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Article

Effects of Biochar Application on Nitrogen Fixation and Water Use Efficiency of Understorey *Acacia* Species as well as Soil Carbon and Nitrogen Pools in a Subtropical Native Forest

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Abstract

This study aimed to examine how biochar and *Acacia* species would affect biological nitrogen fixation (BNF) and water use efficiency (WUE) of understorey *Acacia* species as well as soil carbon (C) and nitrogen (N) pools 15 months after biochar application in the suburban native forest of subtropical Australia. This experiment was established with wood biochar applied at 0, 5, and 10 t ha⁻¹ at 20 months after prescribed burning. We collected foliar and soil samples 15 months after biochar application and used N isotope composition ($\delta^{15}\text{N}$) and carbon isotope composition ($\delta^{13}\text{C}$) to assess the BNF and WUE of two understorey *Acacia* species (*Acacia leiocalyx* and *Acacia disparrima*). We also characterised soil C and N pools and their $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. Biochar did not influence *Acacia* plant BNF and WUE 15 months after biochar application. However, the BNF of *A. leiocalyx* was significantly greater compared with that of *A. disparrima*. The soil under *A. leiocalyx* had greater $\text{NH}_4^+\text{-N}$ (i.e., 10–20 cm) but lower $\delta^{15}\text{N}$ than *A. disparrima*. This study represents one of the few attempts to apply the ^{15}N natural abundance ($\delta^{15}\text{N}$) techniques to quantify the soil–plant–microbe interactions for N cycling in a native forest ecosystem. Understorey *A. leiocalyx* was more effective in improving N recovery post-fire via BNF. Soil under *A. leiocalyx* had greater N availability with lower $\delta^{15}\text{N}$, influencing plant available N sources and $\delta^{15}\text{N}$. Thus, *A. leiocalyx* would be able to fix more N_2 from the air compared with that of *A. disparrima* in the suburban native forest ecosystem subject to periodical fuel reduction prescribed burning.

Keywords: prescribed burning; biochar; *Acacia leiocalyx*; *Acacia disparrima*; ^{15}N natural abundance



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1. Introduction

Forests are important for regulating the terrestrial nitrogen (N) cycle and significantly contribute to mitigating climate change [1–3]. In Australia, various forest management practices, particularly prescribed fuel reduction burning, are applied to reduce the risk of wildfires in native eucalyptus forests [4]. However, frequent burning can limit N availability through various mechanisms, leading to N loss via volatilisation during fire, which consequently restricts its availability [5,6]. Therefore, enhancing N input and adopting sustainable N and water management practices are essential for resilient and healthy forests, particularly those undergoing prescribed burnings [1,7,8].

Biological N fixation (BNF) is an important biological process of understorey leguminous species for maintaining and enhancing N input to the soil, especially under stressed environmental conditions, such as limited water and nutrients [9]. Understorey *Acacia* species significantly contribute to N recovery and C sequestration in post-fire, N-limited Australian forests through biological N fixation (BNF) [9–11]. A complementary study has been conducted at adjacent sites, which has examined the rhizobium root nodule bacteria associated with understorey *Acacia* species, specifically, *A. leiocalyx* and *A. disparrima*. This research has identified BNF as a key mechanism for addressing N limitations six years after prescribed burning in a subtropical suburban forest ecosystem [9]. Additionally, in Portugal, *A. longifolia* has also been found to promote post-fire recovery of *Acacia* spp., *Eucalyptus* spp., and *Pinus* spp. trees [12]. Studies have highlighted that fire directly affects the bacterial diversity and functionality of the BNF mechanisms of *Acacia* species [12]. Hence, *Acacia* species can be considered well-adapted plants that effectively recover from post-fire disturbances and environmental changes [9,12].

Incorporating various organic amendments such as crop residues, cover crops, straw mulching, and wood strand mulching can persist for a longer span and offer multiple benefits in forest ecosystems. These practices can effectively modify soil properties, leading to improved soil N transformations, retention, and soil water holding capacity [13,14]. For example, wood strand mulching application in a coniferous forest management system as a fuel reduction treatment [15] has been shown to enhance mineral N after its application. However, the high C:N ratio of wood strand mulch immobilises N rather than stimulating mineralisation, ultimately limiting the N availability to support long-term revegetation [16]. These considerations are important when selecting organic amendments for effective forest management.

Biochar is an effective C-rich product produced through the pyrolysis of organic materials [17]. The pyrolysis temperature changes the original biochar feedstock materials chemically and biologically during the manufacturing processes and makes biochar more stable and recalcitrant compared with non-pyrolysed materials (i.e., straw mulching, wood strand mulching, and/or plant materials) to decomposition and degradation [18,19]. Biochar plays a vital role in enhancing soil N retention and water use and significantly improves the N cycling between plants and soils through various mechanisms [17,20–22]. Biochar improves water availability and soil pH, which accelerates BNF [23,24]. Biochar reduces N losses, enhances the availability of inorganic N via surface adsorption, and increases the residence time of inorganic N in soils [17,20,25]. This enhanced N retention and improved water availability stimulate plant roots to grow towards the biochar surface for symbiotic relationships [26–29]. After the application of biochar, changes in soil properties modify root growth and activity while increasing root nodulation by rhizobia, thereby impacting BNF directly [26,30,31]. However, biochar application rates can significantly influence plant–soil N cycling processes and N pools [17,21,32], affecting BNF by altering soil pH, enhancing N immobilisation, or changing soil N availability [33,34]. For instance, a study has demonstrated that increasing biochar amounts leads to higher BNF [31], while others have reported opposing results [34–36]. It is important to note that the beneficial effect of biochar application depends on factors such as soil type, biochar characteristics, application rate, local conditions, and interactions with soil and plants [37]. Therefore, the characteristics of the used biochar and environmental factors are parameters that guarantee its maximum benefits.

