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Appraisal of ^{15}N enrichment and ^{15}N natural abundance methods for estimating N_2 fixation by understorey *Acacia leiocalyx* and *A. disparimma* in a native forest in subtropical Australia

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Abstract

Purpose: It is anticipated that global climate change will increase the frequency of wildfires in native forests of eastern Australia. Understorey legumes such as *Acacia* species play an

important role in maintaining ecosystem N balance through biological nitrogen fixation (BNF). This is particularly important in Australian native forests with soils of low nutrient status and frequent disturbance of the nutrient cycles by fire. This study aimed to examine ^{15}N enrichment and ^{15}N natural abundance techniques in terms of their utilisation for evaluation of N_2 fixation of understorey acacias and determine the relationship between species ecophysiological traits and N_2 fixation.

Materials and methods: A trial was established at two Sites 1 and 2 located at Toohey Forest, Queensland, Australia, a eucalypt-dominated native forest, to examine the determination of BNF using ^{15}N enrichment and ^{15}N natural abundance methods. Toohey Forest is an urban forest and subjected to frequent fuel reduction burns to protect the adjacent properties. Plant physiological status was measured to determine the relationship between physiological and N_2 fixation activities.

Results and discussion: Both ^{15}N enrichment and ^{15}N natural abundance techniques may be used to estimate N_2 fixation of acacia tree species. The estimation of BNF using ^{15}N enrichment was higher than those of the ^{15}N natural abundance method. A grass reference plant, *Themeda triandra,* as well as tree reference plants provided an appropriate $\delta^{15}N$ signal. Potential *B* values for *Acacia* spp. between -0.3‰ and 1.0‰ provided an acceptable BNF estimation. This suburban forest has been located nearby busy highways leading to the N deposition over time with negative $\delta^{15}N$ signal. This N deposition may explain the separation between acacias $\delta^{15}N$ signal and that of the reference plants which would be led to the successful use of the ^{15}N natural abundance technique. *A. leiocalyx* demonstrated greater N_2 fixation as well as photosynthesis and instantaneous water use efficiency (iWUE) than *A. disparimma*. However, no strong relationship between plant photosynthesis and N_2 fixation was observed in this study. A high-within treatment variation may have masked the relationships between plant BNF activities and photosynthesis

Conclusions: The ^{15}N natural abundance technique is preferred to be used for future studies as it is simple and inexpensive compared to^{15}N enrichment method. The dependence of both species on BNF at Site 2, where fuel reduction burning had not taken place for 8 years, suggests that the frequent burning impoverished the soil and this has wider implications as higher fire frequencies are to be expected in other Australian ecosystems as a result of global climate change.

Keywords ^{15}N enrichment method • ^{15}N natural abundance method • *Acacia leiocalyx, A. disparimma* • N_2 fixation

1 Introduction

Global climate change, particularly rising atmospheric carbon dioxide (CO_2) concentration and temperature can alter rainfall patterns and distribution (Xu et al. 2009; McKeon et al. 2009); and reduce soil moisture across Australia (Jung et al. 2010). These alterations may be linked to more extreme environmental conditions such as prolonged drought and drier growing seasons and consequently more frequent fires. Prescribed burning is widely used as a forest management tool in Australia and may result in the loss of N through volatilisation (Crutzen and Andreae 1990; May and Attiwill 2003; Reverchon et al. 2011) and also increase the release of terrestrial carbon (C) to the atmosphere (Thonicke et al. 2010). Therefore, after a fire, fast growing understorey legumes such as *Acacia* species may play an important role in post-fire recovery by adding N through N_2 fixation (Hamilton et al. 1993; Guinto et al. 2000; Reverchon et al. 2011) and increasing C sequestration. Nitrogen input to the soil through N_2 fixation of *Acacia* species, in Australia, may exceed 50 Kg N ha^{-1} $year^{-1}$ depending on acacia density in the ecosystem (May and Attiwill 2003; Adams et al. 2010). With the exception of a study conducted by Guinto et al. (2000), who determined the ability of *A. leiocalyx* to fix atmospheric N_2, there is limited published information of *Acacia* spp. particularly *A. leiocalyx* and *A. disparimma* in subtropical Australia. Quantifying the ability of these species to fix N_2 will increase understanding of their contribution to Australian native forest ecosystems.

The most commonly used methods to determine N_2 fixation in legumes are: the acetylene reduction assay (ARA); ^{15}N enrichment; and ^{15}N natural abundance (Forrester et al. 2006). Different studies have shown that the accuracy of ARA to estimate BNF may be uncertain when used in the field (Peoples et al. 1989; Unkovich and Pate 2000). ^{15}N enrichment and ^{15}N natural abundance are considered to be time integrated methods to determine N_2 fixation under natural conditions (Boddey et al. 2000; Unkovich and Pate 2000). However, both methods have limitations under natural conditions including: alteration in the pattern of N uptake by trees; the different potential capacity for nodulation of plants within species; and uneven plant distribution leading to variation in the availability of resources (Hansen and Pate 1987; Ladha et al. 1993; Peoples et al. 1996). Furthermore, the determination of the N_2 fixation in large plants has always been challenging due to difficulties associated with existing methodologies for large trees which could underestimate the N input to ecosystems (Hopmans et al. 1993). The use of ^{15}N natural abundance method

in agricultural ecosystems has been well established (Unkovich and Pate 2000; Somado and Kuehne 2006; Houngnandan et al. 2008; Naab et al. 2009). In Australia, this method has also been used successfully for shrub and woody legumes including: western Australia for *Chamaecytisus proliferus* (Unkovich et al. 2000); north Queensland for *Calliandra calothyrsus* (Peoples et al. 1996); and Victoria for three species of acacias (Hamilton et al. 1993; May and Attiwill 2003). However, the method was unsuccessful when applied to *A. leiocalyx* in subtropical Queensland (Guinto et al. 2000). Using ^{15}N natural abundance method to estimate the percentage of BNF derived from the atmosphere (%Ndfa) of woody plants in subtropical Australia therefore requires further investigations.

