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Long-term effects of biochar application on biological nitrogen fixation of acacia species and soil carbon and nitrogen pools in an Australian subtropical native forest

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Abstract

Purpose Biological nitrogen (N) fixation (BNF) of understory acacia species presents a potential way for effectively restoring N in forest systems. This study aimed to quantify the impact of acacia species and biochar application rates on BNF and soil mineral N in a suburban native forest of subtropical Australia in the first 4–5 years after prescribed burning.

Method Plant growth values and BNF were measured to assess the impact of biochar rates at 0, 5, and 10 t ha^{-1} on different acacia species. Soil NH_4^+-N and NO_3^--N along with their N isotope composition ($\delta^{15}N$) were determined to investigate soil–plant interactions in response to acacia species and biochar application.

Results The application of 10 t ha^{-1} biochar significantly enhanced the growth of acacia species, and concurrently reduced the loss of NO_3^--N at soil depths of 0–5 and 5–10 cm. Compared with *Acacia disparimma* (percentage of N derived from the atmosphere or %Ndfa: 78.2%), *A. leiocalyx* demonstrated significant higher BNF ability (%Ndfa: 91.3%). Similarly, *A. leiocalyx* had better growth, in terms of height (269.1 cm versus 179.6 cm), diameter at ground level (2.62 cm versus 1.94 cm), basal area (6.49 cm^2 versus 3.43 cm^2) and volume (692.2 cm^3 versus 258.0 cm^3). This was associated with its ability to promote organic matter mineralization, resulting in the accumulation of ^{15}N-depleted NH_4^+-N. NH_4^+-N, acting as a substrate, was transformed into NO_3^--N through nitrification. From regression analysis, the efficient absorption of NH_4^+-N by *A. leiocalyx* significantly mitigated NH_4^+-N leaching with increasing soil moisture concentration (SMC), resulting in lower $\delta^{15}N$ of NH_4^+-N, which was more negatively related to SMC ($R^2 = 0.401$), compared to that of *A. disparimma* ($R^2 = 0.250$) at soil depth of 0–5 cm. The production of NO_3^--N was reduced, leading to lower NO_3^--N concentrations of *A. leiocalyx* than *A. disparimma* at soil depth of 0–5 cm (8.06 μg N g^{-1} versus 9.61 μg N g^{-1}) and that of 5–10 cm (8.24 μg N g^{-1} versus 9.21 μg N g^{-1}) respectively.

Conclusions As an effective soil amendment, biochar exhibited promise in reducing mineral N loss and stimulating plant growth in long-term applications of exceeding three years. Higher BNF capacity and greater plant growth were observed with *A. leiocalyx*, compared with those of *A. disparimma*. The retention and utilisation of mineral N by *A. leiocalyx* can be considered as strategy to restore forest soils.

Keywords N deposition · Mineral nitrogen · $\delta^{15}N$ · BNF · SMC · Biochar

1 Introduction

In recent years, climate factors and land management have highly affected soil labile carbon (C) and nitrogen (N) pools (Wang et al. 2019). It has been reported that precipitation affects soil organic matter decomposition and N turnover processes by changing soil moisture content (SMC) (Jackson et al. 2011; Li et al. 2022a). Both ammonia N (NH_4^+-N) and nitrates N (NO_3^--N) are highly soluble and able to cause soil N losses via N leaching, denitrification, and even nitrous

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oxide (N_2O) emission (Kasper et al. 2019; Taresh et al. 2021; Li et al. 2022a). Furthermore, changes in SMC also alter microbial activity and BNF (Warshan et al. 2016). In order to effectively reflect plant photosynthesis and water availability, foliar C isotope composition ($\delta^{13}C$) was used to indicate water use efficiency (WUE) (Huang et al. 2008; Taresh et al. 2021). Prescribed burning as a management measure to reduce wildfires in Australia forests also depletes soil C and N stocks (Reverchon et al. 2020; Yang et al. 2023).

N deposition and biological N fixation (BNF) are two crucial pathways for N input in ecosystems (Cusack et al. 2009; Bai et al. 2012, 2015a). As an effective source of N, BNF is particularly important for soil ecosystems in nature (Bai et al. 2015a, b; Reverchon et al. 2020). The process is primarily realized through a symbiotic relationship between legumes and rhizobia (Farhangi-Abriz et al. 2021a, 2022). This relationship transforms atmospheric N_2 into forms directly assimilable by plants such as NH_4^+-N, NO_3^--N, and organic N (Franche et al. 2008). These N compounds are partially released into the soil via plant root exchange (Iannetta et al. 2016; Yoseph and Shanko 2017). This process further attracts microbial growth, thereby promoting soil N mineralization (Adjesiwor and Islam 2016; Abdalla et al. 2019). Therefore, BNF is considered as a pollution-free pathway in enhancing soil fertility and supporting sustainable development.

The widespread acacia species in Australia are crucial for promoting the health of soil ecosystems by increasing litter and N_2 fixation (Witt et al. 2017; Reverchon et al. 2020). The symbiosis between acacia species and rhizosphere microorganisms results in the fixation of atmospheric N_2 into organisms and soil through root nodules (Farhangi-Abriz et al. 2021a, 2022). The acacia species not only promotes N cycling and maintains biodiversity but also contributes a lot to the C cycle (Seymour and Huyser 2008; Hosseini Bai et al. 2013). This contribution is closely linked to plant WUE and the N supplied by BNF for photosynthesis, potentially enhancing plant biomass accumulation (Kiers et al. 2003; Hosseini Bai et al. 2013). These species exhibit strong tolerance with environmental stress, beneficial for forest ecosystem recovery (Yang et al. 2009; Reverchon et al. 2012).

As a C-rich material, biochar addition can effectively increase the stable organic C content in soil and improves soil conditions (Blanco-Canqui 2017; Nguyen et al. 2017; Swagathnath et al. 2019; Nessa et al. 2021). This addition potentially lead to net N mineralization, thereby increasing the mineral N availability for plant uptake (Nelson et al. 2011; Wang et al. 2012; Asadyar et al. 2021). Furthermore, biochar significantly enhances the size and BNF of legumes under different climatic conditions, demonstrating its broad applicability (Macil et al. 2020; Farhangi-Abriz et al. 2021b; Das et al. 2022). Biochar with high porosity and high cation exchange capacity (CEC) has high adsorption capacity, which helps in the reduction of mineral N loss and enhance SMC and soil nutrient effectiveness (Dempster et al. 2012; Mukherjee et al. 2014; Sika and Hardie 2014; Chen et al. 2019). This includes the direct adsorption of NH_4^+ and highly mobile NO_3^- iron, extending their availability time for plant and microbial use (Mukherjee et al. 2014; Bai et al. 2015a). However, Liang et al. (2006) and Thies et al. (2015) have noted that biochar might limit the assimilation of NH_4^+ by microbes or plants. Consequently, the impact of biochar-soil interactions on N mineralization and the growth of leguminous plants remains limited.

