



Impact of biochar addition on water use efficiency, biological nitrogen fixation and growth of understory *Acacia leiocalyx* and *Acacia Disparimma* in a suburban native forest of subtropical Australia

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Abstract

Purpose This study evaluated the responses of water use efficiency (WUE), biological nitrogen fixation (BNF) and growth of understory *Acacia leiocalyx* and *A. disparimma* to the biochar addition and prescribed burning for 9–22 months in a suburban native forest of subtropical Australia.

Materials and methods Foliar and surface soil (0–10 cm) samples were collected from two understory species of acacia (*A. leiocalyx* and *A. disparimma*) and reference plants (*Eucalyptus psammitica*) shortly before biochar application at 0, 5 and 10 t ha⁻¹ and at 1, 2, 3, 6 and 9 months after biochar application or 22 months after prescribed burning. Soil and plant samples were analysed to determine total carbon (C), total nitrogen (N) and C and N isotope compositions ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). Plant growth was measured in the first 9 months after biochar application.

Results and discussion Foliar total N was significantly higher ($P < 0.05$) for *A. leiocalyx* than *A. disparimma* 9 months after biochar application, while foliar total N was significantly lower at 6 months after biochar application for *A. disparimma*. There were significant differences ($P < 0.05$) in BNF between *A. leiocalyx* and *A. disparimma* at all the sampling times. The BNF of *A. disparimma* was significantly higher at 2 months after biochar application than those of *A. leiocalyx*, while the lower BNF was found in *A. leiocalyx* at 3 months after biochar application. The BNF ranged from 52.4 to 78.7% for *A. leiocalyx* and from 65.0 to 84.2% for *A. disparimma* at 2 months after biochar application. The WUE, as reflected in foliar $\delta^{13}\text{C}$, was higher for *A. disparimma* than that of *A. leiocalyx* at 1, 2, 3, 6 and 9 months after biochar application. There was a significantly higher plant growth for *A. leiocalyx* than that of *A. disparimma* when biochar was applied at 10 t ha⁻¹, while the lowest height was found in the control without biochar application.

Conclusions Biochar application improved foliar total N, BNF and growth of understory acacia species in the first 9 months of biochar application in the suburban native forest of subtropical Australia. Both acacia species showed an improved foliar total N, BNF, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ after biochar application at the 3 and 9 months after biochar addition compared with those of the control.

Keywords Biochar · Biological nitrogen fixation · Understorey *Acacia* Spp · Water use efficiency · Plant growth

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1 Introduction

Biochar is a carbon-rich black solid material that is a by-product of energy generation during biomass pyrolysis intended specifically for soil application (Lehmann et al. 2011; Lehmann and Joseph 2015). Biochar, a product formed by the incomplete combustion of biomass, has been shown to improve soil quality and increase crop growth (Adams et al. 2013). Biochar plays an important role as a soil additive in reducing N loss and improving

physical and chemical soil fertility (Joseph and Lehmann 2009; Joseph et al. 2013). Biochar has been shown to affect soil bio-chemical and physical properties, which increases soil productivity (Glaser et al. 2002; Lehmann et al. 2006; Lehmann 2007). As a result, biochar application has been shown to increase plant growth and crop yield (Biederman and Harpole 2013; Bai et al. 2022). Biochar has attracted much attention due to its promising role in many environmental management issues (Lehmann et al. 2006; Kumar et al. 2011). Prescribed burning can be used as an effective tool for protecting both native and plantation forests from the risk of damaging wildfires (Guinto et al. 1999a, b; 2000; Reverchon et al. 2012). Fire is able to increase the short-term availability of some nutrients (including nitrogen (N), which may benefit regenerating plants shortly after the fire (Carter and Foster 2004). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus the effect of fire on carbon (C) and N cycling and climate (Hurteau and Brooks 2011; Bai et al. 2012; Bai et al. 2015). Prescribed burning has played an important role in changes in land use and the effectiveness achieved in suppressing wildfires, which leads to a general increase in biomass in many ecosystems (Morgan et al. 2003). Some studies showed that prescribed fires have the ability to reducing wildfire hazard, thus avoiding negative effects on different ecosystem components, such as soil, seeds, shrubs and trees (Fernandes and Rigolot 2007; Catalanotti et al. 2010). Prescribed burning can be used as an effective tool for protecting both native and plantation forests from the risk of damaging wildfires (Guinto et al. 1999a, b; 2000; Reverchon et al. 2012). The nutrients may be available temporarily after the fire, which can benefit plants, although nutrient leaching could also contribute to nutrient losses (Yildiz et al. 2010). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus there would be the effect of fire on C and N cycling and climate (Hurteau and Brooks 2011; Bai et al. 2012; Bai et al. 2015).