For the ^{15}N enrichment technique, a ^{15}N-enriched fertiliser is applied to the soil and the dilution of ^{15}N enrichment in the plant by atmospheric ^{15}N reveals the extent of N_2 fixation (Warembourg 1993). To attain the greatest accuracy, soils need to be homogeneously labelled by the fertiliser solution to provide a stable $^{15}N/^{14}N$ ratio (Danso et al. 1992; Warembourg 1993; Parrotta et al. 1994).

The ^{15}N natural abundance method uses the differences between the ^{15}N enrichment of soil and atmospheric N_2 (Shearer and Kohl 1986). The ^{15}N natural abundance technique requires well matched reference plants and accurate *B* values, the ^{15}N abundance of the N in the plant that is derived from the air, to quantify BNF(Boddey et al. 2000; Guinto et al. 2000; Gehring and Vlek 2004). Guinto et al. (2000) found no significant difference in $\delta^{15}N$ signal between *Acacia* spp. and the selected reference plants which led to unsuccessful use of the ^{15}N natural abundance technique. The experiment was also conducted in a natural forest with high spatial variability. Reference plants are selected from species that naturally occur at the experimental sites and the availability of suitable plants may be a condition to using the ^{15}N natural abundance method to evaluate BNF.

N_2 fixation is an energy consuming process which may be directly linked to plant photosynthetic capacity (Lambers et al. 2008). Under frequently water limiting conditions, alteration in plant water use efficiency strategies (WUE), including stomatal closures, leaf to air vapour pressure and transpiration, may influence both N_2 fixation and plant photosynthetic capacity. Hence, these parameters may be linked to each other and the ability of the plant to fix C through photosynthesis may control the rate of N_2 fixation (Fujita et al. 1988; Imsande 1988; Aranjuelo et al. 2007).

This study aimed to (1) examine ^{15}N enrichment and ^{15}N natural abundance techniques in terms of their utilisation for evaluation of N_2 fixation of understorey acacias; (2) evaluate the utility of the ^{15}N natural abundance technique for future study due to its low

cost and simplicity; and also (3) determine the relationship between species ecophysiological traits, including leaf-level photosynthesis and WUE, and N_2 fixation.

2 Materials and methods

2.1 Site description

The experimental sites were situated at Toohey Forest (27°32'53 S;153°03'21E) located in Brisbane, south-east Queensland, Australia. Toohey Forest is a remnant island of native vegetation of about 640 ha, completely surrounded by suburban sprawl. The vegetation is dry sclerophyll forest dominated by rough-barked eucalypts with an understorey usually less than 2 m tall consisting of grasses and shrubs. *Xanthorrhoea johnsonii* (Xanthorrhoeaceae) may be quite dense in the shrub layer in areas where sandstone is the parent material. Soils are red-yellow podzolics with a silty loam A horizon, generally less than 10 cm (Catterall et al. 1987). Soil chemical and physical properties are given in Table 1. The area is classified as sub-tropical with a mean annual temperature of 27℃ and an average annual precipitation of 1350 mm, with the data for the study period presented in Fig. 1.

The two experimental sites have been subjected to fuel reduction burns over a period of 20 years. Site 1 was burnt in 1991, 1993 and 2008 and Site 2 was burnt in 1991 and 2001. The experiments were designed as a randomised complete block. At Site 1, treatments were two methods (^{15}N enrichment and ^{15}N natural abundance) and two species (*A. disparimma* and *A. leiocalyx*) with 5 replications per treatment. At Site 2, treatments were species (*A. disparimma* and *A. leiocalyx*) and plant sizes (<2.5 m and 2.5 to 4.5 m height) categorised as small and medium, with 5 replications per treatment. At Site 2, only the ^{15}N natural abundance was examined and the ^{15}N enrichment was not practiced due to difficulties associated with making barriers for large trees. Sampling was undertaken at both Sites in April 2009, August 2009 and March 2010, defined as Seasons 1 to 3.

2.2 ^{15}N enrichment technique

A. leiocalyx and *A. disparimma* seedlings and with *Eucalyptus planchoniana* (Myrtaceae) as reference plant, all plants with height under 1 m, were randomly selected at Site 1. A micro-plot was established in the soil around each seedling. Each micro-plot was established using plastic barriers to a depth of 0.30 m and enclosing an area 50 cm × 50 cm. In March 2009, two weeks after installing the barriers, the soil within each micro-plot was labelled by 50 ml fertiliser solution containing ammonium sulphate (10.0 atom% ^{15}N) at 10 kg N ha^{-1}. The ^{15}N fertiliser solution was applied to an area 20 cm × 20 cm in an area around basal diameter that

had been equally divided into 25 squares. Each square received 2 ml of the fertiliser solution injected to depth of 2 cm in the soil using a 3-ml syringe with a terumo spinal needle 18G × 3½ " (1.20 × 90 mm). The ^{15}N natural abundance micro-plots received 50 ml water using the same method. To avoid N volatilisation, the ^{15}N fertiliser solution was applied on a cool and cloudy day.