N isotope composition ($\delta^{15}N$) is a critical indicator of N losses in both soil and plant systems, closely linked to microbial-mediated N transformations, such as N mineralization, nitrification as well as denitrification (Craine et al. 2009; Wang et al. 2015, 2020; Nessa et al. 2021). It is influenced by factors affecting microbial processes, which are soil moisture and plant species (Collins et al. 2019). Fractionation of NH_4^+-N and NO_3^--N occurs in the absorption of plant rhizosphere, which affects the distribution of N in the plant and the isotopic differences in nitrogenous exudates (Ariz et al. 2011; Gauthier et al. 2013; Yousfi et al. 2013). However, the mechanisms of natural ^{15}N variations in plants under complex environmental conditions is not fully understood (Tcherkez 2010; Succarie et al. 2020, 2022).

In the past, the relationship between plant BNF and soil inorganic N availability has been relatively underexplored. This study focused on the impact of understory acacia species on soil mineral N in the soil profiles with the addition of biochar. We selected *Acacia leiocalyx* and *A. disparimma* in this study to assess soil NH_4^+-N and NO_3^--N concentrations, along with their $\delta^{15}N$, after 5 years of prescribed burning in the Toohey Forest. At the same time, the long-term impact of biochar application on plants and soil interactions over 3.5 years was explored.

2 Materials and methods

2.1 Study site and experiment design

This research was conducted at Toohey Forest (27°32′53"S; 153°03′21"E) in Brisbane, Southeast Queensland, Australia. Spanning an extensive area of approximately 680 hectares, this forest ecosystem is located in the subtropical climate zone, with an annual average precipitation of 1350 mm (Bai et al. 2012). Prescribed burning has been in effect in this forest since 1993 until the most recent burn at the study site in August 2017 (Reverchon et al. 2020). The study plots were established in May 2019, and field trials were conducted at Site 7 (S7). A mixed cover of understory acacia species, including *Acacia leiocalyx* and *A. disparimma*, and overstorey *Eucalyptus psammitica* are typical of the area.

To delineate the study plot, each plot was demarcated with an area of 4 m^2 (2 m × 2 m). The trial employed a randomized complete block design with four circular blocks, each covering an area of 500 m^2 containing 13 plots (Reverchon et al. 2020). These plots comprised two understory acacia species, *A. leiocalyx* and *A. disparimma*, with six individuals of each, and one *E. psammitic* as a reference plant. The biochar derived from pine wood (*Pinus radiata*) used in this study was produced with a pyrolysis temperature of 600 °C. Different rates of pine biochar, namely control 0 t ha^{-1}, biochar 5 t ha^{-1}, and biochar 10 t ha^{-1}, were applied to the soil of *A. leiocalyx* and *A. disparimma.* This addition was artificially conducted on the plot surface in May 2019. *E. psammitica,* serving as a reference plant, received no biochar applied. According to Bruckman et al. (2015), these application rates were chosen to support sustainable development in forest systems at optimal and financially feasible levels. The physicochemical properties of biochar, including pH, total C, total N, $\delta^{13}C$, and $\delta^{15}N$, were measured, as reported by Yang et al. (2023).

2.2 Soil and foliage sample collection

After 42 months of field treatment establishment, soil samples were collected from three soil depths (0–5, 5–10 and 10–20 cm) using soil cores from various locations in each plot in November 2022. Subsequently, soil was sieved and homogenized evenly and stored at 4 °C to extract and analyze soil physicochemical properties in one week.

Foliage samples were collected and oven-dried thoroughly at 60 °C over 72 h. The dried foliage samples were then ground using the RocklabsTM ring grinder before being weighed for mass spectrometry analyses. The total C, total N and their isotope compositions ($\delta^{13}C$ and $\delta^{15}N$) were evaluated following the method of Xu et al. (2000).

2.3 Measurements of foliage physicochemical properties

Approximately 6–7 mg of the foliage powder was weighed and transferred into tin capsules. Samples were analysed for their total C, total N, $\delta^{13}C$ and $\delta^{15}N$ using a high-precision isotope ratio mass spectrometer (IRMS, Elementar, Langenselbold, Hesse, Germany) follow procedure (Bai et al. 2015a).

$\delta^{13}C$ and $\delta^{15}N$ values would be determined by the following formula as reported previously (Sun et al. 2024):

$$\delta^{13}C_{sample} = \frac{[R_{sample} - R_{VPDB}]}{R_{VPDB}} \times 1000 \quad (1)$$

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

where, R = the isotope ratio, R_{sample} = the ratio of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ of sample respectively, R_{VPDB} = the ratio of $^{13}C/^{12}C$ of the international standard (Vienna Pee Dee. Belemnite (VPDB)), R_{std} = the ratio of $^{15}N/^{14}N$ of the international standard (atmospheric N_2).

The percentage of N derived from atmospheric N_2 (%Ndfa) was determined using the following formula (Bai et al. 2012):

$$\%Ndfa = \left[\frac{(\delta^{15}N_{ref} - \delta^{15}N_{acacia})}{\delta^{15}N_{ref} - B\ value}\right] \times 100 \quad (3)$$

where $\delta^{15}N_{ref}$ and $\delta^{15}N_{acacia}$ are the $\delta^{15}N$ values of the reference plants and acacia species respectively.

B value: Isotopic abundance of acacia species growing without N.

B values in previous extensive research have been reported within a range of -2.9 ‰ to 1.0 ‰ for woody species (Boddey et al. 2000; Bai et al. 2012). Various B values were assessed from -1.5 ‰ to 1.0 ‰ to confirm a suitable B value for acacia species based on this trial design. For the purpose of this study, we employed a B value of -1.5 ‰ to present the BNF results.

Freshly collected field soil was oven dried at 105 °C for 24 h to determine the soil moisture content (SMC). The SMC values were calculated using the formula below (Voroney 2019):

$$\text{Soil moisture content } (\%) = \frac{[W_{wet\ soil} - W_{dry\ soil}]}{W_{dry\ soil}} \times 100 \quad (4)$$

2.4 Measurements of soil properties

The concentration of NH_4^+-N and NO_3^--N, along with their $\delta^{15}N$ in soil samples, were determined by microdiffusion technique (Stark and Hart 1996). Fresh soil samples (about 8 g dry weight) and 2 M KCl (40 ml) solution were mixed at a ratio of 1:5 (w/w). After centrifuging the mixture, 10 ml of supernatant was extracted, and NH_4^+-N was released as NH_3 by adding 100 µl of $(NH_4)_2SO_4$ spiked solution and 0.4 g of MgO. The filter paper discs were added with 2.5 M $KHSO_4$ to absorb the NH_3 for seven days, and then dried in concentrated H_2SO_4 for 28 days. To the same solution, 100 µl of standard KNO_3 spiking solution and 0.2 g of Devarda's alloy were added. Filter paper discs were prepared in the same manner for NO_3^--N collection.