Biological nitrogen fixation (BNF) represents a primary source of new N input into terrestrial ecosystems (Sullivan et al. 2014). Also, the rate of BNF, at both regional and global scales, has decreased during recent decades, as a result of an increasing atmospheric N deposition due to human activities (Sullivan et al. 2014). Forest type is an important factor regulating the effects of external N input on BNF, and that elevated atmospheric N deposition in recent decades has suppressed BNF in mature forests with legume trees (Zheng et al. 2016). Biochar application has shown to influence N_2 -fixing bacteria (diazotrophs), including both free-living and symbiotic soil bacteria; and, thus, that biochar influences BNF (Giller 2001; Thies et al. 2015). Increased biochar application rate leads to

increased nodulation of rhizobia due to increased availability of boron (B), molybdenum (Mo), potassium (K), phosphorous (P), and calcium (Ca) (Rondon et al. 2007).

The ^{15}N natural abundance ($\delta^{15}N$) in soil and plant is considered an indicator of N cycling for plants at different locations, on a local, regional, or global scale, and is used to examine extractable soil mineral N pools (NH_4 and NO_3) and soil N turnover rates (Kahmen et al. 2008; Craine et al. 2009). Although many of the mechanisms driving the variability of foliar and soil $\delta^{15}N$ values within and across ecosystems are still unclear, $\delta^{15}N$ in plants and soil is a simple tool for assessing ecosystem N dynamics (Houlton et al. 2008; Kahmen et al. 2008; Cheng et al. 2010). Emmett et al. (1998) suggested that the ^{15}N signal of vegetation may provide a useful tool in evaluating the past and current N status of forested ecosystems.

Water use efficiency (WUE) is considered the link between wood production and plant water usage in forest ecosystems (Cernusak et al. 2007). It has been found that WUE is related to the optimum water use of a plant and enhances plant responses to the competition for water under xeric conditions (Robinson 2001; Schulze and Drevon 2005). Previous studies have noted the relation between stable carbon isotope compositions ($\delta^{13}C$) of plants and WUE linearly, which is used to investigate WUE of plants (Sun et al. 2010; Succarie et al. 2020; Taresh et al. 2021). The higher $\delta^{13}C$ value generally means higher WUE, and this technique has been used in different forest ecosystems of subtropical Australia (Huang et al. 2008a, b; Bai et al. 2014a, b; Tutua et al. 2014). Water use efficiency or stomatal conductance is considered useful information for photosynthesis and plant ecophysiology. Foliar $\delta^{13}C$ can be a useful index for plant WUE because this method enables researchers to assess long-term physiological processes (Huang et al. 2008a). Bai et al. (2013) observed no significant difference in long-term WUE, as shown by foliar $\delta^{13}C$, between the two species of *A. leiocalyx* and *A. disprinna*, regardless of measuring times. Adams et al. (2013) found that biochar application improved soil quality and increased crop growth. Biochar, produced through pyrolysis of a wide range of biomass sources, may improve soil water availability.