The percentage of N_2 fixed from atmosphere (%Ndfa) was calculated using the following equation (Fried and Middelboe 1977):

$$\%Ndfa = 100 \times [1 - (atom\%\ ^{15}N_{excess\ sample} / atom\%\ ^{15}N_{excess\ ref})] \quad (1)$$

Where:

atom% $^{15}N_{excess\ sample}$ = the weighted average ^{15}N enrichment of *Acacia* spp.

atom% $^{15}N_{excess\ ref}$ = the weighted average ^{15}N enrichment of reference plants

2.3 ^{15}N natural abundance technique

The experiment examined *A. leiocalyx* and *A. disparimma* of different sizes. Nine different species of non-N_2-fixing reference plants of a medium size at Site 2 and small size at Site 1 were used. The reference plants were categorised into two groups; consisting of eight tree species *Eucalyptus planchoniana*, *E. psammitica*, *E. carnea*, *E. tindaliae*, *E. resinifera*, *Corymbia henryi*, *Angophora woodsiana* (all Myrtaceae), *Xanthorrhoea johnsonii* and one herbaceous species, *Themeda triandra* (Poaceae). Each species of reference plant chosen at each plot had two replications. *Angophora woodsiana* was selected as reference plant for all BNF estimation using the ^{15}N natural abundance because of its availability at both sites. Furthermore, using one reference plant for all estimations could minimise the potential error.

The value of $\delta\ ^{15}N$ was determined using the following equation:

$$\delta\ ^{15}N = 1000 \times [(R_{sample} - R_{std})/R_{std}] \quad (2)$$

Where

R is the isotopic ratio $^{15}N/^{14}N$ of sample and standard (std) which refers to the atmospheric N_2.

The percentage of N derived from atmospheric N_2 (%Ndfa) was calculated using the following equation (Shearer and Kohl 1986):

$$\%Ndfa = [(\delta\ ^{15}N_{ref} - \delta\ ^{15}N_{acacia})/(\delta\ ^{15}N_{ref} - B_{value})] \times 100 \quad (3)$$

Where:

$\delta\ ^{15}N_{ref}$ = the $\delta\ ^{15}N$ value of the reference plants

$\delta\ ^{15}N_{acacia}$ = the $\delta\ ^{15}N$ value of the *Acacia* spp.

B_{value} = the relative isotopic abundance of *Acacia* spp. growing under a N-free nutrient condition.

The *B* values reported from a large array of sources vary from -2.9‰ to 1.0‰ for woody plants (Shearer and Kohl 1986; Boddey et al. 2000). In the present study, different *B* values varying from -1.3‰ to 1.0‰ were examined to determine an applicable *B* value for *Acacia* spp. under the experimental condition.

2.4 Plant sampling and analysis

Three fully expanded leaves of each selected *Acacia* species from different sizes and also from each of the reference plants were collected. The leaves were kept in separate paper bags and transferred to the laboratory. These samples were then oven dried at 50°C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al. 2000). The resulting homogenised powder was weighed into 8 mm × 5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime, Manchester, UK). A minimum of 10% replication was used to verify the accuracy of the results. These samples were assessed for total N (TN), N isotope composition ($\delta\ ^{15}N$) and C isotope composition ($\delta\ ^{13}C$) as described by Prasolova et al. (2000) and Xu et al. (2000; 2003).

2.5 Gas exchange measurements

Gas exchange was determined for each plant using a portable photosynthesis system (Model LI-6400, Li-Cor). Data were collected for each selected plant with three replicates per plant using a constant CO_2 concentration 380 µmol mol^{-1} and a blue-red light-emitting diodes (Model 6400-02B) adjusted at photosynthetically active radiation (PAR) 1400 µmol s^{-1}. Parameters derived from survey data were net photosynthesis (A_n), transpiration (E), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i). The photosynthetic response to light (A/Q response curves) was also determined at various PAR varying from 1700 to 0 in 12 steps as 1700, 1400, 1100, 800, 500, 300, 150, 100, 80, 50, 20 and 0 and maximum photosynthesis (A_{max}) derived from this curve. Instantaneous water use efficiency (iWUE) at leaf level was determined as A_n/E (Farquhar and Richards 1984). All measurements were taken on sunny days between 09:00 and 12:00.

2.6 Statistical analysis

Analysis of variance (ANOVA) was conducted to detect the differences in N_2 fixation with respect to seasonal variation, age classes, species and also physiological parameters. The

Tukey HSD test at $P<0.05$ was used to determine a comparison among treatment means. A paired T-test was carried out to compare means of two techniques of N_2 fixation. Linear regression was carried out to indicate the relationships among N_2 fixation and photosynthesis or WUE. Statistix software (Version 8) was used for all the statistical analyses.

3 Results

3.1 ^{15}N enrichment and ^{15}N natural abundance methods

The BNF estimation using the two methods indicated a significant difference at each sampling season ($P<0.05$). A paired-samples T-test demonstrated that mean %Ndfa determined using the ^{15}N enrichment technique was significantly ($P<0.05$, $n=58$) higher than that derived from the ^{15}N natural abundance technique. This difference existed irrespective of acacia species or sampling season, and was about 31% higher on average using the ^{15}N enrichment technique, compared with that of the ^{15}N natural abundance technique.

