Binkley 2000, May and Attiwill 2003). Nitrogen transfer between species that fix N_2 and those that do not may also occur via root exudation or connections between root systems when both species form symbioses with the same mycorrhiza (He et al. 2003). For example, several mixtures of *Eucalyptus* (non-N_2 fixing) and *Acacia* (N_2 fixing) were found to be more productive than monocultures of either species (Forrester et al. 2006). *Eucalyptus* spp. (Gardina and Malajczuk 1988, Bellei et al. 1992, Adjoud-Sadadou and Halli-Hargas 2000) and *Acacia* spp. (McGee 1986, Bellgard 1991, Brundrett and Abbott 1991, Reddell and Milnes 1992) can both form symbioses with arbuscular mycorrhizal fungi, and so N may have been transferred between species via mycorrhizal connections. However, there is no evidence of such mycorrhizal connections occurring in mixtures of eucalypts and acacias. In addition, because N_2-fixing plants can rely heavily on fixed N_2, more soil N may be available to associated non-N_2-fixing trees before the fixed N_2 is cycled and transferred to the non-N_2-fixing trees.

The time it takes for the N fixed by N_2-fixing species to benefit associated non-N_2-fixing species has received little attention. Earlier work in the plots studied here found that individu-

als of *Eucalyptus globulus* subsp. *pseudoglobulus* (Naudin ex Maiden) J.B.Kirkp. planted with *Acacia mearnsii* de Wildeman were, from as early as 25 months of age, taller than individuals grown in monoculture, and *E. globulus* in mixture had higher N concentrations in senescent foliage and fine roots compared with monocultures at 25 and 31 months of age, respectively (Khanna 1997). In addition, in-situ soil mineralization rates were about 28 and 53 kg ha^{-1} $year^{-1}$ in 2-year-old *E. globulus* monocultures and 1:1 mixtures, respectively. In mixtures of *Eucalyptus* × *robusta* J.E. Smith and *Casuarina equisetifolia* J.R & G. or *Leucaena leucocephala* (Lam.) de Wit, Parrotta et al. (1996) found that *E.* × *robusta* individuals were taking up nitrogen fixed by both N_2-fixing species after two years. This was demonstrated by the ^{15}N labeling method. Based on ^{15}N natural abundance, Van Kessel et al. (1994) found that, after 1 year, the $\delta^{15}N$ of the non-leguminous understory of a *L. leucocephala* plantation was about 7.3‰ compared with less than 4‰ for *L. leucocephala,* but after 4 and 6 years, $\delta^{15}N$ of both the understory and *L. leucocephala* was about 1‰, leading Van Kessel et al. (1994) to conclude that the understory relied as much on previously fixed N_2 as *L. leucocephala.* Similarly, in mixtures of the N_2-fixing species *Sesbania sesban* (L.) and the non-N_2-fixing species *Senna spectabilis* (D.C.) Irwin and Barneby, *Eucalyptus saligna* (Sm.) or *Grevillea robusta* (A. Cunn.), Ståhl et al. (2005), using the ^{15}N labeling method, found that $\delta^{15}N$ values declined in plant material of each species by age 12 months.

Previous studies have shown that mixed stands of *E. globulus* and the N_2-fixing *A. mearnsii* are significantly more productive, as assessed by stem volume or aboveground biomass, than *E. globulus* monocultures at ages three (Khanna 1997), six (Bauhus et al. 2000), nine (Bauhus et al. 2004) and 11 years (Forrester et al. 2004). Increased N availability due to nitrogen fixation by *A. mearnsii* leading to an increase in N availability is likely the major factor responsible for the enhanced growth observed in these mixtures. The aim of this study was to provide estimates of N_2 fixed by *A. mearnsii*, and to assess how fixed N_2 is cycled through the plant–soil system and transferred to the eucalypts in the mixed species plots.

Materials and methods

Site characteristics

The experimental site is situated 5 km southeast of Cann River in East Gippsland, Victoria, Australia (37°35′ S, 149°10′ E). Dry sclerophyll forest dominated by *Eucalyptus sieberi* L. Johnson was cleared in 1991 and the trial was established in 1992. The soil is a yellow Podzol with a high coarse sand content (Stace et al. 1968). Initial soil N concentration was 1.10 g kg^{-1} (0–5-cm layer) and Bray I-P (Bray and Kurtz 1945) concentration was 1.6 mg kg^{-1} (0–5-cm layer). Mean annual rainfall at Cann River (1951–1973) is 1009 mm, evenly distributed throughout the year. Mean daily minimum and maximum temperatures are 7.8 and 20.5 °C, respectively.