Two batches of filter paper discs were encapsulated in tin capsules, and the ^{15}N atom% in NH_4^+-N and NO_3^--N was determined by mass spectrometry, respectively (Zhang et al. 2018). The NH_4^+-N and NO_3^--N in the samples were converted into NH_3 and absorbed by the filter paper discs.

2.5 Statistical analyses

The statistical analyses involved the application of a two-way analysis of variance (ANOVA) to investigate the significant impact of acacia species and biochar application rates on foliar total C, total N, $\delta^{13}C$, $\delta^{15}N$, BNF, as well as plant height, diameter at ground level (DGL), basal area (BA) and volume. These analyses were executed utilizing the statistical software SPSS 26.0 (IBM SPSS Statistics Inc., Chicago, USA). The same software was employed to perform a three-way ANOVA to evaluate the effects of different species, biochar rates, soil depths and their interactions on soil NH_4^+-N, NO_3^--N, $\delta^{15}N$ of NH_4^+-N and $\delta^{15}N$ of NO_3^--N. The threshold for statistical significance was established at $\alpha = 0.05$.

Multiple regression was conducted to investigate the relationships between foliage $\delta^{13}C$ and total N, as well as between SMC and soil mineral N using the software Origin Pro 9.0 (OriginLab, Northampton, MA, USA).

3 Results

3.1 Initial chemical and physical properties of plant samples

Significant differences were observed in foliar total C with biochar application with increases for 5 t ha^{-1} (49.16%) and 10 t ha^{-1} (49.64%) compared to the control (48.23%) ($P < 0.05$) (Table 1). Significantly differences in total C, $\delta^{13}C$ and $\delta^{15}N$ were noted between two understory acacia species. The foliar total C of *A. disparimma* (50.03%) was significantly higher than *A. leiocalyx* (48.66%) (Table 1). *A. leiocalyx* displayed a notably lower foliar $\delta^{13}C$ value (-33.03 ‰) in comparison to *A. disparimma* (-32.31 ‰), and had a significantly lower foliar $\delta^{15}N$ (-0.98 ‰) in contrast to *A. disparimma* (-0.20 ‰) (Table 1). It is worth noting that *A. leiocalyx* exhibited a significantly greater %Ndfa (nitrogen derived from the atmosphere) at 91.30% compare to *A. disparimma* at 78.22% (Table 1).

With the increase in biochar application rates, plant diameter at ground level (DGL), basal area (BA), and volume increased. With 10 t ha^{-1} of biochar treatment, DGL (2.97 cm), BA (7.90 cm^2), and volume (835.0 cm^3) exhibit the highest level of significance ($P < 0.05$) (Table 2). A similar trend was observed for plant height, although the increase was not statistically significant ($P > 0.05$) (Table 2).

Furthermore, distinct differences in growth data were observed between two acacia species. *A. leiocalyx* exhibited significantly greater plant height (269.1 cm), DGL (2.62 cm), BA (6.49 cm^2), and volume (692.2 cm^3) compared to those of *A. disparimma* ($P < 0.05$) (Table 2).

3.2 Soil mineral N and its $\delta^{15}N$

NH_4^+-N values in the 0–5 and 10–20 cm soil depth were 16.4 μg g^{-1} and 16.3 μg g^{-1}, significantly higher than that in the 5–10 cm depth (14.0 ug g^{-1}) ($P < 0.05$) (Table 3). Soil NO_3^--N values were 8.91 μg g^{-1} and 8.93 μg g^{-1} in the 0–5 cm and 5–10 cm depth respectively, and significantly higher values were observed in the 10–20 cm depth (9.93 ug g^{-1}) ($P < 0.05$) (Table 3). The $\delta^{15}N$ of NH_4^+-N at the 10–20 cm soil (13.3 ‰) was significantly higher than the other two depths ($P < 0.05$) (Table 3). The $\delta^{15}N$ of NO_3^--N values in the 5–10 cm and 10–20 cm soil depth were 10.5 ‰ and 11.9 ‰ respectively, significantly higher than the 6.21 ‰ in the 0–5 cm layer ($P < 0.05$) (Table 3). With the 5 t ha^{-1} biochar application rate, NO_3^--N (9.91 μg g^{-1}) was significantly higher than that of 10 t ha^{-1} (8.66 μg g^{-1}) ($P < 0.05$) (Table 3). The $\delta^{15}N$ of NO_3^--N at 10 t ha^{-1} was significantly lower ($P < 0.05$) (Table 3).

The *A. leiocalyx* had significantly lower NO_3^--N values (8.74 μg g^{-1}) compared to that of *A. disparimma* (9.58 μg g^{-1}).

Table 1 Impacts of understory *Acacia* spp. and pine biochar application rates on foliar total C, total N, C and N isotope composition ($\delta^{13}C$ and $\delta^{15}N$), and percentage of N derived from the atmosphere (%Ndfa) of *Acacia leiocalyx* and *A. disparimma* in Toohey Forest after 3.5 years of biochar application

Treatments	Total C (%)	Total N (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%Ndfa (%)
Biochar rates (t ha^{-1})					
0	**48.23 b**	2.08 a	-32.48 a	-0.10 a	76.42 a
5	**49.16 a**	2.07 a	-32.40 a	-0.38 a	81.13 a
10	**49.64 a**	1.90 a	-32.93 a	-0.59 a	84.68 a
Species					
Acacia leiocalyx	**48.66 b**	2.08 a	**-33.03 b**	**-0.98 b**	**91.30 a**
Acacia disparrima	**50.03 a**	2.12 a	**-32.31 a**	**-0.20 a**	**78.22 b**

The lower case letters for each variable among biochar rates and understory *Acacia* spp. demonstrate significant difference at the level $P < 0.05$

Table 2 Impacts of biochar application rates and understory *Acacia* spp. on plant height (cm), diameter at ground level (DGL, cm), basal area (BA, cm^2) and volume (cm^3) of *Acacia leiocalyx* and *A. disparrima* after 3 years of biochar application in Toohey forest, Australia