At Site 1, BNF of *A. leiocalyx* and *A. disparimma* based on ^{15}N enrichment technique indicated a significant difference at three sampling times ($P<0.05$, Fig. 2a). BNF of *A. leiocalyx* was significantly higher than those of *A. disparimma*. The estimated BNF based on ^{15}N enrichment technique varied from 94.8% to 96.5% for *A. leiocalyx* and was from 83.3% to 91.8% for *A. disparimma*. At Site 1, no significant difference was detected in BNF estimation using the ^{15}N natural abundance method for *A. leiocalyx* and *A. disparimma* with the only exception at Season 3 when BNF of *A. leiocalyx* was significantly higher than that of *A. disparimma* (see Fig. 2b). The estimated BNF based on ^{15}N natural abundance technique was from 59.6% to 82.5% for *A. leiocalyx* and ranged from 35.8% to 72.0% for *A. disparimma*. At Site 2, the BNF did not vary significantly in response to plant size. The BNF of *A. leiocalyx* was significantly higher than that of *A. disparimma* at Seasons 2 and 3 ($P<0.05$). The determination of BNF based on ^{15}N natural abundance technique was from 75.6% to 86.2% for *A. leiocalyx* and varied from 80.6% to 54.5% for *A. disparimma* (see Fig. 2c).

3.2 δ^{15}N values of acacias and reference plants

The δ^{15}N values of *A. leiocalyx*, irrespective of sampling season, were less negative than those of *A. disparimma*, -0.73‰ *vs.* -1.06‰ respectively. However, this difference was only significant at the last sampling ($P<0.05$). At Site 1, the δ^{15}N differences were smaller than 2.0‰ (Table 2) on a few occasions. At Site 2, δ^{15}N differences between acacia species and reference plants was >2.0‰ for all selected reference plants and there was a significant

difference in $\delta^{15}N$ between acacias and all selected reference plants, regardless of sampling seasons (P<0.05).

3.3 *B* values

Using a *B* value of -1.3‰ resulted in an estimation of %Ndfa that was greater than 100%. At Site 2, assuming *B*=-1.3‰ resulted in 68% of all cases having the %Ndfa greater than 100% (n=33 out of 48) while this percentage dropped to 6.25% when *B* was assumed to be -0.3‰ (n=3 out of 48). None of the cases were greater than 100% when *B* values were either 0.0‰ or 1.0‰. Therefore, potential *B* value for these two acacias could be from -0.3‰ to 1‰. In our study, we chose B value of 0.0‰ to report the results of BNF determination based on the ^{15}N natural abundance method.

3.4 Physiological traits of both species

A. leiocalyx had significantly higher net photosynthesis (A_n) and maximum photosynthesis (A_{max}) than *A. disparimma* (P<0.05, Fig. 3a and Table 3), Transpiration (E) of *A. leiocalyx* was greater when compared with that of *A. disparimma* but it was significant only at Season 1 (P<0.05, Fig. 3b). Instantaneous water use efficiency (iWUE) did not vary significantly in response to plant species with the exception observed at Season 3 when iWUE of *A. leiocalyx* was significantly greater than *A. disparimma* (P<0.05, Fig. 3c). No significant difference of C isotope composition ($\delta^{13}C$) between two plant species (main effect) was observed (Table 3). Total N (TN) of *A. leiocalyx* was slightly higher than that of *A. disparimma*. However, the difference in foliar TN between two species was only significant at Season 3 (P<0.05, 2.50% *vs.* 2.05% respectively, Table 3).

3.5 Relationships among physiological traits

A linear regression was carried out between net photosynthesis A_n and iWUE, E or C_i. iWUE, E or C_i explained 20%, 35% and 17% of the variation in A_n (n=18, P<0.05). There was a positive correlation between TN and A_n and between TN and %Ndfa (R^2= 0.17 and R^2=0.31 respectively, n=16, P<0.05, Fig. 4). A linear regression was performed between %Ndfa and physiological traits including: A_n, E and iWUE for each species separately and there was a poor or no significant relationship among those variables.

4 Discussion

4.1 ^{15}N enrichment and ^{15}N natural abundance methods

Our results demonstrated that both ^{15}N enrichment and ^{15}N natural abundance were valid methods to provide quantitative estimation for N_2 fixation of understorey trees, *A. leiocalyxs*

and *A. disparimma*. The difference in the BNF estimation between the two methods, with the ^{15}N enrichment method giving consistently higher estimates of BNF was similar to the findings of Bouillet et al. (2008). Fertilisation may have stimulated soil microbial activity and the ^{15}N may have been mineralised or immobilised. Uptake of the newly mineralised N could have caused the higher estimation of BNF in the ^{15}N enrichment (Høgh-Jensen and Schjoerring 1994). Small within-treatment variation in the ^{15}N enrichment method may demonstrate an even distribution of $\delta^{15}N$ available in the soil of reference plants.

The determination of %Ndfa based on the natural abundance method for *A. leiocalyxs* and *A. disparimma* was in agreement with values for other acacia species reported in Australia a 43% for *A. melanoxylon*, 48% for *A. mucronata* (Hamilton et al. 1993) and 59% for *A. dealbata* (May and Attiwill 2003) based on the ^{15}N natural abundance method. There was limited information regarding %Ndfa of *A. leiocalyxs* and *A. disparimma* in Australia.