The current study sought to determine the impacts of biochar addition on biological nitrogen fixation (BNF), WUE, growth by understorey acacia species in Toohey Forest, a suburban forest in subtropical Australia. We hypothesised that biochar addition would influence C and N pools as well as BNF, WUE, and plant growth of understory acacia species in short time under specific fire regimes that are followed in a native forest ecosystem of southeast Queensland, Australia. The addition of biochar to soil may offer a chance to mitigate climate change by increasing soil C stocks, improving soil fertility and enhancing plant growth.

Fig. 1 Monthly air temperatures and rainfall during the study period of 2019 in Toohey Forest

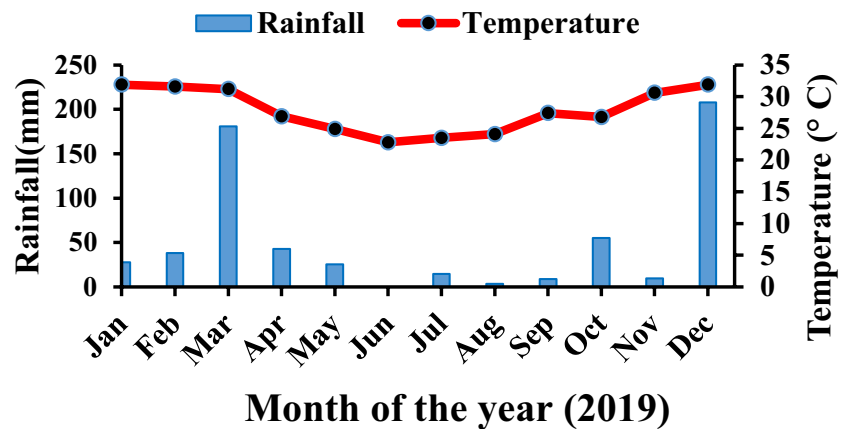


Table 1 Soil characteristics of the study site shortly before (September 2016), and 3 years after burning (April 2019) respectively in Toohey Forest. Samples for sampling depths of 0–5, 5–10, and 10–20 cm

Soil depths	Soil chemical properties before burning (September 2016)				After burning 29 April 2019			
	TC (%)	TN (%)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	TC (%)	TN (%)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
0–5 cm	6.08 (0.63)a	0.24 (0.02)a	–25.9 (0.1)b	0.10 (0.25)b	7.60 (0.70)a	0.30 (0.01)a	–26.9 (0.2)b	0.90 (0.10)c
5–10 cm	3.93 (0.52)b	0.15 (0.01)b	–25.3 (0.1)a	0.44(0.32)ab	5.20 (0.30)b	0.20 (0.02)a	–25.8 (0.1)a	1.70 (0.10)b
10–20 cm	3.58 (0.38)b	0.13 (0.01)b	–25.2 (0.0)a	1.49 (0.21)a	4.00 (0.16)b	0.20 (0.02)a	–25.4 (0.1)a	3.00 (0.15)a

Our specific objectives were to quantify whether biochar addition could reduce N loss and improve biological, physical and chemical properties of surface soils. However, the study aimed to examine the short-term effect of biochar addition in typical Toohey Forest on BNF, WUE and growth of understory acacia species at 1, 2, 3, 6 and 9 months after three rates of biochar application (0 t ha^{-1} , 5 t ha^{-1} , and 10 t ha^{-1}) following about 12 months of prescribed burning at a site in the Toohey Forest of southeast Queensland, Australia.

2 Materials and methods

2.1 Biochar characterisation

In this field work, pine wood (*Pinus radiata*) was used to produce the biochar material through slow pyrolysis at temperature 600°C . Biochar chemical properties are: pH (1:2) $7.27(\pm 0.06)$, total C $79.4\% (\pm 0.4)$, total N $0.145\% (\pm 0.014)$, $\delta^{13}\text{C}$ $-27.52\text{‰} (\pm 0.02)$, and $\delta^{15}\text{N}$ $1.81\text{‰} (\pm 0.84)$.