Treatments	Height (cm)	DGL (cm)	BA (cm^2)	Volume (cm^3)
Biochar rates (t ha^{-1})				
0	194.2 a	**1.83 b**	**3.11 b**	**254.6 b**
5	202.4 a	**1.97 b**	**3.60 b**	**305.6 b**
10	268.0 a	**2.97 a**	**7.90 a**	**835.0 a**
Species				
Acacia leiocalyx	**269.1 a**	**2.62 a**	**6.49 a**	**692.2 a**
Acacia disparrima	**179.6 b**	**1.94 b**	**3.43 b**	**258.0 b**

The lower case letters for each variable among biochar rates and understory *Acacia* spp. demonstrate significant difference at the level $P < 0.05$

Table 3 Effects of soil depths, biochar application rates and understory *Acacia* spp. on soil mineral nitrogen (NH_4^+-N and NO_3^--N) and their N isotope composition ($\delta^{15}N$ of NH_4^+-N and $\delta^{15}N$ of NO_3^--N) after 3.5 years of biochar application in Toohey forest, Australia

Treatments	NH_4^+-N (ug g^{-1})	NO_3^--N (ug g^{-1})	$\delta^{15}N$ of NH_4^+-N (‰)	$\delta^{15}N$ of NO_3^--N (‰)
Depth				
0–5	**16.4 a**	**8.91 b**	**9.55 b**	**6.21 b**
5–10	**14.0 b**	**8.93 b**	**7.15 b**	**10.5 a**
10–20	**16.3 a**	**9.93 a**	**13.3 a**	**11.9 a**
Biochar rates (t ha^{-1})				
0	15.9 a	**9.22 ab**	10.4 a	**10.9 a**
5	15.6 a	**9.91 a**	8.94 a	**9.93 a**
10	15.2 a	**8.66 b**	10.4 a	**7.08 b**
Species				
Acacia leiocalyx	15.2 a	**8.74 b**	**8.58 b**	**11.9 a**
Acacia disparrima	15.5 a	**9.58 a**	**11.7 a**	**7.94 b**

The lower case letters for each variable among biochar rates and understory *Acacia* spp. demonstrate significant difference at the level $P<0.05$

The $\delta^{15}N$ of NH_4^+-N values of *A. leiocalyx* (8.58 ‰) were significantly lower than that of *A. disparimma* (11.7 ‰) ($P<0.05$) (Table 3). Conversely, $\delta^{15}N$ of NO_3^--N values were significantly higher for *A. leiocalyx* (11.9 ‰) compared to that of *A. disparimma* (7.94 ‰) ($P<0.05$) (Table 3).

After three and half years of biochar application, in the 0–5 cm soil, soil NH_4^+-N with biochar rate of 5 t ha^{-1} (17.5 μg g^{-1}) was higher than that with 10 t ha^{-1} (14.9 μg g^{-1}) ($P<0.05$) (Table 4). Soil NO_3^--N was significantly higher in the soil with application rate of 5 t ha^{-1} (10.5 μg g^{-1}) compared to 0 and 10 t ha^{-1} biochar rates (8.55 μg g^{-1} and 7.86 μg g^{-1}) ($P<0.05$) (Table 4). Soils without biochar application had higher $\delta^{15}N$ of NO_3^--N values than those with biochar application ($0.05<P<0.10$) (Table 4). In the 5–10 cm soil, the $\delta^{15}N$ of NO_3^--N value without biochar application were significantly higher than that of 10 t ha^{-1} ($P<0.05$) (Table 4).

In the 0–5 cm and 5–10 cm soil depths, NO_3^--N concentration of *A. leiocalyx* (8.06 μg g^{-1} and 8.24 μg g^{-1}) were significantly lower than those of *A. disparimma* (9.61 μg g^{-1} and 9.21 μg g^{-1}). Conversely, $\delta^{15}N$ of NO_3^--N values of *A. leiocalyx* (10.5 ‰ and 13.7 ‰) were significantly higher than those of *A. disparimma* (3.70 ‰ and 8.14 ‰) ($P<0.05$) (Table 4). Furthermore, in the 0–5 cm soil, soil $\delta^{15}N$ of NH_4^+-N of *A. leiocalyx* (7.88 ‰) was significantly lower than that of *A. disparimma* (11.7 ‰) ($P<0.05$) (Table 4). In the 10–20 cm soil, soil NH_4^+-N of *A. leiocalyx* (14.0 ‰) was significantly lower than that of *A. disparimma* soil (18.6 ‰) ($P<0.05$) (Table 4).

3.3 Relationships between plant physiological variables and soil properties

Plant foliar total N and $\delta^{13}C$ showed stronger relationship in *A. leiocalyx* ($R^2=0.352$, $P<0.05$) than that of *A. disparimma* ($R^2<0.001$, $p=0.986$) (Fig. 1). Soil $\delta^{15}N$ of NH_4^+-N was negatively related with SMC ($R^2=0.228$, $P=0.001$) at 0–5 cm depth (Fig. 2). Under different biochar treatments, increases in the biochar application rates led to a more significant decline in $\delta^{15}N$ of NH_4^+-N with SMC at 0–5 cm depth, as indicated by the negative slopes for each regression equation (Fig. 3). At biochar application rate of 5 t ha^{-1}, the relationship between SMC and $\delta^{15}N$ of NH_4^+-N was significant, and it approached significance at 10 t ha^{-1} (Fig. 3). However, the effect was not significant when no biochar was applied (0 t ha^{-1}) (Fig. 3).

A significant statistical relationship ($P<0.05$) was observed between SMC and soil $\delta^{15}N$ of NH_4^+-N under two acacia species at 0–5 cm depth (Fig. 4). Among them, *A. leiocalyx* showed a higher relationship ($R^2=0.401$) compared to that of *A. disparimma* ($R^2=0.250$) (Fig. 4). It was also noticeably observed that the values of soil $\delta^{15}N$ of NH_4^+-N with *A. leiocalyx* were generally lower than those with *A. disparimma* (Fig. 4). At 5–10 cm soil depth, the concentration of NH_4^+-N was significantly positively related with SMC ($R^2=0.308$, $P=0.002$) (Fig. 5).

SMC was more closely related to soil NH_4^+-N concentration in *A. disparimma* ($R^2=0.373$, $P=0.035$) compared to that of *A. leiocalyx* ($R^2=0.228$, $P=0.115$) at 5–10 cm soil (Fig. 6a). Conversely, SMC was significantly positively related with soil NO_3^--N concentration in *A. leiocalyx*, while in *A. disparimma*, the relationship was not significant ($R^2=0.098$, $P=0.166$) (Fig. 6b). At a depth of 10–20 cm, the regression for *A. leiocalyx* between SMC and NH_4^+-N concentration ($R^2=0.624$, $P<0.001$), as well as the regression between SMC and $\delta^{15}N$ of NH_4^+-N ($R^2=0.467$, $P=0.006<0.05$), both demonstrated significant quadratic relationships (Fig. 7). With increasing SMC, both NH_4^+-N and $\delta^{15}N$ of NH_4^+-N initially increased to a peak value, followed by a decline (Fig. 7).