Despite the fact that %Ndfa determination of two methods were within an acceptable range, the relationship between the two methods was poor. This was consistent with other studies (Androsoff et al. 1995; Stevenson et al. 1995). Galiana et al. (2002) reported a range of N_2 fixation for *A. mangium* from 20% to 90% based on^{15}N natural abundance method. The range in our study was much narrower for both species and this variability may have been due to the within-treatment variation caused by spatial variation of mutualistic organisms (Boddey et al. 2000) or non-homogenised N and water availability in the soil (Stevenson et al. 1995).

Our experiment indicated that the ^{15}N enrichment technique provided accurate and reliable estimations and demonstrated a smaller range of values compared to the ^{15}N natural abundance method. However, it was associated with technical barriers and costs when applied to trees and tends to give higher estimates of BNF. The ^{15}N natural abundance method was simple and inexpensive and our results clearly indicated that this method can be successfully used to determine BNF in Australian native forests despite the within-treatment variation which was probably inevitable under natural conditions.

Negative $\delta^{15}N$ values, observed in this study, were unusual for Australian native forests which normally demonstrated a level of enrichment as a result of isotopic discrimination (Blumfield and Xu 2003; Blumfield et al. 2006; Burton et al. 2007). Two forests in Netherland exposed to elevated N input for 40 years had negative $\delta^{15}N$ soil values (Koopmans et al. 1997) and negative $\delta^{15}N$ values have also been found in forest soil near a highway in Switzerland (Guerrieri et al. 2009). In the present study, negative $\delta^{15}N$ values were probably the result of atmospheric N deposition as this forest was an urban forest that

was bisected by a busy motorway. Fuel combustion in vehicles was one of the major sources for NO_x (Xiao and Liu 2002; Xiao et al. 2010) and the $\delta^{15}N$ in NO_x emitted from vehicles has a range from -13‰ to -2‰ (Heaton 1990). Long-term deposition of atmospheric NO_x, which was depleted in ^{15}N (Choi et al. 2005; Kwak et al. 2011) would lead to the negative soil ^{15}N values observed in our experiment (Choi et al. 2005; Savard et al. 2009). The more negative $\delta^{15}N$ signal in soil led to a good differentiation between the $\delta^{15}N$ signal derived from the atmosphere in the acacias and the soil derived signal in the reference plants. This could also explain the narrower range of BNF estimation in our study than reported elsewhere when using the ^{15}N natural abundance method.

Our results also suggested that taxonomic proximity between legume and reference plant may not be necessary as *T. triandra*, a grass, was able to provide an appropriate $\delta^{15}N$-signal. At Site 2 where mature plants were examined, in addition to *T. triandra*, all other reference plants also gave an appropriate $\delta^{15}N$-signal ($\geq$2‰). This was within the range recommended by Sanford et al. (1994) but lower than the 5‰ suggested by Hogberg (1997). Taxonomic proximity between legume and reference plant was considered as an important factor to obtain accurate results for BNF estimation (Boddey et al. 2000; Gehring and Vlek 2004). However, the successful use of shrubs and herbaceous plants as reference has been also observed in limited studies such as Pareek et al. (1990) and Peoples et al. (1996). Danso et al. (1992) argued that trees and grass take up nutrients from the same soil zone and consequently, the source of N uptake of both trees and grass could be similar. Therefore, woody reference plant and grass give the same $\delta^{15}N$-signal even though both have different root structures. Different mycorrhizae species associated with plant may be resulted in providing different $\delta^{15}N$ signals in plants. If woody plants and grasses have the same mycorrhizae species, they may be providing similar $\delta^{15}N$ signals (Evans 2001). Further work was required to clarify this observation, using different species of grasses and shrubs and examining soil N availability in time and space.

Our study indicated that potential *B* values ranging from -0.3‰ to 1.0‰ could be used to provide an acceptable %Ndfa estimation for *Acacia* species based on ^{15}N natural abundance method in sub-tropical native forests. To our knowledge, a range of *B* values from 0.0‰ to -1.3‰ have successfully been already used to estimate N_2 fixation for *Acacia* spp. (Hamilton et al. 1993; Galiana et al. 2002; May and Attiwill 2003).because the $\delta^{15}N$ abundance of atmospheric N was assumed to be 0.0‰ (Okito et al. 2004). This assumed that the amount of fractionation occurring was negligible when atmospheric N_2 was fixed. Hamilton et al. (1993) successfully applied a *B* value of 0.0‰ to quantify BNF in *A.*

mucronata and *A. melanoxylon* and accepted a minor error when *B* value of a species had not been determined. Guinto et al. (2000) reported a *B* value of -0.04‰ for *A. leiocalyx* which would support our decision to choose *B* value of 0.0‰ for BNF determination.BNF activities of legumes may be increased in less fertile soil (Galiana et al. 2002) to compensate for soil N limitation. In the present study, the high BNF activity indicated at Site 2 suggests a low N availability despite the fact that this area had not been burned for 8 years. Soil N availability slowly increased following fire through litter-fall decomposition and %Ndfa in legumes decreased as plants start taking up N from soil (van Kessel et al. 1994). However, our study indicated a continuing reliance on BNF, which may suggest that this ecosystem requires a longer period to recycle N and to recover from fire. Increasing fire frequency, which has been predicted as a consequence of global climate change, may result in decreasing soil fertility and increasing ecosystem vulnerability.