Experimental design

Eucalyptus globulus subsp. *pseudoglobulus* and *A. mearnsii* were planted in monocultures and in mixed-species plots that contained 50% *E. globulus* + 50% *A. mearnsii*. Trees were planted at 3 × 3.3-m spacing in 23 × 28-m plots including a surrounding row of buffer trees (35 trees per plot, excluding buffer trees). Plots were arranged in a randomized block design consisting of four replicate blocks. In the mixed-species plots, each row contained both species planted alternately, resulting in a checker board arrangement of species within the plot. *Eucalyptus globulus* and *A. mearnsii* seedlings were planted in early July and October 1992, respectively. In November 1992, 25 kg P ha^{-1} superphosphate was applied to each plot. More details about the site and plantation establishment are provided by Khanna (1997) and Bauhus et al. (2000). Tree sizes and stand characteristics at age 11 years are shown in Table 1. Additional details are provided in Forrester et al. (2004). No root barriers were used and sample trees were located near the plot centers. It was assumed that the bulk of fine roots of a given tree would generally not extend beyond those of its neighbors (Ammer and Wagner 2002), and thus that sampled trees did not use resources (such as fixed N) from neighboring plots. However, this assumption was not tested so we cannot rule out the possibility that temporal changes in *E. globulus* foliar $\delta^{15}N$, or differences in foliar $\delta^{15}N$ between treatments, may have been influenced by the growth of *E. globulus* roots into plots containing *A. mearnsii* or *A. mearnsii* roots growing into *E. globulus* monocultures.

Estimation of N_2 fixation

Nitrogen fixation was estimated by the N accretion method (Silvester 1983) by comparing the N content of soil, litter and biomass in monocultures and mixtures of *A. mearnsii* and *E. globulus* at age ten years. We also estimated N_2 fixation by

Table 1. Stand characteristics for *Eucalyptus globulus* and *Acacia mearnsii* in mixed (50% of each species) and monoculture plots at age 11 years. Data are means for four 23 × 28 m plots in a randomized block design in which trees are spaced at 3 × 3.3 m. Standard deviations of means are given in parentheses.

Parameter	*E. globulus*	Mixture		*A. mearnsii*
		E. globulus	*A. mearnsii*	
Height (m)	15.6 (1.1)	17.7 (0.9)	11.5 (0.2)	11.2 (0.1)
Diameter (cm)	11.9 (1.5)	14.7 (1.4)	15.9 (0.5)	13.2 (0.3)
Basal area (m^2 ha^{-1})	11.5 (2.6)	9.0 (1.6)	9.7 (0.7)	14.0 (1.5)
Volume (m^3 ha^{-1})	71.7 (18.3)	60.0 (12.1)	54.1 (4.6)	78.7 (10.3)

the ^{15}N natural abundance method (Kohl et al. 1980, Rennie and Rennie 1983, Shearer and Kohl 1986, Handley and Raven 1992, Handley and Scrimgeour 1997, Boddey et al. 2000).

Nitrogen accretion method

Soil N concentrations were measured by Pares (2002). As a result of site preparation by soil ripping and mounding, the plantation plots were characterized by mounded and flat microsites. Trees were planted on the mounds. In each plot, a total of 12 soil cores (7.5-cm-diameter and 30-cm-length) were collected, which covered all microsites (48 cores per treatment; Pares 2002). Concentrations of N were estimated for each biomass component (foliage, live branches, dead branches, bark and stem) and allometric equations (for wood, bark, leaf, twig and dead branch) developed for *E. globulus* at another site in Gippsland, Victoria (Bennett et al. 1997) and for *A. mearnsii* at this site (Forrester 2004). These allometric equations were developed from trees covering the same size range as in this study, and Bennett et al. (1997) found no significant effect of site or fertilizer on these equations. Similarly, DeBell et al. (1997) found that biomass equations for *Eucalyptus saligna* Sm. and *Falcataria molucanna* (Miquel) Barneby & Grimes could be applied to mixtures and monocultures. Thus, it was assumed that these equations were applicable across treatments in the present study. Leaf and branch N concentrations were determined from the leaf and litter samples described below. Nitrogen concentrations of bark and wood (sapwood and heartwood) were measured on material from wood cores (5-mm-diameter) taken from about eight trees per treatment.

Coarse root biomass was estimated based on the allometric equations developed by Misra et al. (1998). Fine root (< 2 mm) N was estimated by Bauhus et al. (2000) at age six years. Fine roots were sampled with 16 cores (0 to 30-cm depth and 7.5-cm diameter) per plot. It was assumed that the site was fully occupied by age three years, so fine root biomass would have stabilized and be similar at ages six and ten years. Forest floor litter N was estimated by Forrester et al. (2005) at age ten years from three forest floor litter samples collected from each microsite (nine samples per plot), based on 0.25 m^2 quadrats. The forest floor litter mass (and N content) per plot was estimated from the weighted mean of each microsite.