Water use efficiency (WUE) is a key indicator of photosynthesis and plant ecophysiology assessment in forested ecosystems. This is because increased photosynthesis and reduced stomatal conductance originating from rising CO₂ can result in higher plant WUE [1,38,39]. Generally, foliar carbon isotope composition ($\delta^{13}\text{C}$) is considered an index

of WUE, and a higher $\delta^{13}\text{C}$ indicates higher WUE [40,41]. The use of $\delta^{13}\text{C}$ has been documented to investigate the WUE of plants in suburban native Australian forest ecosystems, as evidenced by the linear relationship between the $\delta^{13}\text{C}$ of plants and their WUE [1,8]. Biochar can enhance WUE [42,43] due to its high internal porosity and large surface area, which decreases soil bulk density and increases porosity and water retention [44–49]. This makes biochar particularly valuable in addressing water limitations caused by climate change, and biochar's potential to improve WUE has received much attention [50–54].

Even though biochar holds considerable potential for revitalising disturbed forest soils, its application in forested ecosystems may not be economically viable yet. However, recent advancements in biochar production, particularly the conversion of slash piles from thinning or overstocking into biochar, have resulted in economically viable products [55]. Furthermore, biochar produced during wildfires can affect post-fire N cycling [56–58]. Despite this, there is still a significant lack of information regarding the application of biochar in forest ecosystems and its impact on various tree species under field conditions [59–61]. Our study focused on investigating the mechanisms driving BNF and WUE of two understorey *Acacia* species following biochar application in a subtropical natural forest. The findings aim to enhance our understanding of the differences and suitability of two *Acacia* species (*A. leiocalyx* and *A. disparrima*) in N recovery and ecosystem resilience of a burned subtropical native forest after the application of biochar.

Understanding N cycling as well as C and N pools in forest soils after biochar application in natural forest ecosystems is therefore critical [61–66]. Hence, this study was designed to (a) assess the biochar surface application on BNF and WUE for two understorey *Acacia* species (*A. leiocalyx* and *A. disparrima*) and (b) assess the effects of biochar and *Acacia* species on soil C and N pools 15 months after biochar application in the post-fire Toohey natural forest to simulate the effects of prescribed burning. This is because prescribed burning can itself create some natural charcoal that may affect the overall N cycling processes. We therefore hypothesize that even at our experimental site (a) biochar application would enhance BNF and WUE of *Acacia* species and the BNF process would be the main mechanism of N input by modifying soil N availability post-fire with variations between the *Acacia* species due to their different growth responses to the prescribed burning 15 months after biochar application and (b) biochar application and *Acacia* species would change soil C and N pools through surface adsorption and N immobilisation in the N limited soil-plant ecosystem 15 months after biochar application.

2. Materials and Methods

2.1. Site Description

The experimental site is situated in Site 7 Block 12B of Toohey Forest (27°32'45" S; 153°02'31" E) (Figure 1), a dry sclerophyll forest in Brisbane, southeast Queensland, Australia. This forest is one of the native eucalypt-dominated forests in Australia, with an understorey comprising grasses, shrubs, and various *Acacia* species [7,9,11]. The landscape of Toohey Forest features hills, valleys, and creeks, resulting in elevations. These elevations vary between 35 m and 195 m above sea level [9]. Toohey Forest is classified as having a subtropical climate, characterised by hot, wet summers and cool, dry winters [7,9,67]. At the time of the establishment of this experimental plot, temperatures ranged from 17.1 to 27.8 °C in 2019, with an annual rainfall of approximately 613.4 mm for that year [7]. During the sampling time of this study in early August 2020, 15 months after biochar application, the mean minimum and maximum temperatures were 11.4 °C and 23.4 °C, respectively, while the total rainfall was 19.4 mm (<http://www.bom.gov.au/climate/current/month/qld/archive/202008.summary.shtml>, accessed on 1 February 2022).

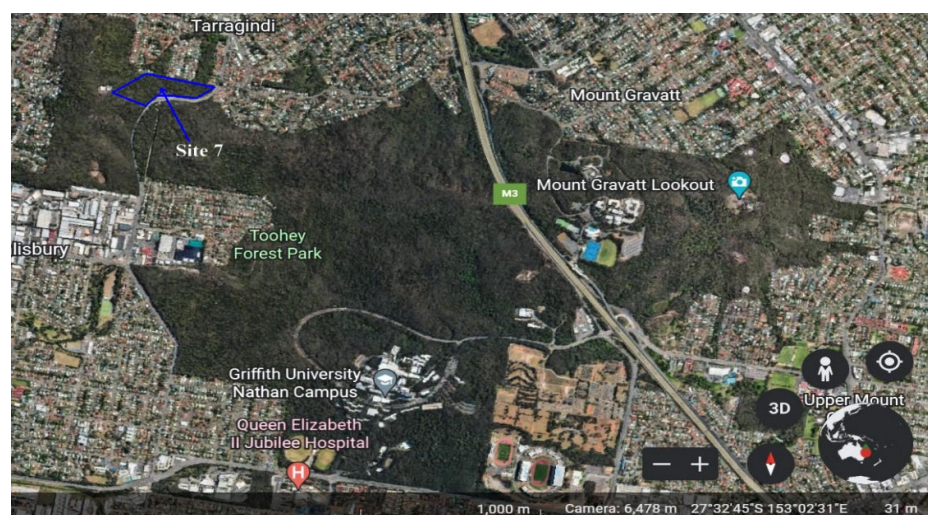


Figure 1. Toohey Forest satellite view. The labelled zone Site 7 is our experimental site.

Since 1993, prescribed burning has been periodically practised in Toohey Forest to reduce the risk and severity of bushfires effectively [68]. The present experimental site was last burnt in August 2017 [7]. The common soil types of Toohey Forest include lithosols, red-yellow podzolic soils, red earth, and alluvial soils; most of these soils are shallow and have poor water storage capacity [8]. The soil in our experimental site is classified as lithosols [69].

2.2. Biochar Production and Characterisation

The biochar used in this study was produced from pine wood (*Pinus radiata*) with slow pyrolysis at 600 °C with a particle size of 25 mm, a rotation speed of 2 rpm for 8 h, and a residence time of 25 min. It exhibits chemical properties including a pH of 7.27, a total C content of 79.4%, a total N content of 0.145%, and isotope compositions of $\delta^{13}\text{C}$ of -27.5‰ and $\delta^{15}\text{N}$ of 2.3‰ . Detailed information regarding the biochar production process and the determination of its isotopic composition has been documented in a complementary study [70].