4.2 Plant physiological status and N_2 fixation

Plant physiological status may be used as another indicator of N_2 fixation because *A. leiocalyx* consistently indicated greater A_n, *E*, iWUE and TN as well as BNF than those of *A. disparimma* throughout the sampling period. Other studies have determined that there was a positive linear relationship between foliar A_n and BNF activities (Kitaoka and Koike 2004; Kaschuk et al. 2009). Higher BNF activity in *A. leiocalyx* may have provided more N for photosynthesis. N is a vital component of Rubisco, a pivotal enzyme to fix CO_2 in the photosynthetic system and enhanced foliar N concentration with increases photosynthetic capacity in plants (Evans 1989). In our study, there was a poor correlation between %Ndfa and physiological traits such as A_n and WUE. A high-within treatment variation may have masked the relationships between plant BNF activities and photosynthesis or WUE.

A. leiocalyx showed greater WUE, reflected through higher $\delta^{13}C$ values and higher iWUE, than *A. disparimma*. Higher WUE may suggest that *A. leiocalyx* would be able to alter the strategy of water use with drought stress as argued by Otieno et al. (2005) and Raddad & Lunkkanen (2006), using this factor as a tool of adaptation to climate change. Increased N could enhance A_n and WUE. Therefore, higher BNF in *A. leiocalyx* may have played an important role to increase A_n and WUE and resulted in higher growth rates (data not presented) and consequently higher C sinks from atmosphere to the earth. Enhanced temperature and prolonged drought may decrease photosynthesis due to increased stomatal closure (Otieno et al. 2005) and plants that have the ability to alter their nutrient and water use strategies may be better able to deal with the adverse impacts of climate change.

5 Conclusions

This study demonstrated that both ^{15}N enrichment and ^{15}N natural abundance techniques may be used to estimate N_2 fixation of acacia tree species. The ^{15}N natural abundance technique was preferred to be used for future studies as it was simple and inexpensive and the use of this method facilitated in the present study due to identifying a wider range of reference plants and narrowing down of *B* values for acacias in native forest. The atmospheric N deposition could be a main reason for successful use of the ^{15}N natural abundance technique leading to provide a good difference of $\delta^{15}N$ signal between acacias and reference plants. This could also explain a narrower range of BNF estimation in our study when using the ^{15}N natural abundance method. The present study also demonstrated that foliar physiological traits such as A_n, A_{max}, iWUE and *E* of plants may be used to indicate plant ability to fix N_2. *A. leiocalyx* with its greater growth and establishment after fire and higher %Ndfa in comparison to *A. disparimma*, may also be able to accelerate N input to the impoverished soils of the Australian urban forests and improve soil fertility. The immediate impacts of environmental extremes such as prolonged drought and frequent fires on the understorey legumes would be expected less severe on *A. leiocalyx* than *A. disparimma*. Therefore, species in which the adaptive capacity is within narrow eco-physiological limits such as *A. disparimma*, would be more likely to become extinct with a consequent reduction in ecosystem biodiversity in response to global climate change and the anticipated more frequent fires.

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Table 1: Soil chemical properties and particle component at Sites 1 and 2 located at Toohey Forest collected from experimental plots at depth 0-10 cm in April 2009.

	Soil chemical properties[a]					Soil particle component		
	pH	TC (%)	TN (%)	C:N	δ^{15}N (‰)	Clay (%)	Silt (%)	Sand (%)
Site 1	5.07 (0.05)	2.71 (0.20)	0.10 (0.00)	13.5 (1.0)	-0.83 (0.42)	25.9 (0.66)	26.0 (0.42)	47.9 (0.46)
Site 2	4.92 (0.10)	3.32 (0.14)	0.12 (0.02)	12.4 (1.3)	-0.92 (0.30)	19.9 (1.20)	23.6 (0.40)	56.4 (0.84)

[a] Soil chemical properties include: soil total C (TC), total N (TN), C:N ratio and N isotope composition (δ^{15}N) Numbers in the brackets denote standard errors.

Table 2: Nitrogen isotope composition (δ^{15}N ‰) of *Acacia* spp. and reference plants during three seasons.

Plant Species	δ^{15}N (‰)					
	Site 1			Site 2		
	Season 1	Season 2	Season 3	Season 1	Season 2	Season 3
A. leiocalyx	-0.73(0.17)	-0.53(0.22)	-0.56(0.16)	-0.58(0.16)	-0.93(0.15)	-0.70(0.07)
A. disparimma	-0.73(0.14)	-1.06(0.19)	-1.26(0.15)	-0.82(0.13)	-1.53(0.11)	-1.28(0.10)
E. planchoniana	-1.87(1.39)*	-5.73(0.65)*	-3.78(0.54) *			
E. psammitica	-2.42(0.65)*	-5.22(0.99) *	-2.72(0.55) *			
E. carnea				-5.40(0.18)*	-6.48(0.07) *	-3.39(0.16) *
E. tindaliae	-0.65(0.05)	-4.39(0.00) *	-5.26(0.00) *	-3.50(0.00) *	-5.74(0.00) *	-3.45(0.00) *
E. resinifera	-0.30(2.9)	-4.40(0.00) *	-2.04(0.72) *	-4.25(0.75) *	-6.42(0.68) *	-4.31(0.00) *
Corymbia henryi				-4.93(0.43) *	-7.05(0.18) *	-3.94(0.24) *
Angophora woodsiana	-1.81(0.42)*	-4.35(0.75) *	-1.91(0.35) *	-4.06(0.53) *	-5.78(0.53) *	-2.95(0.46) *
Xanthorrhoea johnsonii	-1.53(0.32)	-3.24(0.22) *	-1.20(0.00)			
Themeda triandra	-3.45(0.26)*	-6.60(0.23) *	-3.53(0.76) *	-4.21(0.00) *	-5.60(1.02) *	-3.23(0.74) *