Table 4 Effects of soil depths, biochar application rates and understory *Acacia* spp. on soil mineral nitrogen (NH_4^+-N and NO_3^--N) and their N isotope composition ($\delta^{15}N$ of NH_4^+-N and $\delta^{15}N$ of NO_3^--N) at different soil depths (0–5, 5–10, 10–20 cm) after 3.5 years of biochar application in Toohey forest, Australia

Treatments	NH_4^+-N ($ug\ g^{-1}$)	NO_3^--N ($ug\ g^{-1}$)	$\delta^{15}N$ of NH_4^+-N (‰)	$\delta^{15}N$ of NO_3^--N (‰)
		0 – 5 cm		
Biochar rates (t ha^{-1})				
0	**16.8 ab**	**8.55 b**	9.64 a	**8.19 A**
5	**17.5 a**	**10.5 a**	7.93 a	**6.54 AB**
10	**14.8 b**	**7.86 b**	11.0 a	**2.91 B**
Species				
Acacia leiocalyx	16.7 a	**8.06 b**	**7.88 b**	**10.5 a**
Acacia disparrima	15.2 a	**9.61 a**	**11.7 a**	**3.70 b**
		5 – 10 cm		
Biochar rates (t ha^{-1})				
0	14.4 a	9.09 a	6.77 a	**12.3 a**
5	13.6 a	9.04 a	6.71 a	**10.5 ab**
10	14.0 a	8.58 a	8.16 a	**7.85 b**
Species				
Acacia leiocalyx	14.8 a	**8.24 b**	7.15 a	**13.6 a**
Acacia disparrima	12.9 a	**9.21 a**	8.20 a	**8.14 b**
		10 – 20 cm		
Biochar rates (t ha^{-1})				
0	16.4 a	10.0 a	14.8 a	12.1 a
5	15.7 a	10.2 a	12.2 a	12.8 a
10	16.9 a	9.53 a	12.0 a	10.5 a
Species				
Acacia leiocalyx	**14.0 b**	9.92 a	10.7 a	11.5 a
Acacia disparrima	**18.6 a**	9.91 a	15.1 a	12.0 a

The lower case letters for each variable among biochar rates and understory *Acacia* spp. demonstrate significant difference at the level $P < 0.05$; if different upper case letters, the difference between the two values is small/insignificant ($0.05 < P < 0.10$)

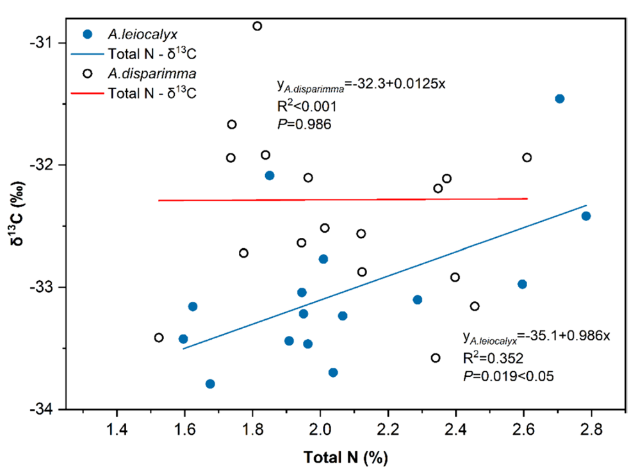

Fig. 1 Linear relationship between foliar total N (%) and $\delta^{13}C$ (‰) of *Acacia leiocalyx* and *A. disparrima*

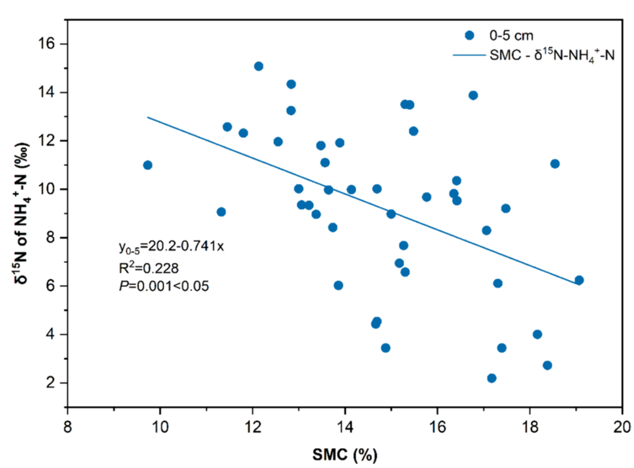

Fig. 2 Liner relationship between soil moisture concentration (SMC) and soil $\delta^{15}N$ (‰) of NH_4^+-N at soil depth of 0–5 cm

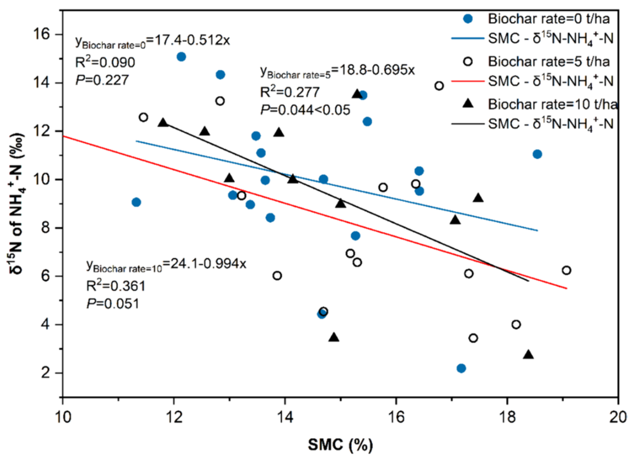

Fig. 3 Liner relationship between soil moisture concentration (SMC) and soil δ^{15}N (‰) of NH_4^+-N of three biochar rates (0, 5 and 10 t ha^{-1}) at soil depth of 0–5 cm