2.2 General site description

A series of studies has been conducted within Toohey Forest, located 10 km south of Brisbane, in southeast Queensland of subtropical Australia. This dry sclerophyll forest is approximately 400 ha and is dominated by different species of acacia and eucalyptus. About half of this ecosystem

is a local government conservation reserve surrounded by suburban areas (Catterall et al. 2001; Farmer et al. 2004). The climate of the region is subtropical, with annual rainfall about 613.4 mm for 2019 (Fig. 1). Annual minimum and maximum temperatures ranged between 17.1 and 27.8°C in 2019 (Fig. 1). In general, summer is hot and wet, whereas winter is cool and dry (Catterall and Wallace 1987; Stock et al. 1987).

Toohey Forest soil type is a combination of lithosols, podzolic and alluvial (Catterall and Wallace 1987). The lithosols soil, which contains both stony components and loam, to silty loam, may be rich with humus. Conversely, the podzols are mainly less than 60 cm deep in the soil and are low in nutrients. Horizon-B is sandy clay, while horizon-A is sandy loam with coarser particles (Catterall and Wallace 1987; Stock et al. 1987). Soil properties before and 3 years after prescribed burning are described in Table 1.

The trial plots were randomly located within one site at Toohey Forest, which was last burnt in August 2017 and contained a mixture of understory legumes, such as *A. leiocalyx* and *A. disparimma*, and overstorey such as *E. psammitica*, typical of the area. In each plot, there are six plants of *A. leiocalyx*, six plants of *A. disparimma* and one plant of *E. psammitica* as a reference plant. These four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2 , were randomly located within the site, giving a total sampling area of one-fifth of a hectare. A randomised complete block design was used in this study within the main study

site. Each plant area was defined by four steel pegs, providing an area of 2 m² for each. Biochar treatment was applied on 13 May 2019. Three biochar treatments (0 t ha⁻¹, 5 t ha⁻¹, and 10 t ha⁻¹) were applied. Foliar samples were taken from the upper part of the two species of acacia (*A. leiocalyx*, *A. disparimma*) and *E. psammitica* from each plot within the study site for sampling months of April, June, July, August, November for 2019, and March for 2020. Soil samples were collected before and immediately after biochar application at 1, 2, 3, 6, and 9 months within two square metres of each plant.

2.3 Soil sampling and analysis

Soil samples was taken after identifying each area. Each plant area was defined by four steel pegs providing an area of 2 m². Soil sampling was carried out before and 1, 2, 3, 6, and 9 months immediately after biochar application within the 2 m² for each plant. The biochar was applied on 13 May 2019 within the three biochar treatments (control 0, 5 t ha⁻¹, and 10 t ha⁻¹) in each of the 2 m² for six plants of *A. leiocalyx* and six plants of *A. disparimma*. Two soil cores were randomly collected within 2 m² for each plant in each plot within the study site, at three depths (0–5, 5–10, 10–20 cm) before, 3, 6 and 9 months, while eight soil cores were collected randomly from each plant area at 1 and 2 months after biochar application for one depth (0–5 cm), using a 2.5 cm diameter auger. The soil samples were kept in separate plastic bags and transferred to the laboratory to measurement of soil properties.

2.4 Foliar sampling and analysis

Three leaves per plant (upper canopy position) were collected from two species of acacia and eucalyptus as reference plants within the study site. The leaves were kept in separate paper bags and transferred to the laboratory to represent a comprehensive measurement of BNF, WUE and plant growth. There were four replicated plots at the study site, with foliar samples collected from two individuals of *A. leiocalyx*, and *A. disparimma* for each of the 3 biochar rates (0, 5 and 10 t ha⁻¹) and one reference plant of *E. psammitica* (with no biochar added). These foliar samples were collected at six sampling months (April, June, July, August, November of 2019, and March 2020). In summary, the sampling regime consisted of six *A. leiocalyx*, six *A. disparimma* and one *E. psammitica* from each plot over 9 months. Overall, 156 foliar samples were collected for each sampling month (*A. leiocalyx*=72, *A. disparimma*=72), as well as 12 samples of *E. psammitica* and 936 foliar samples were collected over 6 sampling months (156×6 sampling month=936). Three leaves per plant were collected and kept in separate paper bags and transferred to the laboratory, then