Mean rates of N_2 fixation over ten years were estimated by calculating the difference in plant biomass, forest floor litter and soil N between the *E. globulus* plots and plots containing pure and mixed stands of the N_2-fixing species, *A. mearnsii*. Nitrogen in understory plants was not examined, which may have led to underestimates of N_2 fixation. However, it is well established that the highly energy-demanding process of symbiotic N fixation is closely related to light availability, and thus occurs only at low rates in the understory of closed stands (Gordon and Wheeler 1983). Leaching and nitrification processes were not measured. However, N mineralization at age two years was predominantly in the form of NH_4-N (5.78 kg ha^{-1} $month^{-1}$) rather than NO_3-N (0.05 kg ha^{-1} $month^{-1}$) (Khanna unpublished data) so there was little nitrate available for leaching or denitrification. It was therefore assumed that differences among treatments resulted largely from N_2 fixation and not from different rates of other processes such as N leaching or denitrification.

Leaf and litter samples were collected at 2 (August 1994) and 10 years after plantation establishment (October 2002). At 2 years of age, two leaf samples from opposite sides of the top third of the canopy were collected from each of 10 trees of *E. globulus* and *A. mearnsii* in each plot (Khanna 1997). About 10 senescent leaves were collected per tree from 10 trees of each species per plot (Khanna 1997). These senescent leaf samples collected after 2 years into the study are described as leaf litter because litterfall was not measured at age two years.

^{15}N natural abundance method

Several criteria should be met when using the ^{15}N natural abundance technique to measure N_2 fixation (see Witty 1983, Fried et al. 1983, Shearer and Kohl 1986, Handley and Raven 1992, Handley and Scrimgeour 1997, Boddey et al. 2000): (1) a significant difference between the $\delta^{15}N$ of the non-N_2-fixing reference plants and the N_2-fixing plants should exist; (2) N_2-fixing and reference plants should not vary in their discrimination between ^{15}N and ^{14}N during transport of N between plant parts; (3) N_2-fixing and reference plants should access the same soil N pools; and (4) discrimination between ^{15}N and ^{14}N during transport of N from mycorrhizae to host plants should not differ between N_2-fixing and reference plants. Because it is difficult to predict whether a non-N_2-fixing species will access N from the same sources, in the same proportions and with the same temporal and spatial patterns as the N_2-fixing plants, it is desirable to use several reference species (Boddey et al. 2000).

Ten years after plantation establishment, leaf material was collected from *E. globulus*, *A. mearnsii* and understory species (listed in Table 2) that were common to most plots to determine a suitable reference species. Mycorrhizal status of these species was inferred from published work (Table 2). In addition, the mistletoe *Amyema pendula* (Sieb. ex Spreng.) Tiegh, which grew parasitically on *A. mearnsii*, was sampled. We lacked means to collect foliage samples from the upper canopy. Therefore, foliage was collected from the middle to lower canopy of three trees per plot. Mature foliage of understory plants was collected from about three individuals of each species in every plot where that species occurred. Samples were bulked to plot level before analysis. Leaf litter at 10 years was collected from three litter traps per plot (< 1 mm mesh, 0.25 m^2 traps) emptied about monthly (more detail provided in Forrester et al. 2005). All leaf and leaf litter samples at a given age were bulked for analysis so that there was one sample per species per plot.

Additional soil samples were collected in March 2003 to determine soil $\delta^{15}N$ values. Sampling depth was 0–5 cm because organic matter and N were concentrated at the soil surface. In each plot, three samples were collected from each microsite: mound and flat (a total of 24 samples per treatment). Samples were bulked so that there was a single sample per microsite per

Table 2. Mycorrhizal status and $\delta^{15}N$ values of species sampled in the mixed-species plots of *Eucalyptus globulus* and *Acacia mearnsii*. Mycorrhizal status was determined from published work on the same species or others in the same genus and family.