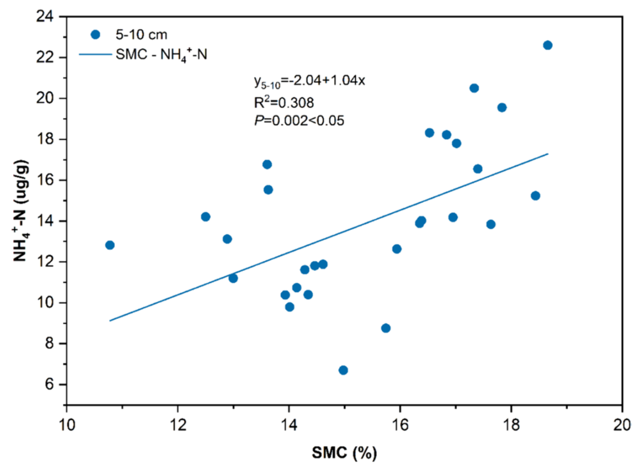

Fig. 5 Liner relationship between soil moisture concentration (SMC) and soil NH_4^+-N (ug g^{-1}) at soil depth of 5–10 cm

4 Discussions

4.1 The effect of biochar application on acacia plants

Previous studies have noted an increase in growth in leguminous species with biochar application (Xiao et al. 2020; Farhangi-Abriz et al. 2021b). Our results showed foliar total C values and growth-related measurements increased with the amount of biochar applied (Tables 1 and 2). This supports the notion that biochar addition significantly improved rhizosphere soil conditions and nutrient availability (Schulz et al. 2013; Das et al. 2022). Biochar has been reported to enhance nodule growth and BNF levels to some extent (Güereña et al. 2015; Farhangi-Abriz et al. 2022). While, in this study, %Ndfa was not observed to be statistically significant with increasing biochar application. The reason is that biochar properties change over time, leading to the diminishing effect of enhanced BNF (Mia et al. 2017, 2018).

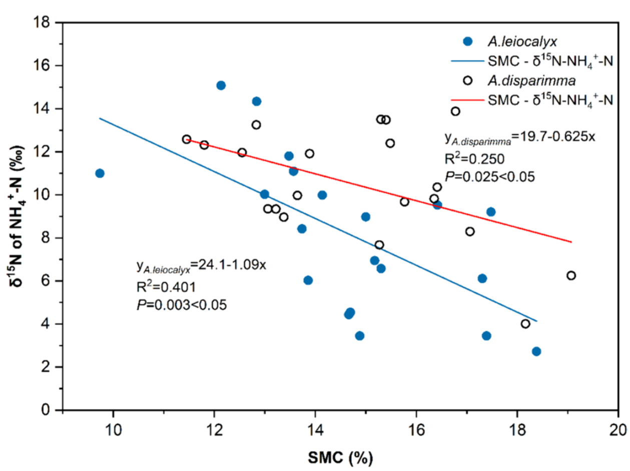

Fig. 4 Liner relationship between soil moisture concentration (SMC) and soil δ^{15}N (‰) of NH_4^+-N of *Acacia leiocalyx* and *A. disparrima* at soil depth of 0–5 cm

The BNF is a strategy for plant growth at the cost of consuming a large amount of photosynthetically accumulated C, especially under N limitation (Hosseini Bai et al. 2013). The foliar total N values measured for both species were within the range observed for acacia species (1.66–2.38N%) (Niinemets et al. 2009). *A. leiocalyx* exhibited significantly higher %Ndfa in this study. This is consistent with the findings of studies by Hosseini Bai et al. (2013) and Taresh et al. (2021), supporting that *A. leiocalyx* is a species with strong N fixation ability. N is an crucial element of C-fixing enzymes in photosynthesis (Wilson et al. 2000; Evans 2001). BNF can supply N for photosynthesis, supporting plant biomass accumulation and rapid growth (Bai et al. 2012). The lower foliar total C values and larger plant size of *A. leiocalyx* can therefore be attributed to more C being converted to biomass than accumulated in the foliage (Kiers et al. 2003). At the same time, high BNF leads to high photosynthesis rates, enabling faster C fixation and allocation to underground organs (such as roots and nodules) of *A. leiocalyx* (Bai et al. 2012). This in turn provided additional energy and C for the BNF process.

The δ^{13}C of plant is generally regarded as an index of WUE, and higher δ^{13}C values usually indicate higher WUE (Xu et al. 2000). Previous studies have demonstrated that *A. leiocalyx* exhibited higher δ^{13}C values compared with those of *A. disparimma*, reflecting a better WUE and strategy under drought stress (El Amin and Luukkanen 2006; Bai et al. 2012; Hosseini Bai et al. 2013). However, in this study, it was observed that *A. leiocalyx* exhibited greater growth, yet its foliar δ^{13}C value was significantly lower. It suggested that WUE was not the determining factor for plant growth in Toohey forest soil. Lower δ^{13}C has been shown

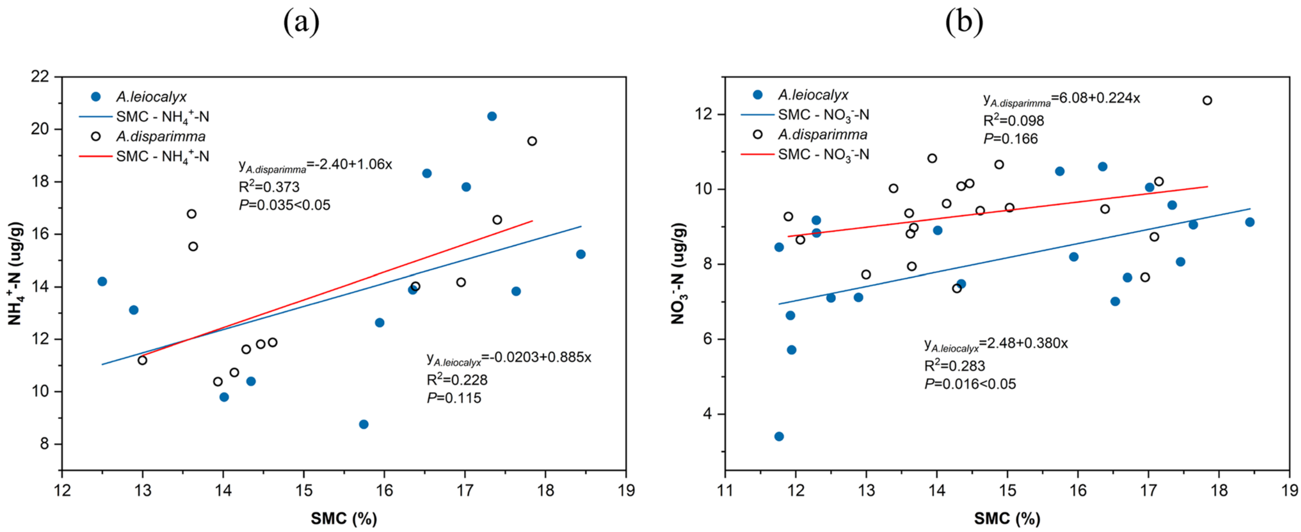

Fig. 6 Liner relationship between soil moisture concentration (SMC) and soil NH_4^+-N (**a**) and NO_3^--N (**b**) of *Acacia leiocalyx* and *A. disparrima* at soil depth of 5–10 cm

to support adequate water and N supply for the growth of *A. leiocalyx* (Whitehead et al. 2011). The significant regression observed between foliar δ^{13}C and soil total N in *A. leiocalyx* can be attributed to the crucial role of N as a limiting resource for plant growth (Farooq et al. 2021). *A. leiocalyx* exhibited a high water demand to support vigorous growth and metabolic activities, and this demand was limited by soil N supply. While soil total N is not a limiting factor for *A. disparimma* due to its slow growth.