oven-dried at 60 °C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al. 2000). The homogenised powder was weighed and placed in 8×5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK). Total N (TN), total C (TC), C and N isotope composition (δ¹³C and δ¹⁵N) were assessed using an isotope ratio mass spectrometer, as described by Prasolova et al. (2000) and Xu et al. (2003), 6–7 mg plant material was packed into tin capsule to determine foliar total C and N as well as δ¹³C and δ¹⁵N by isotope ratio mass spectrometer with Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy) (Sollins et al. 1999; Molero et al. 2011), δ¹³C or δ¹⁵N was determined by ¹³C/¹²C ratio, and ¹⁵N/¹⁴N ratio as:

$$\delta^{13}C \text{ or } \delta^{15}N (\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{std}}} - 1 \right) \times 1000 \quad (1)$$

BNF was estimated by ¹⁵N natural abundance for two acacia species. Also, one reference of non-N fixing plant of *E. psammitica* was identified for all estimations which could minimise the potential error. The value of δ¹⁵N was determined using the following equation:

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

Where

R_{sample} is the isotopic ratio ¹⁵N/¹⁴N of sample and R_{std} is ¹⁵N/¹⁴N ratio of the atmospheric N₂.

The percentage of N derived from atmospheric N₂ (%Nd_{fa}) was calculated using the following equation (Shearer and Kohl 1986):

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

Where:

δ¹⁵N_{ref}: The δ¹⁵N value of the reference plants.

δ¹⁵N_{acacia}: The δ¹⁵N value of the Acacia spp.

B_{value} : The relative isotopic abundance of Acacia species. growing under a N-free nutrient condition.

The present study used different B values varying from −0.3‰ to 1.0‰ in this study to determine an applicable B value for acacia species to estimate BNF at the site. In our study, using B value of −0.3‰ resulted in an estimation of %Nd_{fa} that was a better and more reasonable than that of 1.0‰ for the study site.

Water use efficiency (WUE) was calculated based on expression of C isotope composition (δ¹³C) (Farquhar et al. 1982; McCarroll and Loader 2004). WUE was defined as the ratio of CO₂ assimilation (A) to stomatal conductance for water vapor (g_w):

$$A/g_w = (C_a - C_i) / 1.6 \quad (4)$$

The relation between the isotope ratios in plants ($\delta^{13}\text{C}_p$) and the source of CO_2 for photosynthesis and the atmospheric CO_2 ($\delta^{13}\text{C}_a$) is described by the equation:

$$\delta^{13}\text{C}_p = \delta^{13}\text{C}_a - a - (b - a) C_i/C_a \quad (5)$$

Xu et al. (2014) calculated WUE by using the equation:

$$A/gw = C_a(\delta^{13}\text{C}_p - \delta^{13}\text{C}_a + b)/(b - a)/1.6$$

Where

C_a and C_i are the CO_2 concentrations in the atmosphere and the leaf intercellular space, respectively.

$a = (4.4\text{‰})$ refers to the enrichment during CO_2 diffusion, and $b = (27\text{‰})$ is the fractionation by Rubisco against $^{13}\text{CO}_2$. Historical annual atmospheric CO_2 concentrations were obtained by using the direct measurements at the South Pole. As for the trend in $\delta^{13}\text{C}_a$, the exponential functions adjusted for the compiled data by Feng (1998) were used.

2.5 Plant growth measurement

Plant height (H) and diameter at ground level (DGL) were measured in August 2019 and November 2019. All understory plants such as acacia species, as well as eucalyptus species for all plots were included for growth measurements, in total, four plots containing an average of 52 plants, 24 plants for each *Acacia* species (*A. leiocalyx*=24, *A. disparimma*=24) and four plants of *E. psammitica*.