2.3. Experimental Design and Treatments

The experimental site was established in May 2019 using a randomised complete block design, twenty months after the last burning event. This site was divided into four experimental plots (Figure 2), and each experimental plot had thirteen individual tree plots. We randomly selected twelve *Acacia* plants (e.g., six plants of *A. leiocalyx* and six plants of *A. disparrima*) along with one *E. psammitica* plant. Each plot was marked with steel pegs at the corners, delineating an area of 4 m^2 ($2\text{ m} \times 2\text{ m}$). Earlier studies have highlighted that individual *Acacia* species grow densely after fuel reduction prescribed burning and, on average, at a spacing of 2 m by 2 m if the large overstorey eucalypt species plants are excluded [7]. Our experimental plots comprised a combination of two understorey *Acacia* species (*A. leiocalyx* and *A. disparrima*) $\times$ three biochar rates ($0, 5, \text{ and } 10\text{ t ha}^{-1}$) $\times$ two plants of each *Acacia* species + one eucalyptus species without biochar added = 13 individual tree plots for each of the four plots. We specifically chose *Acacia leiocalyx* and *Acacia disparrima* due to their natural presence and their ability to rapidly re-establish in disturbed areas following fires [4,7,9,71,72]. One eucalyptus plant plot (i.e., *Eucalyptus psammitica*) was used as a reference plant for estimating BNF rates, acting as a non- N_2 -fixing plant for assessing the N_2 fixation by understorey *Acacia* species, and comparing plant growth data in this study. This reference plant was chosen based on the best available information and field

observations from other experimental sites within the same native forest [8,11]. Unlike agricultural settings, the application of biochar in forest soils is different and challenging due to uncertainties related to its performance, technological requirements, and economic feasibility [73]. Therefore, biochar was applied manually on the surface of the forest floor after removing the surface litter and debris to minimise soil disturbance and protect the established root systems [61].



Figure 2. Map of Tooley Forest showing the position of four plots of Site 7, designated as Block 12B.

The experimental treatments included three biochar application rates combined with two *Acacia* species, resulting in a total of 52 plants (13 tree plots $\times$ 4 experimental plots). The biochar application rates of 0 t ha^{−1}, 5 t ha^{−1} and 10 t ha^{−1} corresponded to 0 kg, 2 kg and 4 kg of dry biochar, which were applied on an area of 2 m $\times$ 2 m = 4 m² per plant. We selected 5 t ha^{−1} and 10 t ha^{−1} biochar rates based on the recommendations and findings of previous studies, as this rate is economically feasible [74,75]. Biochar was applied at three different rates of 0, 5, and 10 t ha^{−1} to two plants of each of the two *Acacia* species (*A. leiocalyx* and *A. disparrima*), and no biochar (0 t ha^{−1}) was applied to the reference plant of non-N₂-fixing *E. psammitica*. The soil properties in this study area were analysed before burning, after burning, and before biochar application in 2019 and are reported comprehensively in a complementary study [7].

2.4. Sample Collection and Analysis

2.4.1. Foliar Sample Collection and Analysis

Three fully expanded leaf samples (upper canopy position, ranging from 1–2 m in height) from each *Acacia* plant and one *E. psammitica* plant from each plot were collected 15 months after biochar application. These leaf samples were then dried in an oven at 60 °C until reaching a constant weight and subsequently ground into a fine powder using a RocklabsTM ring grinder (Griffith University Stable Isotope Laboratory, Gold Coast, Australia), resulting in one sample per plant. Approximately 7 mg of the ground plant sample was placed into tin capsules for the analyses of total C, total N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$. These analyses were carried out using an isotope mass spectrometry analysis through an isotope ratio mass spectrometer (GV Isoprime Manchester, UK) [40].

The use of the ¹⁵N natural abundance method has also been successfully employed for determining N₂ fixation in woody legumes of suburban native Australian forest ecosystems following prescribed burning [4,7,9,11]. Earlier studies undertaken in native forest ecosystems have reported a comparison of the two isotope dilution methods, e.g., ¹⁵N enrichment and ¹⁵N natural abundance methods for determining N₂ fixation of understorey

leguminous species after fire conditions [11,76,77]. Between these two methods, ^{15}N natural abundance is considered a valid method for providing quantitative estimates of N_2 fixation in understory leguminous plant species (i.e., *A. leiocalyx* and *A. disparrima*), as this method is relatively simple, less expensive, and reliable than the ^{15}N enrichment method [11]. This is because the ^{15}N abundance of the N in the plant is derived from the air and is expressed as %Ndfa to quantify BNF [76,78]. To estimate the BNF rate of leguminous species through the ^{15}N natural abundance, a compatible reference plant species is required that naturally occurs at the experimental sites, which would need to be of a similar age to the tested *Acacia* species, as well as the most reasonable fitted B values are essential.

In this study, to measure the BNF of *Acacia* plant species, we selected *E. psammitica* for all BNF estimations to minimise potential errors, as this species is a typical non- N_2 fixing reference plant in the experimental area. We selected only one *E. psammitica* species from each plot, ensuring that both tested *Acacia* species were of comparable age to the reference *E. psammitica*. The *E. psammitica* demonstrated a total C of 48.2 (1.50) %, a total N of 1.16 (0.07)%, and exhibited C and N isotope compositions ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of -31.7 (0.83)‰ and -2.14 (0.52)‰, respectively. This study is among the few that have successfully applied ^{15}N natural abundance techniques to quantify soil–plant–microbe interactions within native forest ecosystems, regardless of biochar application [4,8,9,11].

The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values were calculated using the following formula and expressed in parts per thousand (‰) [79]:

$$\delta^{15}\text{N}_{\text{sample}} (\text{‰}) = [(\text{R}_{\text{sample}} - \text{R}_{\text{std}}) / \text{R}_{\text{std}}] \times 1000 \quad (1)$$

$$\delta^{13}\text{C}_{\text{sample}} (\text{‰}) = [(\text{R}_{\text{sample}} - \text{R}_{\text{VPDB}}) / \text{R}_{\text{VPDB}}] \times 1000 \quad (2)$$

where R represents the isotope ratio.

R_{sample} represents the ratio of $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ of the samples.

R_{std} represents the ratio of $^{15}\text{N}/^{14}\text{N}$ of the international standard (atmospheric N_2).

R_{VPDB} represents the ratio of $^{13}\text{C}/^{12}\text{C}$ of the international standard (Vienna Pee Dee Belemnite (VPDB)).