Family	Species	Mycorrhizal status	Leaf $\delta^{15}N$	Reference
Cyperaceae	*Caustis flexuosa* R.Br.	Arbuscular mycorrhiza	1.76	Logan et al. 1989, Bellgard 1991
	Gahnia sieberiana Kunth	No mycorrhiza	4.16	
Dennstaedtiaceae	*Pteridium esculentum* (G.Forst.) Cockayne	Arbuscular mycorrhiza	−0.72	Logan et al. 1989
Dilleniaceae	*Hibbertia empetrifolia* (DC.) Hoogland	Arbuscular mycorrhiza	−0.41	Logan et al. 1989, Bellgard 1991, Brundrett and Abbott 1991
Epacridaceae	*Epacris impressa* Labill.	Ericoid mycorrhiza	−2.71	Hutton et al. 1994
Fabaceae	*Dillwynia glaberrima* Sm.	Ectomycorrhiza	−1.43	Bellgard 1991
	Gompholobium latifolium Sm.	Arbuscular mycorrhiza	1.18	Bellgard 1991, Brundrett and Abbott 1991
	Platylobium formosum Sm.	Unknown	−1.04	
Goodeniaceae	*Goodenia ovata* Sm.	Arbuscular mycorrhiza	−0.72	Logan et al. 1989, Brundrett and Abbott 1991
Loranthaceae	*Amyema pendula* (Sieber ex Spreng.) Tiegh.	Not applicable (mistletoe on *A. mearnsii*)	−0.60	
Mimosaceae	*Acacia mearnsii* De Wild.	Arbuscular mycorrhiza	−0.63	Bellgard 1991, Brundrett and Abbott 1991, Reddell and Milnes 1992
	Acacia terminalis (Salisb.) J.F.Macbr.	Arbuscular mycorrhiza	−0.51	
Myrtaceae	*Eucalyptus globulus* subsp. *pseudoglobulus* (Naudin ex Maiden) J.B.Kirkp.	Ectomycorrhiza (and Arbuscular mycorrhiza when seedlings)	−2.59	Gardina and Malajczuk 1988, Bellgard 1991, Brundrett and Abbott 1991, Bellei et al. 1992, Reddell and Milnes 1992
Proteaceae	*Banksia marginata* Cav.	No mycorrhiza	3.78	Logan et al. 1989, Bellgard 1991, Brundrett and Abbott 1991
	Banksia serrata L.f.	No mycorrhiza	−1.73	
	Banksia spinulosa Sm.	No mycorrhiza	1.26	
	Persoonia mollis subsp. *leptophylla* S.Krauss & L.A.S. Johnson	No mycorrhiza	3.02	Reddell and Milnes 1992
Tremandraceae	*Tetratheca thymifolia* Sm.	Arbuscular mycorrhiza	−1.58	Brundrett and Abbott 1991

plot. The percentage of fixed soil N was estimated as:

$$N_{dfa} = \frac{\delta^{15}N_{ref} - \delta^{15}N_{sample}}{\delta^{15}N_{ref} - \beta}$$

where N_{dfa} is the percentage of soil N that was derived from the atmosphere, $\delta^{15}N_{ref}$ is the soil $\delta^{15}N$ in *E. globulus* plots, $\delta^{15}N_{sample}$ is the $\delta^{15}N$ in mixed or *A. mearnsii* plots and β is the $\delta^{15}N$ of legumes grown with air as their only source of N (Shearer and Kohl 1986). Stock et al. (1995) estimated $\beta = -1.3$ for *Acacia* spp. It was assumed that this β value would be appropriate for our study because soil N from N_2 fixation originated from plant material.

Chemical analysis

The $\delta^{15}N$ values and total N concentrations of plant and soil samples were determined with a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT 252 mass spectrometer (Finnigan, MAT GmbH, Bremen, Germany). The $\delta^{15}N$ samples were run against known standards and the results are expressed relative to the atmospheric standard. For leaf and litter samples, 2 mg from each N_2-fixing species and 2.5 mg from each non-N_2-fixing species were weighed into aluminum cups. For soil samples, 25 or 100 mg (depending on the N concentration of the soil) was weighed into aluminum cups. The samples were loaded into the Carlo-Erba system, where they were combusted at 1600 °C and the resultant gases cleaned on-line before being introduced to the mass spectrometer.

Statistical techniques

Differences between plant and soil $\delta^{15}N$ values and total N (between treatments with different species mixtures and plants with different mycorrhizal associations) were tested by Residual Maximum Likelihood (REML; Genstat 5 Committee 1997). Treatment effects in the REML analysis were assessed by Wald statistics, which are distributed as Chi squared. The Standard Errors of Difference (SED) for comparison of treatment means are provided.

Results

Acacia mearnsii contained higher quantities of N in biomass than *Eucalyptus globulus* (Table 3) because the *A. mearnsii* trees had higher N concentrations (Table 4) and greater biomass than the *E. globulus* trees. Quantities of N in stand biomass were highest in the mixed and pure *A. mearnsii* stands and lowest in the pure *E. globulus* stand (Table 3). Quantities of soil N were highest in the pure *A. mearnsii* stand and lowest in the pure *E. globulus* stand. If differences in soil, forest floor litter layer and biomass N resulted from N_2 fixation by *A. mearnsii,* then after 10 years, 862 and 383 kg N ha^{-1} had been fixed in the pure *A. mearnsii* and mixed stands, respectively.