The DGL measurement was determined in August 2019 and November 2019 by using diameter tape and H measurement by hypsometer (Vertex IV).

2.6 Statistical analysis

Three-way analysis of variance (ANOVA) was conducted to evaluate the differences in BNF, WUE and growth with respect to the biochar application rates, understory acacia species and sampling time after biochar application. Statistical analyses were conducted with Statistix software (Version 8). Differences among means of all variables over time were determined with post-hoc pairwise comparison using the Tukey HSD test at $P < 0.05$ (Idol et al. 2002; Schafer and Mack 2010; Köster et al. 2014). Multiple regressions were carried out to quantify the relationships among plant properties such as $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and total TN.

3 Results

3.1 Foliar total N, %Ndfa and $\delta^{15}\text{N}$

Foliar total N concentrations of *A. leiocalyx* and *A. disparimma* were significantly different ($P < 0.05$) between the

two species after 9 months of biochar application, while there were no significant differences in foliar total N concentrations between the two acacia species at 1, 2, 3 and 6 months after biochar was applied. Our result showed that foliar total N was significantly higher ($P < 0.05$) for *A. leiocalyx* (2.63%) after 9 months (March 2020) (Table 2), while foliar total N concentration was significantly lower at 6 months after biochar application (November 2019) for *A. disparimma* (2.04%) (Table 2).

Our result showed that foliar total N concentrations were significantly higher ($P < 0.05$) (2.46%) in the treatment of 10 t ha^{-1} at March 2020 compared with those of the 5 t ha^{-1} and control, while foliar total N concentrations were significantly lower at month 6 (November 2019) (1.97%) in the control (Table 3).

Meanwhile, the highest foliar total N was found in the autumn sampling (March 2020) (2.38%), 9 months after biochar application, while the lowest foliar total N was detected in November 2019, 6 months after biochar application (Table 4) (2.00%).

Our results showed that BNF of *A. disparimma* was significantly higher at month 2 (July 2019) than those of *A. leiocalyx* (Fig. 2a) (84.17%), while the lower BNF was found in *A. leiocalyx* 3 months after biochar application (Fig. 2a) (52.4%). The BNF ranged from 52.4 to 78.7% for *A. leiocalyx* and from 65.0 to 84.2% for *A. disparimma* for all foliar samplings at 2, 3 and 6 months after biochar application for the winter and spring seasons of July, August and November 2019.

Meanwhile, the current study also indicated that BNF was significantly higher for the treatment of biochar applied at 10 t ha^{-1} than those of the 5 t ha^{-1} and the control (Fig. 2b) (90.3%) at 2 months after biochar application, while BNF was significantly lower 6 months after biochar applied for 5 t ha^{-1} (Fig. 2b) (50.7%).

The current study also showed that BNF significantly differed among the sampling periods. The BNF was significantly higher in the winter sampling (July 2019) (87.1%) compared with those of the spring sampling 6 months after biochar application (Table 4) (58.9%).

The current study showed that $\delta^{15}\text{N}$ values of *A. disparimma* was significantly higher (-0.53‰) in July 2019 (winter-dry season), 2 months after biochar application, than those of *A. leiocalyx* (Fig. 3a). Foliar $\delta^{15}\text{N}$ of *A. leiocalyx* was significantly lower (-1.90‰) in November 2019 (spring season), 6 months after biochar application.