We used the following equation to calculate the N percentage derived from atmospheric N_2 (% Ndfa) [11,80]:

$$\% \text{Ndfa} = [(\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{acacia}}) / (\delta^{15}\text{N}_{\text{ref}} - \text{B}_{\text{value}})] \times 100 \quad (3)$$

where $\delta^{15}\text{N}_{\text{ref}}$ and $\delta^{15}\text{N}_{\text{acacia}}$ represent the $\delta^{15}\text{N}$ value of the sampled reference plant and *Acacia* species, respectively.

To estimate the BNF rate using the ^{15}N natural abundance method, the potential B value of the *Acacia* spp., particularly *A. leiocalyx* and *A. disparrima*, falls within the range of -0.3‰ to 1‰ under similar experimental conditions [11]. In this study, a B value of 0.3‰ was used for the calculations of BNF determinations.

2.4.2. Acacia Species Measurement

The height (H) and diameter at ground level (DGL) of the *Acacia* species were measured 15 months after biochar application. Basal area (BA) was calculated as follows:

$$\text{BA} = \pi \times (\text{DGL})^2 / 40,000 \quad (4)$$

where BA is the tree basal area (m^2), DGL is the diameter at ground level (cm), and π has a fixed value of 3.142.

The volume of trees was calculated based on the following formula:

$$V = 0.33 \times BA \times H \quad (5)$$

where 0.33 is the standard value of trees in a cone shape.

It is important to highlight that the selected plant species (e.g., *Acacia* species and the reference plant) were grown naturally at this site following the fire. At the time of sampling, these plants were approximately 1–3 years old, and some *A. leiocalyx* plants were observed to be very small, broken, or dead. These conditions might affect the plant growth parameters, leading to significant height and diameter variability between the two *Acacia* species.

2.4.3. Soil Sample Collection and Analysis

Soil samples were also collected randomly 15 months after biochar application using an auger with a diameter of approximately 7.5 cm. Samples were taken from four different points from each of the thirteen plants within each of the four plots at a depth of 0–5, 5–10, and 10–20 cm (no litter included). Soil samples collected under the same plant species from each plot were mixed homogeneously after sieving through a 2 mm sieve to constitute two samples for each plot. A sub-sample of the field moist soil was stored at 4 °C, while the remaining portion of the soil was dried in the air and stored in sealed plastic containers for further analysis.

A sub-sample of the air-dried soil was finely ground into powder using a Rocklabs™ ring grinder. Approximately 40 mg of ground soil samples were placed into tin capsules for the 0–5 and 5–10 cm depth ranges, whereas for the analysis at the 10–20 cm depth, around 50 mg of soil was pelletised to facilitate isotope mass spectrometry [8].

Soil mineral N (NH_4^+ -N and NO_3^- -N) was extracted using a 2 M KCl solution. The detailed protocols for soil N extraction and the micro-diffusion method for determining the soil $\delta^{15}\text{N}$ of NH_4^+ -N and $\delta^{15}\text{N}$ of NO_3^- -N have been documented in previous studies [17,70]. It is important to mention that the level of soil NO_3^- -N and $\delta^{15}\text{N}$ of NO_3^- -N were below the detectable limit.

To determine the hot water extractable organic C (HWEOC) and hot water extractable total N (HWETN), 7 g of air-dried soil was added to 35 mL of distilled water (1:5 ratio) in a Falcon tube. For hot water extraction, these soil–water mixed samples were incubated at 70 °C for 18 h and shaken for 5 min by an end-over-end shaker, followed by 10 min centrifuging at 10,000 rpm. The suspension was first filtered through 42 Whatman filter paper (Cytiva, China), followed by a 0.45 µm Millex Syringe filter (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co Cork, Ireland). Water-extractable organic C (WEOC) and water-extractable total N (WETN) were determined by following the same protocol of hot water extraction, excluding the 18-h incubation step. The concentrations of organic C and total N in the filtrate solutions from both hot water extraction and water extraction samples were measured using a Shimadzu TOC-VCSH/CSN and TOC/N Analyser (Shimadzu Corporation, Japan) [81].

2.5. Statistical Analysis

All data were tested for normal distribution. A two-way analysis of variance (ANOVA) with the Tukey test ($p < 0.05$) was performed to determine the significant difference in foliar and soil variables among biochar application rates, *Acacia* species, and their interactions. The analysis was performed using statistical software Statistix (v2003). Regressions were employed to investigate the relationship among leaf physiological variables, BNF, and soil properties by using IBM SPSS (v2019).

3. Results

3.1. Foliar C and N as well as BNF of *Acacia* Species

Foliar total C and total N did not exhibit significant differences with the application of biochar. However, clear variations in foliar total C and total N were observed between the two *Acacia* species. *A. disparrima* recorded a significantly higher foliar total C compared to *A. leiocalyx*, while an opposite trend was observed for foliar total N, with *A. leiocalyx* containing a higher total N than *A. disparrima* (Table 1). There were no significant differences in WUE as indexed by foliar $\delta^{13}\text{C}$ either among the biochar application rates or between the two *Acacia* species (Table 1). Although biochar application did not significantly affect foliar $\delta^{15}\text{N}$, differences were observed between the two *Acacia* species. A greater $\delta^{15}\text{N}$ was found in *A. leiocalyx* than in *A. disparrima* (Table 1). It is also important to highlight that there were no significant interactions between biochar application and *Acacia* species regarding total C, total N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ (Table S1).

Table 1. Effects of biochar application rates and understorey *Acacia* species on foliar total carbon (TC), total nitrogen (TN), carbon (C) and nitrogen (N) isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively), and biological N fixation (BNF) of *Acacia leiocalyx* and *Acacia disparrima* 15 months after biochar application in a sub-tropical native forest, Australia.

Treatments	TC (%)	TN (%)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	BNF (%)
Biochar rates (t ha^{-1})					
0	48.90 a *	2.37 a	−32.25 a	−0.66 a	56.43 a
5	49.07 a	2.31 a	−31.78 a	−0.84 a	49.65 a
10	49.26 a	2.20 a	−32.50 a	−0.73 a	55.43 a
<i>Acacia</i> species					
<i>Acacia leiocalyx</i>	48.36 b	2.51 a	−32.28 a	−0.61 a	60.35 a
<i>Acacia disparrima</i>	49.79 a	2.08 b	−32.08 a	−0.87 b	47.34 b

* Lower case letters indicate significant differences among biochar rates and *Acacia* species at each column at $p < 0.05$.