Soil $\delta^{15}N$ values (average of 1.1‰) decreased with an increasing proportion of *A. mearnsii* (Table 5), whereas soil total N increased with the proportion of *A. mearnsii* (Table 3). Soil $\delta^{15}N$ values indicated that the percentage of N derived from the atmosphere (N_{dfa}) after 10 years was 10 and 63% in the mixed and pure *A. mearnsii* stands, respectively (Table 3). This corresponds to 162 and 1315 kg ha^{-1} of fixed N_2 in the soil over ten years. To test for the influence of the estimated parameter $\beta = -1.3$, we also used values of 0 or –2, which had a considerable influence on N_{dfa}, especially in the pure *A. mearnsii* plots (Table 3). However, based on differences between soil N in the pure *E. globulus* plots compared with plots containing *A. mearnsii*, there was 42 and 506 kg ha^{-1} of fixed N_2 in the soil at 10 years of age in the mixed and pure *A. mearnsii* plots, respectively (Table 3).

Differences in leaf $\delta^{15}N$ of over- and understory species

Table 3. Nitrogen in soil, biomass and forest floor litter from *Eucalyptus globulus* and *Acacia mearnsii* monocultures and 1:1 mixtures of *E. globulus* and *A. mearnsii*. Estimates of N_2 fixation are based on different combinations of these pools using the accretion method, and estimates of N_2 fixation and the proportion of soil N derived from the atmosphere (N_{dfa}) using the ^{15}N natural abundance method. Within a row, means followed by different letters are significantly different at $P < 0.05$ (Residual Maximum Likelihood). Abbreviation: SED, standard error of difference.

		E. globulus	Mixture	*A. mearnsii*	SED
Plant biomass at age 10 years (Mg ha^{-1})					
E. globulus	Foliage	4.9 a	3.9 a		0.42
	Branch	10.3 a	7.6 a		0.70
	Stem and bark	49.0 a	40.5 a		4.42
	Coarse root	15.1 a	11.1 a		1.02
A. mearnsii	Foliage		4.2 a	6.2 a	1.11
	Branch		12.3 a	18.0 a	1.87
	Stem and bark		43.8 a	53.3 a	3.43
	Coarse root		7.9 a	12.2 a	1.00
Fine root[1]		6.9	7.81	6.59	
Total biomass		86.3 a	139.2 b	96.3 a	9.51
N in plant and soil biomass at age 10 years (kg ha^{-1})					
E. globulus	Foliage N	48.4 a	44.6 a		4.62
	Branch N	9.9 a	10.3 a		0.88
	Stem and bark N	40.4 a	39.4 a		4.19
	Coarse root N	14.0 a	10.2 b		0.94
A. mearnsii	Foliage N		128 a	174 a	31.8
	Branch N		56.6 a	87.5 b	3.59
	Stem and bark N		89.5 a	130.4 b	8.24
	Coarse root N		10.5 a	16.0 b	1.32
Fine root N[1]		24.94	90.05	88.03	
Total biomass N		138 a	480 b	496 b	37.9
Soil N (0–30 cm)[2]		1581 a	1623 a	2087 a	299.5
Forest floor litter layer N[3]		50.1 a	49.6 a	48.1 a	
Total biomass + litter + soil N		1769 a	2152 ab	2631 b	303
N_2 fixed by age 10 years (kg ha^{-1})					
N_2 fixed based on biomass, litter and soil N			383	862	
N_2 fixed based on biomass and litter N			341	356	
N_2 fixed based on soil N			42	506	
N_{dfa} (%) ($\beta = -1.3$)			10	63	
N_{dfa} (%) ($\beta = 0$)			17	107	
N_{dfa} (%) ($\beta = -2$)			8	52	
N_2 fixed in soil based on soil $\delta^{15}N$ and $\beta = -1.3$			162	1315	

[1] Bauhus et al. 2000.
[2] Pares 2002.
[3] Forrester et al. 2005.

Table 4. Leaf and leaf litter $\delta^{15}N$ and nitrogen (N) concentrations at age 2 and 10 years in mixed and monoculture plots of *Eucalyptus globulus* and *Acacia mearnsii*. Abbreviation: SED, standard error of difference.