Previous studies indicated that this forest is located near a busy highway, experiencing prolonged high N deposition (Bai et al. 2012; Sun et al. 2024). Both acacia species had negative foliar δ^{15}N values in this study, indicating their capacity to meet N requirements by directly absorbing deposited N from the canopy (Craine et al. 2015). Our results indicated that foliar δ^{15}N value of *A. leiocalyx* is significantly more negative than those of *A. disparrima*. This is consistent with the study of Ma et al. (2015) in Toohey Forest and our study after applying biochar for 2 years (Sun et al. 2024). In general, plants tend to select N forms that are more readily available from the soil, thereby reducing their dependence on BNF (Regus et al. 2017). However,

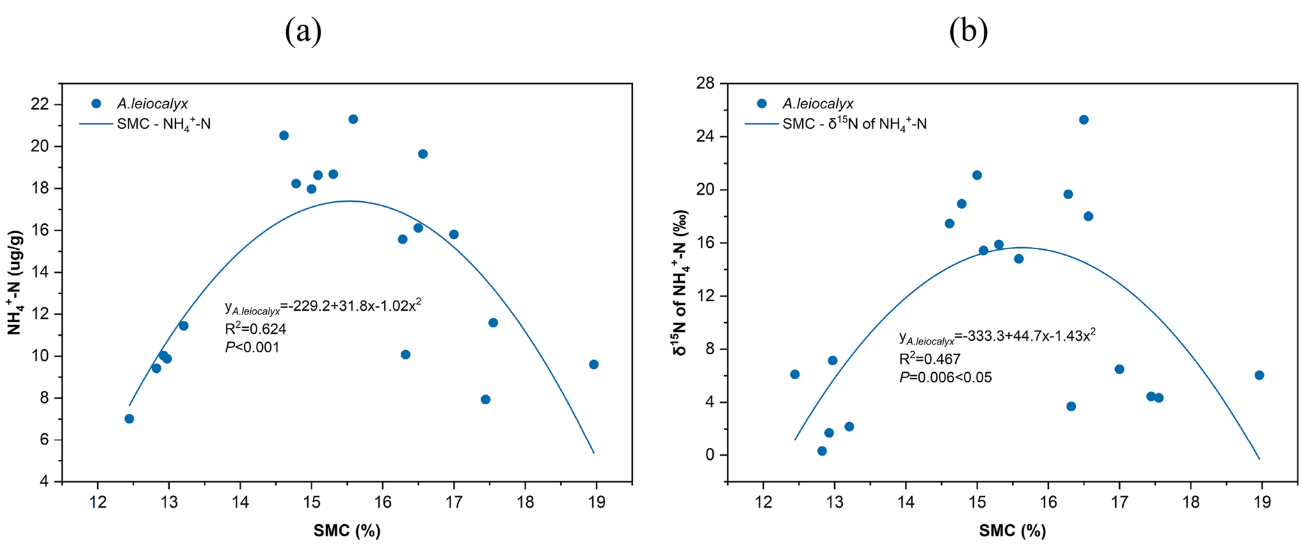

Fig. 7 Non-liner relationship between soil moisture concentration (SMC) and soil NH_4^+-N (ug g^{-1}) (**a**) and δ^{15}N of NH_4^+-N (‰) (**b**) of *Acacia leiocalyx* at soil depth of 10–20 cm

unexpectedly, high %Ndfa in both acacia species suggested that the available N concentration in the soil was insufficient. A possible explanation is the N volatilization loss from this forest due to prescribed burning conducted five years earlier (May and Attiwill 2003; Reverchon et al. 2011). Moreover, it has been reported that persistent heavy rainfall in the south-eastern Queensland in 2022 resulted in multiple floods (Bureau of Meteorology 2023). N leaching caused by precipitation, and nitrous oxide (N_2O) emissions via denitrification further contribute to soil N depletion (Cameron et al. 2013; Di and Cameron 2016). This causes plants to take longer to recycle N into the soil system (Bai et al. 2012). The lower foliar $\delta^{15}N$ values of *A. leiocalyx* suggest that it is more likely to acquire N from air, which is beneficial for soil recovery due to its higher BNF capacity.

4.2 Soil profile mineral N dynamics

N processes in the topsoil involve mineralization, nitrification, and ammonia volatilization, while in the deeper soil, processes include NO_3^--N leaching, denitrification, and microbial and plant assimilation (Choi et al. 2017; Liu et al. 2021). Therefore, the significantly higher NH_4^+-N concentration in 0–5 cm soil come from plant litter accumulation and topsoil mineralization (Mlambo et al. 2007; Hobbie and Högberg 2012). In 10–20 cm soil, significantly higher mineral N (NH_4^+-N and NO_3^--N) concentrations and their $\delta^{15}N$ values were observed. These results are consistent with previous studies, with enriched ^{15}N accumulating in the deep soil (Hobbie and Högberg 2012; Zeng and Han 2020). Soil profile N transfer and fractionation processes, including ammonia volatilization, nitrate leaching, and denitrification, result in the depletion of N-depleted mineral N in the deep soil (Hobbie and Ouimette 2009; Hobbie and Högberg 2012; Gurmesa et al. 2022). Moreover, plant uptake and microbial fixation can also increase $\delta^{15}N$ in deep soil, especially the transfer of N-depleted N into the plant mediated by mycorrhizal fungi (Hobbie and Ouimette 2009).

4.3 Soil mineral N and regression with SMC under biochar application

N turnover can be controlled to some extent by biochar application (Reverchon et al. 2014). Biochar is capable of adsorbing soil mineral N, thereby reducing the N-leaching process (Dempster et al. 2012; Sika and Hardie 2014; Sun et al. 2017). Observations indicated that the lowest soil NH_4^+-N and NO_3^--N concentration were under the 10 t ha^{-1} biochar treatment, especially significant at the 0–5 cm depth (Tables 3 and 4). While adsorbing NH_4^+-N, biochar reduced the production of NO_3^--N and N_2O from nitrification (Liang et al. 2006; Teutscherova et al. 2018). Moreover, biochar directly adsorbs NO_3^--N in soil solution, which can prolong its residence time in soil (Mukherjee et al. 2014). Lower $\delta^{15}N$ of NO_3^--N values indicate that biochar can reduce nitrate N losses, especially with 10 t ha^{-1} addition rate.