The present study indicated that $\delta^{15}\text{N}$ values in the treatment of 10 t ha^{-1} was significantly higher (-0.50‰) for July 2019 (winter-dry season) than those of the control, but did not differ from that of 5 t ha^{-1} , while, foliar $\delta^{15}\text{N}$ was significantly lower (-1.96‰) in November, 6 months after biochar application for 5 t ha^{-1} (Fig. 3b). Foliar $\delta^{15}\text{N}$ was significantly higher (-0.74‰) in July 2019 (winter-dry

Table 2 Impacts of biochar application on total nitrogen (TN), total carbon (TC) and water use efficiency (WUE) for *A. leiocalyx* and *A. disparimma* at 1, 2, 3, 6 and 9 months after biochar application. Different lower case letters in a column indicate significant differences between *A. leiocalyx* and *A. disparimma* at $P < 0.05$

	June 2019			July 2019			August 2019			November 2019			March 2020		
	TN (%)	TC (%)	WUE (μmol mmol ⁻¹)	TN (%)	TC (%)	WUE (μmol mmol ⁻¹)	TN (%)	TC (%)	WUE (μmol mmol ⁻¹)	TN (%)	TC (%)	WUE (μmol mmol ⁻¹)	TN (%)	TC (%)	WUE (μmol mmol ⁻¹)
<i>A. leiocalyx</i>	2.32a	48.03b	34.62b	2.35a	48.39b	34.44b	2.28a	50.78a	35.58b	2.07a	49.76a	43.22a	2.63a	47.13b	32.18a
<i>A. disparimma</i>	2.24a	48.93a	42.17a	2.25a	49.67a	44.14a	2.34a	51.14a	42.53a	2.04a	49.90a	45.07a	2.13b	49.24a	35.36a

Table 3 Impacts of biochar application on total nitrogen (TN), total carbon (TC) and water use efficiency (WUE) for each sampling period at 1, 2, 3, 6 and 9 months after biochar application. Different lower case letters in a column indicate significant differences among biochar treatments at $P < 0.05$

	June 2019				July 2019				August 2019				November 2019				March 2020			
	TN (%)	TC (%)	WUE ($\mu\text{mol mmol}^{-1}$)	TN (%)	TC (%)	WUE ($\mu\text{mol mmol}^{-1}$)	TN (%)	TC (%)	WUE ($\mu\text{mol mmol}^{-1}$)	TN (%)	TC (%)	WUE ($\mu\text{mol mmol}^{-1}$)	TN (%)	TC (%)	WUE ($\mu\text{mol mmol}^{-1}$)	TN (%)	TC (%)	WUE ($\mu\text{mol mmol}^{-1}$)		
0 t ha ⁻¹	2.26a	48.3a	32.39b	2.28a	48.1b	30.34b	2.19a	50.4b	30.43b	1.97a	49.4b	38.68b	2.33a	48.0a	29.73b	2.33a	48.0a	29.73b		
5 t ha ⁻¹	2.28a	48.5a	41.46a	2.29a	49.6a	43.59a	2.31a	51.1a	43.10a	1.99a	50.1a	44.12ab	2.35a	48.3a	36.22a	2.35a	48.3a	36.22a		
10 t ha ⁻¹	2.31a	48.7a	41.34a	2.33a	49.4a	43.93a	2.42a	51.4a	43.64a	2.06a	50.0a	49.63a	2.46a	48.3a	35.36ab	2.46a	48.3a	35.36ab		

0 t ha⁻¹: Control with nil biochar applied5 t ha⁻¹: 2 kg biochar applied within an area of two square metres for each plant10 t ha⁻¹: 4 kg biochar applied within an area of two square metres for each plant