Year	Species	*E. globulus*	Mixture	*A. mearnsii*	SED
Leaf $\delta^{15}N$ (‰)					
2	*E. globulus*	0.22	0.64		0.67
	A. mearnsii		−0.75	−0.15	0.09
10	*E. globulus*	−3.13	−2.05		1.13
	A. mearnsii		−0.73	−0.49	0.33
Litter $\delta^{15}N$ (‰)					
2	*E. globulus*	5.23	5.25		0.46
	A. mearnsii		−1.92	−1.66	0.05
10	*E. globulus*	−2.67	−2.73		0.27
	A. mearnsii		−1.71	−1.41	0.27
Leaf N (mg g^{-1}*)*					
2	*E. globulus*	12.1	13.4		0.3
	A. mearnsii		25.7	24.2	0.67
10	*E. globulus*	9.9	11.3		3.35
	A. mearnsii		30.3	28.1	0.79
Litter N (mg g^{-1}*)*					
2	*E. globulus*	5.4	5.9		0.1
	A. mearnsii		14.4	12.4	0.2
10	*E. globulus*	6.7	10.1		2.6
	A. mearnsii		14.7	18.3	2.9

were related to mycorrhizal status, with non-mycorrhizal species clustered around 3.3‰, arbuscular mycorrhizae (AM) species clustered around −0.4‰ and ericoid or ectomycorrhizal species around −2.5‰ (Table 6, Figure 1). For a given mycorrhizal group, there was no significant difference in leaf $\delta^{15}N$ between the mixed and monoculture plots (Table 6). There was no fractionation of N isotopes between the mistletoe *Amyema pendula* and its host *A. mearnsii*, because its leaf $\delta^{15}N$ reflected that of its host, corroborating similar findings by Bannister and Strong (2001).

Table 5. Soil $\delta^{15}N$ values (‰) at age 10 years in mixed and monoculture plots of *Eucalyptus globulus* and *Acacia mearnsii*. Means followed by different letters (a, b or c) are significantly different at $P < 0.05$ (Residual Maximum Likelihood; REML) across a given microsite (row). Means followed by different letters (x or y) are significantly different at $P < 0.05$ (REML) for a given species proportion (column). Abbreviation: SED, standard error of difference.

Microsite	*E. globulus*	Mixture	*A. mearnsii*	
Flat	1.73 a x	0.79 b y	−0.17 c x	
Mound	2.06 a x	2.36 a x	−0.08 b x	
Average	1.90 a	1.58a	−0.13b	
	SED	Wald	df	χ^2 prob
Species proportion	0.295	19.03	2	< 0.001
Microsite	0.524	14.64	1	< 0.001
Treatment × microsite	0.478	13.88	2	< 0.001

The $\delta^{15}N$ values of *E. globulus* leaf and litter material were higher at 2 years than at 10 years, but there was no significant difference between 2 and 10 years for *A. mearnsii* (Table 4). In *A. mearnsii,* the leaf $\delta^{15}N$ values were higher than litter $\delta^{15}N$ values at both 2 and 10 years. For *E. globulus,* $\delta^{15}N$ values were lower for leaves than litter at 2 years but not at 10 years.

For both *E. globulus* and *A. mearnsii,* leaf and litter N concentrations were higher in mixed plots than in monoculture plots at Year 2, except for *A. mearnsii* leaf N concentrations (Table 4). Leaf N concentrations were significantly higher than leaf litter concentrations for both *E. globulus* and *A. mearnsii* (Table 4).

Discussion

Differences in soil, foliage and litter N among plots showed that *Acacia mearnsii* influenced N dynamics at the site. Several estimates of N_2 fixation were made by N accretion methods. Assuming differences in soil, litter and biomass N resulted from N_2 fixation by *A. mearnsii*, then over the course of 10 years, a mean of 86 and 38 kg ha^{-1} $year^{-1}$ were fixed in pure *A. mearnsii* and mixed plots, respectively. Mean quantities of N_2 fixed in mixed and pure *A. mearnsii* plots were similar at 34 and 36 kg ha^{-1} $year^{-1}$, respectively, when based on N in biomass and litter. However, estimates which included soil N or used $\delta^{15}N$ data were much higher in pure *A. mearnsii* plots, suggesting that, although the more productive mixed treatment appears to have taken up similar quantities of N, higher quantities of N_2 were fixed and stored in the soil in the *A. mearnsii*

Table 6. Leaf $\delta^{15}N$ values of plants from different mycorrhizal groups at age 10 years in mixed and monoculture plots of *Eucalyptus globulus* and *Acacia mearnsii*, n = number of species sampled. Means followed by different letters (a, b or c) are significantly different at $P < 0.05$ (Residual Maximum Likelihood) across a given species composition (column). Abbreviation: SED, standard error of difference.