Mean standard errors are in parentheses
*P<0.05 level indicates a significant difference in δ_{15}N between *Acacia* spp. and reference plants

Table 3: Net photosynthesis (A_n), C isotope composition ($\delta^{13}C$) and foliar total N (TN) of understorey *Acacia leiocalyx* and *A. disparimma* seedlings in a eucalypt-dominated urban forest of subtropical Australia.

Species†	A_n (μmol m^{-2} s^{-1})			$\delta^{13}C$ (‰)			TN (%)		
	Season 1	Season 2	Season 3	Season 1	Season 2	Season 3	Season 1	Season 2	Season 3
A. leiocalyxs	16.2 (0.86)*	15.0 (1.20)*	18.8 (1.25)*	-31.9 (0.29)ab	-32.7 (0.21)b	-31.4 (0.27)a	2.50 (0.11)	2.68 (0.16)	2.50 (0.16)*
A. disparimma	12.8 (0.53)	11.3 (0.46)	11.9 (0.67)	-32.0 (0.25)a	-32.8 (0.20)b	-31.9 (0.18)a	2.40 (0.06)	2.50 (0.07)	2.05 (0.07)

Means followed by the lower case letters within same row for each variable demonstrate the significant difference in seasons at the level $P<0.05$.

Means followed by no lower case letters within same row for each variable demonstrate no significant difference in seasons at the level $P<0.05$

†Interactions between seasons and species were not significant for all variables

*$P<0.05$ level indicates a significant difference between two species at the sampling season

Fig. 1 Monthly rainfall (mm) (grey columns) and monthly mean daily maximum temperature (℃) (■) of the experimental site, Toohey Forest from January 2009 to March 2010.

Fig. 2 Percentage of N derived from atmosphere (%Ndfa) of understorey *Acacia leiocalyx* (white columns) and *A. disparimma* (grey columns) seedlings at Site 1 using ^{15}N enrichment and ^{15}N natural abundance methods (a and b) and at Site 2 using ^{15}N natural abundance (c) in a eucalypt-dominated urban forest of subtropical Australia. * indicates significant differences between two species at the level $P<0.05$. Bars are means ± standard errors.

Fig. 3 Maximum photosynthesis – A_{max} (a), transpiration – E (b) and instantaneous water use efficiency – iWUE (c) of understorey *Acacia leiocalyx* (white columns) and *A. disparimma* (grey columns) seedlings in a eucalypt dominated urban forest of subtropical Australia. The * indicates significant differences between two species at the level $P<0.05$. Bars are means ± standard errors.

Fig. 4 Relationship between foliar total N (TN) and net photosynthesis (A_n) (a) and between TN and percentage of N derived from atmosphere (%Ndfa) (b) in a eucalypt-dominated urban forest of subtropical Australia.