SMC is a key factor regulating nutrient levels in plant and soil, as well as microbial activity (Farooq et al. 2021). Variations in soil moisture conditions promote N mineralization, providing substrates for nitrification and denitrification (Liu et al. 2017; Li et al. 2022b). In the topsoil (0–5 cm), $\delta^{15}N$ of NH_4^+-N decreased with increasing soil moisture, due to enhanced microbial activity producing more ^{15}N-depleted products. Under the biochar treatment, a higher application rate resulted in a faster decline of $\delta^{15}N$ of NH_4^+-N. On the one hand, it benefits from the porous physical structure, which increases soil porosity highlighting the improved soil retention capacity (Zhang et al. 2008; Li et al. 2018). On the other hand, biochar enhances CEC of soil, providing more adsorption sites, thereby reducing NH_4^+-N leaching (Liang et al. 2006; Sun et al. 2017). Notably, the application of biochar at 10 t ha^{-1} appeared to be the optimal biochar addition rate to reduce NH_4^+-N loss and improve N utilization in the soil compared to the control and 5 t ha^{-1}.

4.4 Effects of acacia species on soil mineral N dynamics

The difference in plant N metabolism during growth is closely related to the absorption, utilization, and transformation of N (Nunes-Nesi et al. 2010; Ohyama 2010). Among both species, *A. leiocalyx* significantly decreased soil NO_3^--N concentration and enriched $\delta^{15}N$ of NO_3^--N, especially at 0–5 and 5–10 cm soil. This is attributed to discrimination and fractionation effects in N metabolic processes of different species (Hobbie and Ouimette 2009; Luo et al. 2013). *A. leiocalyx* showed a preference for assimilating more N-depleted compounds. The increase in N-enriched nitrification substrates and gas emissions during nitrification lead to soil $\delta^{15}N$ of NO_3^--N enrichment (Falxa-Raymond et al. 2012; Gauthier et al. 2013).

Legumes supply NH_4^+-N to the soil through BNF and promote microbial N mineralization (Jensen and Hauggaard-Nielsen 2003; Chu et al. 2004). Compared to *A. disparimma*, *A. leiocalyx* exhibited a significant reduction in $\delta^{15}N$ of NH_4^+-N, especially at 0–5 cm, emphasizing more N-depleted NH_4^+-N input. It is noteworthy that at soil depth of 10–20 cm, *A. leiocalyx* showed a significantly lower NH_4^+-N. This suggested that *A. leiocalyx* was more favorable in reducing the leaching of mineral N as the soil profile deepens than *A. disparimma*.

4.5 Regression of soil mineral N and SMC in different acacia species

In addition to regulating the BNF rate of legumes, increased precipitation have either positive or negative impacts on controlling soil litter decomposition and N mineralization levels (Di Blasio et al. 2010). Differences in species physiology may result in varying strategies for utilizing soil water and nutrients in different soil depth (Graciano et al. 2005).

In the topsoil (0–5 cm), $\delta^{15}N$ of NH_4^+-N of both acacia species decreased with increasing SMC. Bobbink et al. (2010) and Abdalla et al. (2019) indicated that when soil moisture is abundant, N_2 fixation increases and enters the soil through plant root exudates exchange, resulting in a decrease in soil $\delta^{15}N$ values. Compared with *A. disparimma*, fast-growing *A. leiocalyx* promoted soil N mineralization and BNF by rhizosphere microorganisms, resulting in a decrease in soil $\delta^{15}N$ of NH_4^+-N (Jensen and Hauggaard-Nielsen 2003; Chu et al. 2004).

At the soil depth of 5–10 cm, *A. disparimma* soil NH_4^+-N concentration was related positively with SMC. This can be attributed to its lower N demands resulting in NH_4^+-N from soil N mineralization more responsive to SMC (Verma and Sagar 2020). *A. leiocalyx* soil NO_3^--N concentration increased with SMC. It indicated stronger soil microbial activity under non-waterlogged conditions, promoting nitrification (Bobbink et al. 2010).

Some studies have indicated that many temperate and tropical legumes have reduced BNF under soil moisture deficiency (Reed et al. 2011; Warshan et al. 2016; Rousk and Michelsen 2017). Due to differences in root distribution, *A. disparimma* caused minimal disturbance in the 10–20 cm soil. While *A. leiocalyx* had the nonlinear relationships between SMC and NH_4^+-N concentration, and the dynamics of $\delta^{15}N$ of NH_4^+-N also exhibited a similar trend. Legume growth and BNF are limited at low SMC (Chalk et al. 2010; Salemaa et al. 2019). SMC limited the uptake of NH_4^+-N by *A. leiocalyx* causing the NH_4^+-N concentration to rise with increasing SMC. At this stage, *A. leiocalyx* preferred to assimilate the lighter isotopic form of NH_4^+-N, enriching the soil with $\delta^{15}N$ of NH_4^+-N. However, once SMC constraints were lifted, greater absorption by *A. leiocalyx* caused soil NH_4^+-N concentrations to decrease. At this time, there was higher BNF capacity of *A. leiocalyx* with more biologically fixed N into soil, which reduced soil $\delta^{15}N$ of NH_4^+-N. These results emphasize that BNF requires a certain level of soil moisture and may not be achieved until meets the basic growth needs of plants.

5 Conclusions

Forest burning management and extreme rainfall have altered soil mineral N dynamics and ecosystem functions in the suburban native forests of subtropical Australia. The long-term biochar application and BNF capacity of legumes can conserve soil mineral N and moisture, thereby reducing N losses from N transformation processes and leaching. This study found that the optimal biochar application rate was 10 t ha^{-1}, which significantly promoted plant growth and limited NO_3^--N leaching. We evaluated the BNF of two acacia species and found that they still relied on this process to supply N 4–5 years after prescribed burning. *A. leiocalyx*, with a higher BNF capacity, surpasses *A. disparimma* in soil moisture retention and improving mineral N utilisation. The *A. leiocalyx* demonstrated a stronger potential for restoring soil N availability.

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Author contribution Zhihong Xu designed the experiment; Yinan Li, Weiling Sun and Yifan Bai carried out the sampling and analyses; Yinan Li, Weiling Sun analyzed the data and wrote the manuscript; and Zhihong Xu and Shahla Hosseini Bai improved the manuscript.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

Declarations

Ethical approval This research does not involve human participants or animals performed by any of the authors.

Competing interests The authors have no competing interests to declare.

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