	E. globulus	Mixture	*A. mearnsii*	Mean
Ectomycorrhiza ($n = 2$)	−2.89 a	−1.77 a	−1.57 b	−2.08
Ericoid mycorrhiza ($n = 1$)	−2.06 ab	−2.74 a	−3.54 a	−2.78
No mycorrhiza ($n = 3$)	3.99 c	3.24 c	2.52 c	3.25
Arbuscular mycorrhiza ($n = 6$)	−0.56 b	−0.19 b	−0.33 b	−0.36
	SED	Wald	df	χ^2 prob
Overstory species proportion	0.80	0.28	2	0.870
Mycorrhizae	0.78	227.85	3	< 0.001
Species composition × mycorrhizae	0.80	8.87	6	0.181

plots. This illustrates that estimating N_2 fixation from individual N pools (biomass or soil) can give useful values, but that this method cannot provide accurate estimates (Shearer and Kohl 1986, Sanginga et al. 1995). The N content of forest floor litter did not differ significantly between treatments in our trial (48–50 kg N ha^{-1}; Forrester et al. 2005) and therefore has little influence on N_2 fixation estimates. Other processes, such as N leaching or denitrification, which were not measured, may have influenced these differences. However, given the low N status and the near absence of nitrification in these acidic soils (Bauhus et al. 1993), the occurrence of these ecosystem losses would have been more likely in *A. mearnsii* plots, which accumulated more soil N, than in *E. globulus* plots. Therefore, the estimates of N_2 fixation rates may be regarded as conservative.

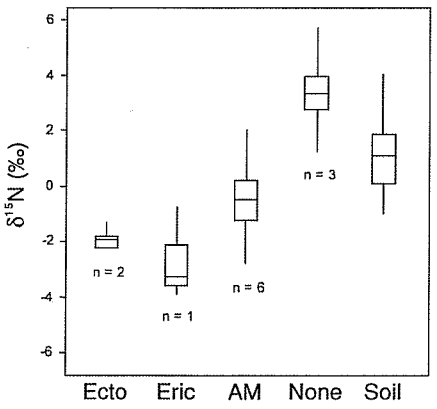

Figure 1. Box and whiskers plot of soil $\delta^{15}N$ values and leaf $\delta^{15}N$ values of plants from different mycorrhizal groups at age 10 years in mixed and monoculture plots of *Eucalyptus globulus* and *Acacia mearnsii*. The lines in the middle of the boxes represent the median values: 25% of the data lie in the box above the line and 25% lie in the box below the line. Each whisker represents 1.5 times the interquartile range (the distance between the bottom and the top of the box) or the furthest observation if this is less than this distance. Abbreviations: Ecto, ectomycorrhizae; Eric, ericoid mycorrhizae; AM, arbuscular mycorrhizae; None, no mycorrhizae; Soil, soil (0–5-cm layer); and n, number of species sampled.

Nevertheless, these values are within the range estimated in other studies on *Acacia* or other N_2-fixing trees (Binkley 1992, Binkley and Giardina 1997, Khanna 1998, Fisher and Binkley 2000, May and Attiwill 2003).

When N fixed from the atmosphere with a lower $\delta^{15}N$ than soil N is recycled and builds up in ecosystems, the $\delta^{15}N$ values of the soil N and of the plants using that N can decrease. Declines in $\delta^{15}N$ values with time, as observed in our study, have been observed and attributed to N_2 fixation in other studies (Van Kessel et al. 1994, Parrotta et al. 1996).

Surface soil (0–5 cm) $\delta^{15}N$ values suggested that N_{dfa} and the quantities of N_2 fixed were higher in *A. mearnsii* plots than in mixed plots. In contrast, other studies have found that competition from a non-N_2-fixing species for soil N can increase the N_{dfa} of the N_2-fixing species. For example, in an 18-month-old pot trial, where N_2-fixing *Casuarina cunninghamiana* Miq. were planted in 1:1 mixtures with *E. globulus*, N_2 fixation and N_{dfa} increased from 125 g N $plant^{-1}$ and 86.7% in monocultures to 153 g N $plant^{-1}$ and 94.7% in mixture (same total plant density as monocultures) (Baker et al. 1994). This was probably the result of competition for soil N from *E. globulus* (Baker et al. 1994). In contrast, competition from *E. robusta* planted with *C. equisetifolia* had no significant effect on the quantity of N fixed or N_{dfa} by *C. equisetifolia* between 6 and 24 months of age (Parrotta et al. 1994). This difference may be due to less intense competition in the plantation than in the pots (Baker et al. 1994). In this study, N_{dfa} estimates were based on the soil N pool. Large quantities of N were also contained in the aboveground biomass, and the N_{dfa} of plant material was not determined because a suitable reference species could not be identified. The difficulty of identifying suitable reference species has been reported by others (e.g., Roggy et al. 1999, Gehring and Vlek 2004). In addition, the available soil N pool may represent only a small proportion of total soil N and thus $\delta^{15}N$ of soil probably does not provide a good estimate of N_2 fixation (Högberg 1997).