The application of biochar did not affect the BNF of the *Acacia* species (i.e., *A. leiocalyx* and *A. disparrima*) 15 months post-application. However, there was a significant difference in BNF between the two *Acacia* species. *A. leiocalyx* exhibited a higher BNF of 60.35%, compared to *A. disparrima*, which recorded a BNF of 47.34% (Table 1). No significant interactions for BNF were observed between biochar application rates and the *Acacia* species (Table S1).

3.2. Plant Growth

There were no differences in plant growth parameters, e.g., plant height, diameter at ground level (DGL), basal area (BA), and volume, between the two *Acacia* species or among biochar rates 15 months after biochar application (Table 2).

Table 2. Effects of biochar application rates and understorey *Acacia* species on plant height (cm), diameter at ground level (DGL, cm), basal area (BA, cm^2), and volume (cm^3) of *Acacia leiocalyx* and *Acacia disparrima* 15 months after biochar application in a sub-tropical native forest, Australia.

Treatments	Height (cm)	DGL (cm)	BA (cm^2)	Volume (cm^3)
Biochar rates (t ha^{-1})				
0	119.56 a *	3.29 a	9.34 a	435.40 a
5	119.31 a	3.36 a	9.61 a	434.18 a
10	137.06 a	3.80 a	12.58 a	639.89 a
<i>Acacia</i> species				
<i>Acacia leiocalyx</i>	119.17 a	3.38 a	10.05 a	467.29 a
<i>Acacia disparrima</i>	131.46 a	3.58 a	10.97 a	539.02 a

* Lower case letters indicate significant differences among biochar rates and *Acacia* species in each column at $p < 0.05$.

3.3. Soil C and N Pools

3.3.1. 0–5 cm Depth

Soil total C, total N, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ were not significantly affected by biochar application rates or the *Acacia* species (Table 3). However, soil $\text{NH}_4^+\text{-N}$ was significantly influenced by biochar application rates compared with that of the control soil. Biochar application reduced soil $\text{NH}_4^+\text{-N}$ compared with the no-biochar amended soil, but no differences were observed between the two biochar application rates (e.g., 5 t ha^{-1} and 10 t ha^{-1}). Soil $\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ was not affected either by biochar rates or *Acacia* species 15 months after biochar application (Table 3).

Table 3. Effects of biochar application rates and understory *Acacia* species on soil total carbon (TC), total nitrogen (TN), carbon (C) and nitrogen (N) isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively), $\text{NH}_4^+\text{-N}$ ($\mu\text{g N g}^{-1}$), and $\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ (‰) at different soil depths (0–5, 5–10, and 10–20 cm) 15 months after biochar application in a sub-tropical native forest in Australia.

Treatments	TC (%)	TN (%)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\text{NH}_4^+\text{-N}$ (μgNg^{-1})	$\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ (‰)
0–5 cm						
Biochar rates (t ha^{-1})						
0	7.89 a *	0.290 a	−26.49 a	0.434 a	6.39 a	1038.40 a
5	8.37 a	0.316 a	−26.51 a	0.289 a	4.38 b	1050.88 a
10	8.52 a	0.320 a	−26.39 a	0.282 a	4.24 b	1045.04 a
<i>Acacia</i> species						
<i>Acacia leiocalyx</i>	8.29 a	0.311 a	−26.54 a	0.242 a	4.53 a	1045.77 a
<i>Acacia disparrima</i>	8.23 a	0.306 a	−26.38 a	0.428 a	5.48 a	1043.75 a
5–10 cm						
Biochar rates (t ha^{-1})						
0	5.03 b	0.189 a	−25.95 a	0.784 a	9.34 a	1018.84 a
5	5.58 a	0.208 a	−25.97 a	0.571 a	7.67 a	1019.23 a
10	5.57 a	0.211 a	−25.96 a	0.588 a	8.11 a	1017.99 a
<i>Acacia</i> species						
<i>Acacia leiocalyx</i>	5.49 a	0.210 a	−26.13 b	0.522 b	8.83 a	1020.11 a
<i>Acacia disparrima</i>	5.27 a	0.195 a	−25.79 a	0.773 a	7.91 a	1017.27 a
10–20 cm						
Biochar rates (t ha^{-1})						
0	3.71 a	0.133 a	−25.46 b	1.96 a	6.93 a	1041.37 a
5	3.60 a	0.137 a	−25.54 b	1.81 a	5.98 b	1034.63 a
10	3.75 a	0.150 a	−25.18 a	2.05 a	6.86 a	1039.33 a
<i>Acacia</i> species						
<i>Acacia leiocalyx</i>	3.55 a	0.139 a	−25.50 b	1.67 b	7.09 a	1034.44 a
<i>Acacia disparrima</i>	3.82 a	0.140 a	−25.29 a	2.21 a	6.09 b	1042.45 a

* Lower case letters indicate significant differences among biochar rates and *Acacia* species in each column at $p < 0.05$.

Neither biochar application rates nor *Acacia* species show any significant differences in HWEOC, HWETN, WEOC, and WETN (Table 4). Additionally, there were no significant interactions between biochar application rates and *Acacia* species for HWEOC and HWETN or WEOC and WETN (Table S3).

Table 4. Effects of biochar application rates and understory *Acacia* species on soil (0–5 cm) hot water extractable organic carbon (HWEOC), hot water extractable total nitrogen (HWETN), water extractable organic carbon (WEOC) and water extractable total nitrogen (WETN) 15 months after biochar application in a sub-tropical native forest in Australia.

Treatments	HWEOC ($\mu\text{g g}^{-1}$)	HWETN ($\mu\text{g g}^{-1}$)	WEOC ($\mu\text{g g}^{-1}$)	WETN ($\mu\text{g g}^{-1}$)
Biochar rates (t ha^{-1})				
0	688.54 a *	74.71 a	294.76 a	15.36 a
5	610.13 a	38.01 a	226.40 a	12.98 a
10	790.67 a	45.59 a	254.54 a	12.09 a

Table 4. Cont.