Fig. 1

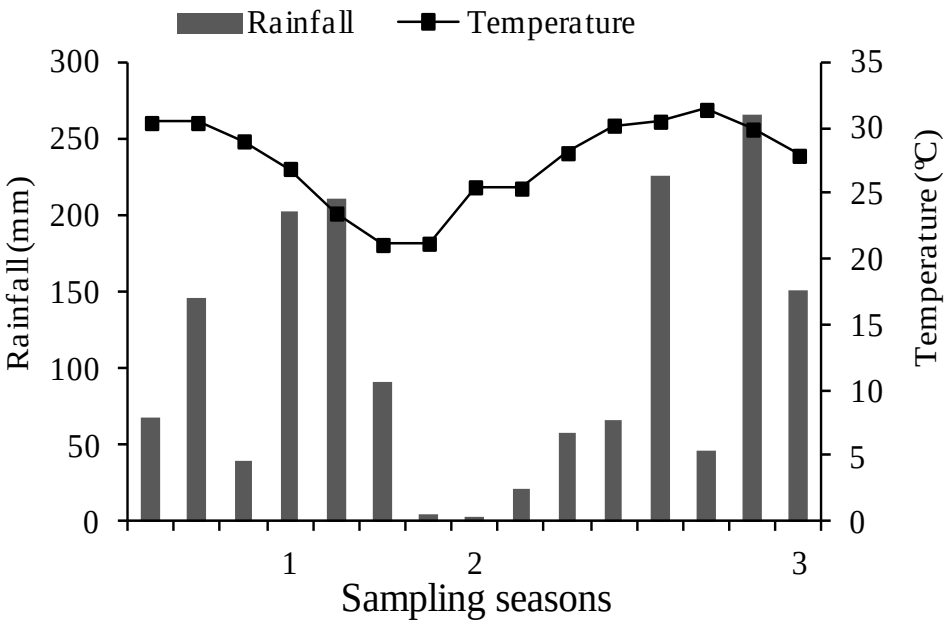
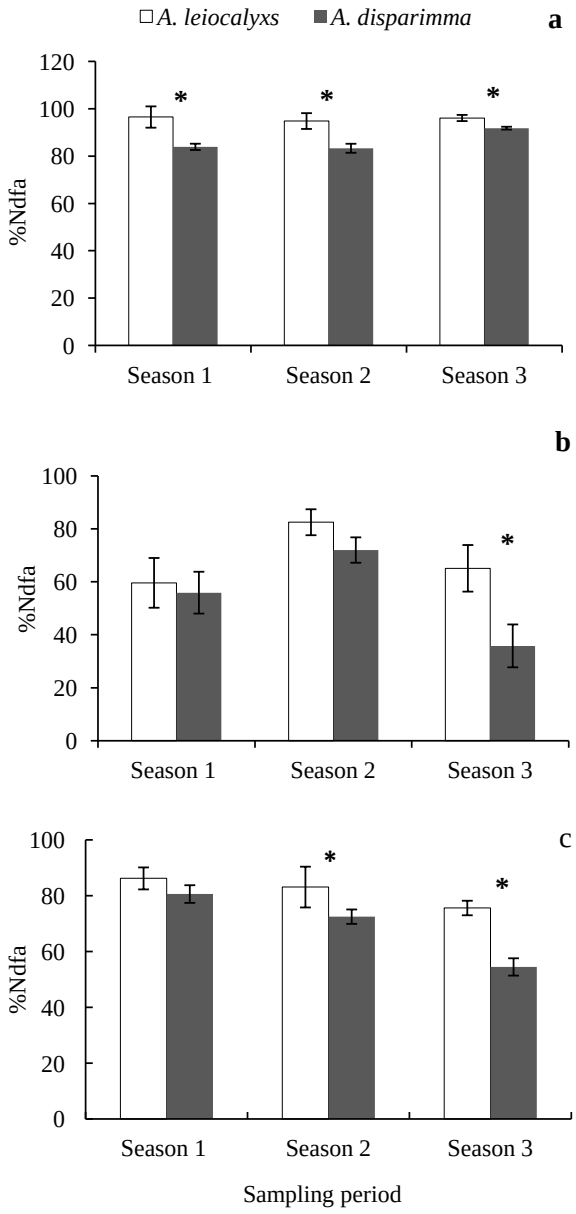

Rainfall
Temperature
300
250
200
150
100
50
0
Rainfall (mm)
35
30
25
20
15
10
5
0
Temperature (℃)
1
2
3
Sampling seasons

Fig. 2


□A. leiocalyxs
■A. disparimma
a
%Ndfa
120
100
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0
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*
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Season 1
Season 2
Season 3
b
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100
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60
40
20
0
*
Season 1
Season 2
Season 3
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*
*
Season 1
Season 2
Season 3
Sampling period

Fig. 3

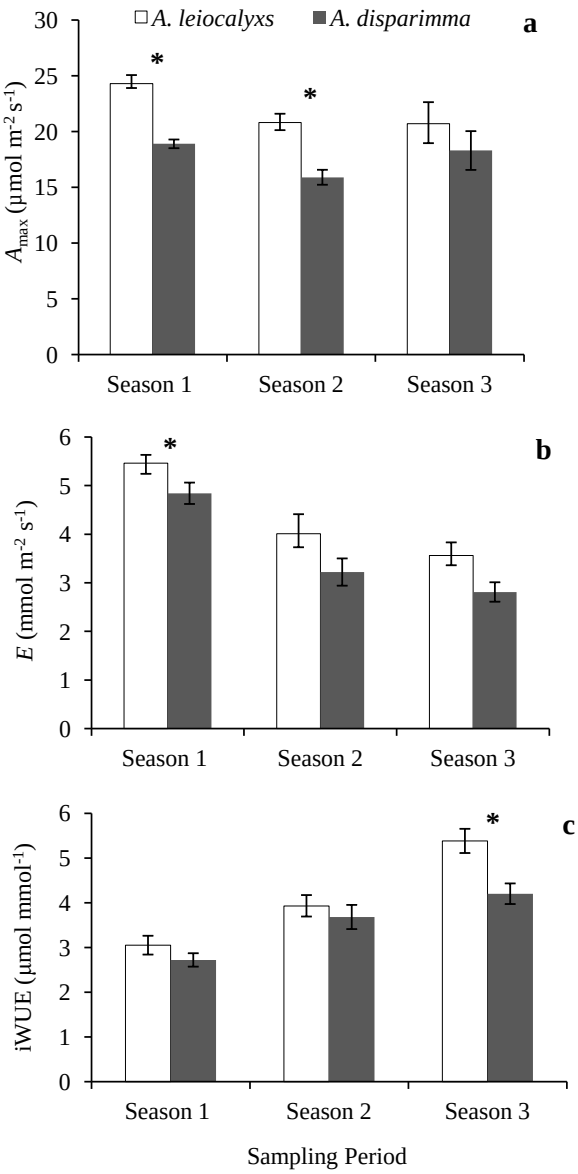
□A. leiocalyxs ■A. disparimma
a
b
c
A_{max} (µmol m^{-2} s^{-1})
E (mmol m^{-2} s^{-1})
iWUE (µmol mmol^{-1})
Season 1
Season 2
Season 3
Sampling Period
*

Fig. 4