Differences in leaf $\delta^{15}N$ were related to mycorrhizal status. Mycorrhizal associations influenced $\delta^{15}N$ values in other studies (Högberg 1990, Handley et al. 1993, Pate et al. 1993, Michelsen et al. 1996, Handley et al. 1999, Spriggs et al.

2003). For example, Spriggs et al. (2003) found that foliar $\delta^{15}N$ values of non-mycorrhizal species were clustered around 1‰, arbuscular mycorrhizal species clustered around –2‰ and ericoid mycorrhizal species were about –1‰ in an N-limited ecosystem in South Africa. This decline in $\delta^{15}N$ values from non-mycorrhizal to arbuscular mycorrhizal to ericoid mycorrhizal species corresponds to the trend that we observed. The temporal changes in $\delta^{15}N$ values may also result from changes in mycorrhizal status, because *Eucalyptus* seedlings can have arbuscular mycorrhizal associations that are replaced by ectomycorrhizal associations as they age (Gardina and Malajczuk 1988, Bellei et al. 1992). The reason for the temporal change is unknown; however, the change in $\delta^{15}N$ with time in *E. globulus* shows that estimates of N_2 fixation could change depending on the relative ages of the N_2-fixing plants and reference plants, even if they are growing together. Therefore, a major assumption of the natural abundance method, that the discrimination of ^{15}N by mycorrhizae of N_2-fixing and reference plants is the same (Shearer and Kohl 1986), may not be satisfied at this site. Thus, $\delta^{15}N$ values could not be used as a quantitative method for examining N_2 fixation. The results also indicate that reference plants with the same mycorrhizal associations as the N_2-fixing species should be used when using ^{15}N methods to quantify N_2 fixation (Spriggs et al. 2003).

The $\delta^{15}N$ values of *A. mearnsii* leaf material were higher than those of litter at both 2 and 10 years, whereas those of *E. globulus* leaf material were much lower than those of litter at 2 years. This suggests that there was some isotopic fractionation during the internal cycling and withdrawal of N from senescing leaves. The direction and magnitude of this discrimination differed not only between species, but also varied with age for *E. globulus,* such that the difference between litter and leaf $\delta^{15}N$ values was significant only at age 2 years. In contrast, changes in $\delta^{15}N$ values during leaf senescence were not observed for mangroves (Kao et al. 2002). Comparisons of $\delta^{15}N$ values of leaf and litter material are rarely given in the literature but have implications for studies on the cycling of fixed N_2 within ecosystems. For example, the $\delta^{15}N$ values of litter influence the $\delta^{15}N$ values of soils as litter decomposes and N is released (Danso et al. 1992, Sanginga et al. 1995). Therefore, the soil $\delta^{15}N$ values may change not only if $\delta^{15}N$ values change with time under N_2 fixing plants, but also because there may be a contrasting change under the non-N_2-fixing species because of the discrimination that occurs during withdrawal of N from senescing litter. This also demonstrates the importance of using reference plants that obtain soil N from the same pools as the N_2-fixing plants (Danso et al. 1992, Parrotta et al. 1996).

In conclusion, nitrogen in biomass, litter and soil were influenced by N_2 fixation by *A. mearnsii,* which amounted to about 38 and 86 kg N ha^{-1} $year^{-1}$ in the 1:1 mixtures and *A. mearnsii* monocultures, respectively. Therefore the greater biomass production of *E. globulus* trees planted with *A. mearnsii* compared to *E. globulus* monocultures (Bauhus et al. 2004, Forrester et al. 2004) can be attributed, at least in part, to increased N availability. Discrimination of ^{15}N during transfer from mycorrhizae to the host plant varies with mycorrhizal status of the plants. Additional differences between $\delta^{15}N$ of litter and foliage suggest that different species may differ in the discrimination of ^{15}N during N withdrawal from senescing leaves. Our data show that, as a result of different mycorrhizal associations, possible temporal changes in the type of mycorrhizae, substantial fractionation with recycling of nutrients during leaf senescence and differences in magnitude of this fractionation between species, caution should be exercised when interpreting ^{15}N natural abundance data in studies of ecosystem N cycling.

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