Treatments	HWEOC ($\mu\text{g g}^{-1}$)	HWETN ($\mu\text{g g}^{-1}$)	WEOC ($\mu\text{g g}^{-1}$)	WETN ($\mu\text{g g}^{-1}$)
		<i>Acacia</i> species		
<i>Acacia leiocalyx</i>	697.97 a	42.92 a	237.98 a	12.41 a
<i>Acacia disparrima</i>	694.92 a	62.62 a	279.14 a	14.54 a

* Lower case letters indicate significant differences among biochar rates and *Acacia* species at each column at $p < 0.05$.

3.3.2. 5–10 cm Depth

Soil total C was significantly greater with biochar-amended soils compared with non-biochar-amended soils, although no differences were found between the *Acacia* species. Soil total N did not show any significant differences among the biochar application rates or between the *Acacia* species (Table 3). Soil $\delta^{13}\text{C}$ was not influenced by biochar application rates, but varied significantly between the *Acacia* species, with greater soil $\delta^{13}\text{C}$ values observed under *A. disparrima* soil compared with that of *A. leiocalyx* soil (Table 3). No significant differences in soil $\delta^{15}\text{N}$ were found among the biochar application rates; however, a higher $\delta^{15}\text{N}$ was observed under *A. disparrima* soil compared with that of *A. leiocalyx* soil (Table 3). Soil $\text{NH}_4^+\text{-N}$ and $\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ were not significantly affected by biochar application rates or the *Acacia* species (Table 3). At this depth (5–10 cm), no significant interactions between the biochar application rates and *Acacia* species were observed in soil properties (Table S2).

3.3.3. 10–20 cm Depth

No significant differences were observed in soil total C and total N for either biochar application rates or by *Acacia* species. However, soil $\delta^{13}\text{C}$ was significantly influenced by both the biochar application rates and the *Acacia* species. The application of 10 t ha^{-1} biochar resulted in higher $\delta^{13}\text{C}$ values compared to soils with lower or no biochar amendments (Table 3). Soil $\delta^{13}\text{C}$ also varied significantly with *Acacia* species, and soils under *A. disparrima* showed higher $\delta^{13}\text{C}$ than that of *A. leiocalyx* soil (Table 3). Biochar application rates did not show any significant differences in soil $\delta^{15}\text{N}$; however, $\delta^{15}\text{N}$ varied with *Acacia* species. Soil $\delta^{15}\text{N}$ was lower under *A. leiocalyx* compared to *A. disparrima*. The concentration of $\text{NH}_4^+\text{-N}$ in the soil was also significantly influenced by both biochar application rates and the *Acacia* species. The $\text{NH}_4^+\text{-N}$ concentration was significantly lower at the 5 t ha^{-1} than in both the control and 10 t ha^{-1} biochar application rates (Table 3). However, soil $\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ was not significantly affected either by biochar application rates or the *Acacia* species (Table 3). Between the two *Acacia* species, *A. leiocalyx* soil had greater $\text{NH}_4^+\text{-N}$ than that of *A. disparrima* (Table 3).

3.4. Relationships Among Foliar Properties, BNF, and Soil Properties

Foliar $\delta^{15}\text{N}$ demonstrated a strong relationship with BNF, with *A. leiocalyx* showing significantly higher BNF compared to that of *A. disparrima* (Figure 3a,b). In most instances, there was no relationship between foliar total N and soil N pools measured 15 months after the application of biochar. Foliar $\delta^{15}\text{N}$ of *A. leiocalyx* showed a significant relationship with soil $\delta^{15}\text{N}$ at the depth of 10–20 cm (Figure 4a); however, foliar $\delta^{15}\text{N}$ of *A. disparrima* did not show any significant relationship with soil $\delta^{15}\text{N}$ (Figure 4b).

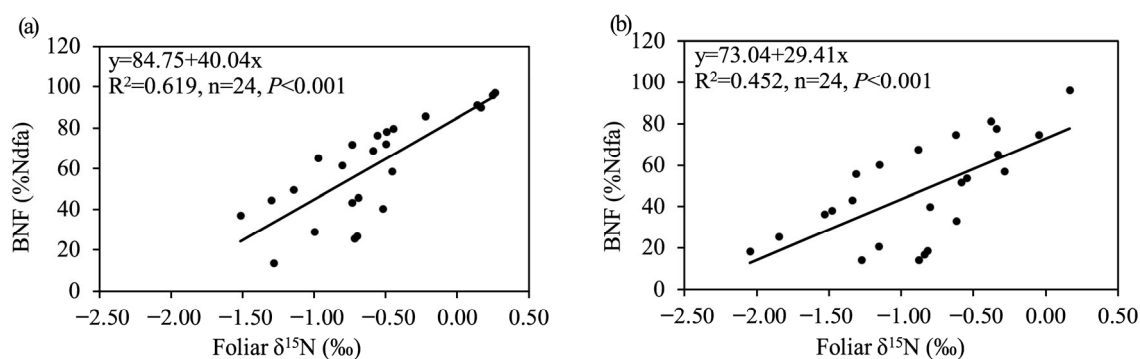


Figure 3. Linear relationships between foliar $\delta^{15}\text{N}$ (‰) and biological N fixation (BNF) rate (%Ndfa) of *A. leiocalyx* (a) and *A. disparrima* (b). The relationships represent the variation between the two *Acacia* species and show the contribution of foliar $\delta^{15}\text{N}$ in changing the BNF rate of *A. leiocalyx* and *A. disparrima*, respectively.

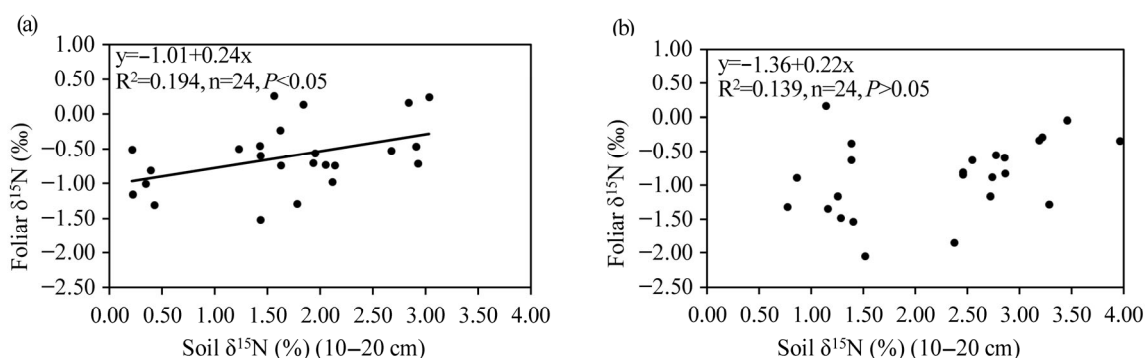


Figure 4. Linear relationships between foliar $\delta^{15}\text{N}$ (‰) and soil $\delta^{15}\text{N}$ (10–20 cm depth) of *A. leiocalyx* (a) and *A. disparrima* (b). These relationships represent the variation between foliar $\delta^{15}\text{N}$ of *A. leiocalyx* and *A. disparrima*, respectively.

4. Discussion

4.1. Biochar Effects on BNF and WUE of *Acacia* Species

Biochar did not affect the BNF of either *Acacia* species 15 months after biochar application. There might be a few reasons for the lack of biochar effects on BNF in the field experiment. For example, biochar is a shelter for microbes, and it can affect the microbes involved in N cycling [17,20,82], including those involved in BNF (i.e., rhizobia) [31,82]. Biochar can enhance exposure to rhizobia by enhancing nodulation and increasing BNF when in contact with plant roots [30,83,84]. We did not incorporate biochar into the soil to mimic natural biochar spread on the forest floor after fires. Therefore, the lack of biochar effects on the BNF in our study could be explained as biochar could not reach the root zone of plants to stimulate root nodules and increase the BNF. Even when biochar has been incorporated into the soil (0–20 cm), biochar may not affect N_2 fixation due to a lack of nitrogenase activity or root colonisation of symbiotic organisms in the rhizosphere [85].

We investigated the impact of biochar after prescribed burning. Earlier studies have shown evidence that burning can impact the rhizosphere microbial activity by influencing the C:N ratio and N availability three years after burning [86]. The lack of biochar effect on BNF in our study could be associated with either the burning history of our experimental site (e.g., we sampled three years after burning), which affected the N cycling processes and plant–soil interactions [23]. Additionally, as we did not apply any fertiliser, biochar application alone could not influence the root growth and root nodulation to stimulate the N_2 fixation 15 months after biochar application [30]. We conclude that in the post-fire forest ecosystem, it may need more time to have the positive effects of biochar application on

the BNF system of the understorey *Acacia* species. However, it is difficult to interpret the mechanisms of why biochar did not influence the BNF rate because most existing studies so far focus on the interaction of biochar and BNF in agricultural crops and controlled environments [33,34,87], whereas the N₂ fixation process in post-fire forest ecosystems may exhibit different responses due to variations in climate, soil, burning history, and vegetation types.

Biochar application rate could be another factor for the insignificant effect of biochar on BNF in our study. An increased BNF of *A. tetragonophylla* has been reported in a glasshouse experiment after six months of biochar application at 37 t ha⁻¹ and 74 t ha⁻¹ [88]. These biochar application rates are much greater than our biochar application rates. However, some earlier studies have reported lower or insignificant changes in N₂ fixation with a greater amount of biochar application [34,36]. Low biochar application rates (e.g., 0.112 t ha⁻¹) in another study have also been associated with the lack of biochar effects on BNF [89]. A meta-analysis has reported the greatest enhancement of N₂ fixation at 10–20 t ha⁻¹ biochar application rates [36]. Given that we suggest that biochar effects on soil N may take a long time to impact when wood biochar is applied, the long-term effects of biochar on soil N in forests remain to be seen.

Similar to BNF, biochar application did not influence the WUE of *Acacia* species 15 months after its application, as shown by the lack of differences in foliar $\delta^{13}\text{C}$. The lack of WUE in response to biochar application in the present study may be due to the biochar type (i.e., wood biochar) [42,90]. Wood biochar normally has low ash content but a higher C:N ratio [90]. The low ash content is associated with hydrophobicity, while a higher C:N ratio (e.g., >20) indicates N immobilisation [90,91]. An earlier greenhouse pot study undertaken for six months at a mine site has also shown that biochar application did not affect the foliar $\delta^{13}\text{C}$ of *Acacia* species (i.e., *A. tetragonophylla*) [88]. The authors have suggested that their experimental pot trial has maintained a consistent water holding capacity (WHC) throughout the experimental period, which likely limited the potential effect of biochar on the water use efficiency of the *Acacia* species [88].

Hence, although biochar has been shown to either increase or decrease WUE in plants, the effectiveness of biochar on WUE can vary depending on factors such as soil type, biochar type, and management practices [42,50,92–94]. Additionally, most studies on this topic have focused on agricultural contexts, so it is reasonable to expect that the influence of biochar on the WUE of understorey *Acacia* species may differ to some extent in forest ecosystems.

4.2. *Acacia* Species Differences in Foliar $\delta^{15}\text{N}$ and BNF

Negative $\delta^{15}\text{N}$ values were observed in *Acacia* species in this study, indicating that these plants were grown close to major highways with a lot of ¹⁵N depleted N deposition from vehicle emissions and with surface soil N to be ¹⁵N depleted or negative $\delta^{15}\text{N}$ [95]. The negative $\delta^{15}\text{N}$ values of *Acacia* species are not uncommon and have been documented in this experimental forest area previously [4,8]. Our findings revealed that *A. leiocalyx* exhibited less negative $\delta^{15}\text{N}$ or closer to '0' of atmospheric $\delta^{15}\text{N}$ compared to *A. disparrima* (Table 1). This observation suggests that *A. leiocalyx* has greater potential for fixing atmospheric N through BNF. As a result, this species appears to rely less on N from the soil, allowing it to release more readily available N for uptake when necessary [96]. We also observed a negative $\delta^{15}\text{N}$ value (−2.14‰) for reference plants, highlighting significant N deposition from highways with ¹⁵N-depleted N into the soil [95]. This finding is consistent with previous studies conducted in Toohey Forest, which also reported the negative $\delta^{15}\text{N}$ values of reference plants, regardless of biochar application [8]. Therefore, the negative $\delta^{15}\text{N}$ values observed in *Acacia* species in our study are mainly influenced by the ¹⁵N depleted N deposition, resulting from vehicle emissions along highways. These findings reflect the

influence of highway-derived N deposition on soil N dynamics and the $\delta^{15}\text{N}$ signatures in the study area.

The estimated BNF rates from this study revealed a significant difference between the two *Acacia* species 15 months after the application of biochar. Our findings showed that the BNF rates were lower, with 60.35% for *A. leiocalyx* and 47.34% for *A. disparrima*, in contrast to the higher rates reported in another complementary study. For example, earlier studies documented BNF percentages ranging from 59.6% to 82.5% for *A. leiocalyx* and 35.8% to 72.0% for *A. disparrima*, irrespective of biochar application [8,11]. Furthermore, BNF can vary seasonally, with greater BNF for *A. leiocalyx* during the winter season, compared to summer [8]. Given that our sampling was conducted during the winter season, the enhanced BNF observed for *A. leiocalyx* may be attributed to these seasonal variations. Despite the lower BNF rates observed in our study compared with previous studies, the significant positive relationship between foliar $\delta^{15}\text{N}$ and BNF rates in *A. leiocalyx* indicates this species is more effective in terms of N recovery and ecosystem resilience in prescribed burned forest ecosystems when compared to *A. disparrima* (Figure 3a,b).

4.3. *Acacia* Species Effects on Soil N Pools

Our findings revealed that the concentration of $\text{NH}_4^+\text{-N}$ in the soil under *A. leiocalyx* was significantly higher than that under *A. disparrima* in the soil profile at a depth of 10–20 cm under the field conditions. The enhanced soil $\text{NH}_4^+\text{-N}$ concentration under *A. leiocalyx* resulted in a significantly lower soil $\delta^{15}\text{N}$ at the same depth, indicating that the BNF of *A. leiocalyx* would effectively be fixed from the atmosphere to the root zone, within the 10–20 cm soil, leading to a lower $\delta^{15}\text{N}$. In contrast, soil enriched $\delta^{15}\text{N}$ suggests an enhanced N availability, which can result in greater N loss, as the lighter isotope form of ^{14}N than heavier ^{15}N during N transformation processes (e.g., ammonia volatilisation, nitrate leaching via nitrification, and denitrification). Consequently, the soil system becomes enriched in heavier ^{15}N substrates due to the rapid movement of these substrates during N loss [97,98]. Thus, the lower $\delta^{15}\text{N}$ with *A. leiocalyx* suggests that its higher rate of BNF enhanced N availability in the soil, leading to a lower $\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ of *A. leiocalyx*. Hence, a lower soil $\delta^{15}\text{N}$ implies an improved soil N recovery by *A. leiocalyx* following prescribed burning.

We did not observe any significant relationships between foliar $\delta^{15}\text{N}$ and soil-available specific N sources (i.e., $\text{NH}_4^+\text{-N}$). However, our study indicated a significant positive relationship between soil $\delta^{15}\text{N}$ and foliar $\delta^{15}\text{N}$ of *A. leiocalyx* at a depth of 10–20 cm (Figure 4a), suggesting that the $\delta^{15}\text{N}$ signature of soil N significantly affected the patterns of foliar $\delta^{15}\text{N}$. One potential mechanism to explain the positive relationship between foliar $\delta^{15}\text{N}$ and soil $\delta^{15}\text{N}$ could be that our experimental site had a short history of prescribed burning with relatively young vegetation. Consequently, the N_2 fixation process not only impacts the plant but also changes the soil properties. This change in soil $\delta^{15}\text{N}$ influenced plant available N sources, leading to the effect of plant $\delta^{15}\text{N}$ on BNF. Our results support the findings of other researchers who have reported that the mechanisms influencing soil $\delta^{15}\text{N}$ also influence foliar $\delta^{15}\text{N}$ through N uptake regardless of biochar application [99]. However, a complementary study has reported that after one rotation of fire, BNF becomes one of the primary mechanisms for N replenishment and recovery [8]. Another study has found that long-term burning has enhanced N_2 fixation in *A. aulacocarpa* compared to plants being grown in an unburnt treatment. Therefore, we expect that even at our experimental site, BNF would be the main mechanism of N input within a few years following the last burning application.

To summarise, this study was designed to assess the effect of biochar surface application on BNF, WUE, as well as soil labile C and N pools 15 months after biochar application

to simulate the effects of prescribed burning. This is because prescribed burning can itself create some natural charcoals that may affect the overall N cycling processes [57]. Effects of wood-based biochar on soil inorganic N are usually evident from one year onwards [22]. The lack of significant biochar effect could be due to multiple factors, including but not limited to the quite dry period experienced in the 15 months of biochar application, large field variations in soil water and N availability as well as low rates of biochar application. It is pertinent to note that although 15 months is not a long enough time to observe the effects of biochar application, it is one of the few steps in the evaluation of the effects of potential biochar application on BNF, WUE, as well as soil labile C and N pools in a suburban native forest. Hence, long-term experiments under similar settings are recommended to answer specific research questions.

5. Conclusions

This study revealed that biochar application had no significant effect on foliar total N, $\delta^{15}\text{N}$, BNF, and WUE of the understorey *Acacia* species 15 months after biochar application. However, BNF varied significantly between different *Acacia* species, with a significantly higher rate for *A. leiocalyx* compared with that of *A. disparrima*. This finding suggested the contribution and suitability of *A. leiocalyx* for the recovery of N and its crucial role in enhancing ecosystem resilience post-fire compared with that of *A. disparrima*. Moreover, the significant positive relationship between soil $\delta^{15}\text{N}$ (10–20 cm) and foliar $\delta^{15}\text{N}$ highlights that the mechanisms influencing soil $\delta^{15}\text{N}$ also influence plant $\delta^{15}\text{N}$ through N uptake. This finding can be used as an indicator to monitor the insights of the N transformation processes and plant–soil interactions of the suburban native forest post-fire. They can also be helpful in accurately modelling ecosystem nitrogen budgets to assess nitrogen conservation and carbon sequestration in natural forest ecosystems. This study represents one of the few attempts worldwide to highlight the important interactions among soil, plant, and microbes under field conditions, as revealed by foliar $\delta^{15}\text{N}$ and soil $\delta^{15}\text{N}$, along with NH_4^+ -N availability in native forest ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f16081350/s1>.

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