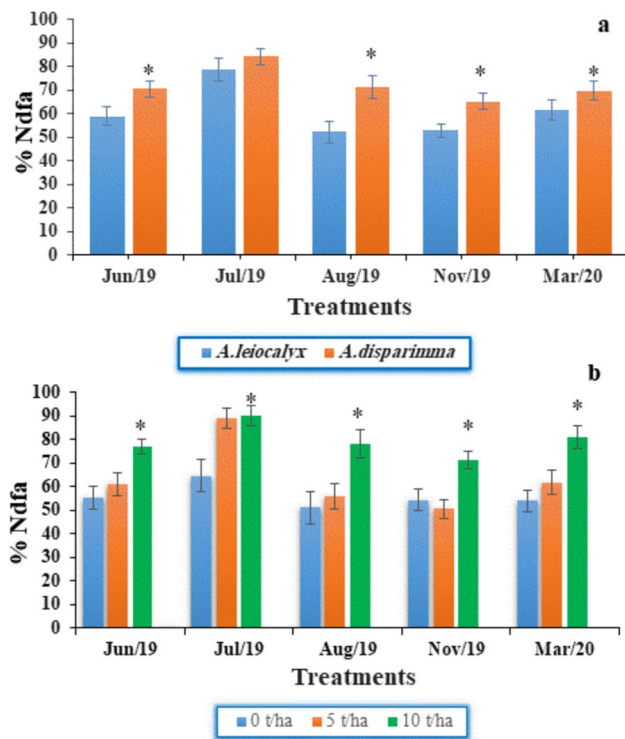


Fig. 2 Biological nitrogen fixation (BNF) for all time since biochar application (months): (a) Percentage of N derived from atmosphere (%Ndfa) of understorey *Acacia leiocalyx* and *A. disparimma*, and (b) Impact of biochar treatments on BNF at 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between BNF of *A. leiocalyx* and that of *A. disparimma*, as well as among biochar treatments at $P < 0.05$

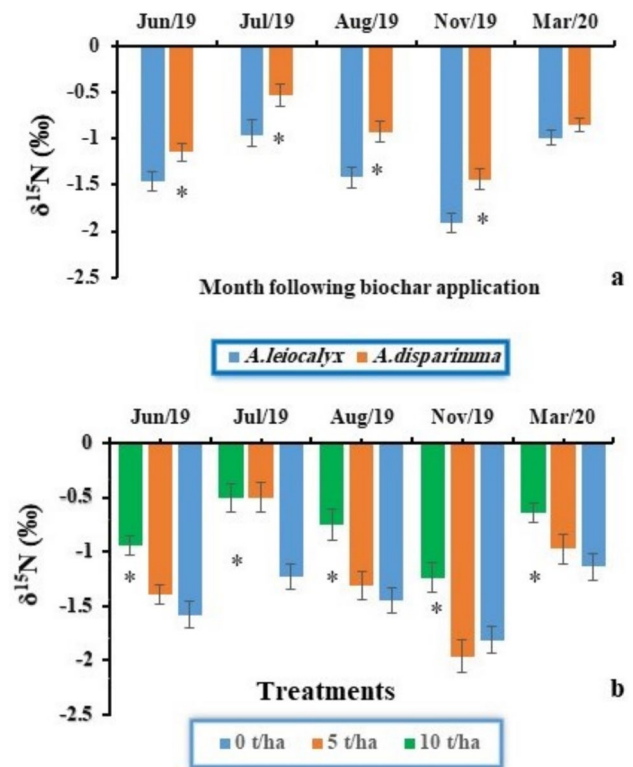


Fig. 3 Nitrogen isotope compositions ($\delta^{15}\text{N}$) for all sampling times since biochar application (months): (a) Foliar $\delta^{15}\text{N}$ of *A. leiocalyx* and *A. disparimma* at 1, 2, 3, 6 and 9 months after biochar application, and (b) Impact of biochar application rates on foliar $\delta^{15}\text{N}$ at 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between $\delta^{15}\text{N}$ of *A. leiocalyx* and that of *A. disparimma*, as well as among biochar treatments at $P < 0.05$

season), while lower foliar $\delta^{15}\text{N}$ was found in the spring sampling (-1.67‰) (November 2019) (Table 4).

3.2 Foliar total C, $\delta^{13}\text{C}$ and WUE

Foliar total C was significantly higher ($P < 0.05$) (51.14%) for *A. disparimma* at 3 months (August 2019) after biochar application, while foliar total C was significantly lower at month 9 (March 2020) after biochar application (47.13%) for *A. leiocalyx* (Table 2).

Foliar total C was significantly higher (51.4%) at 3 months (August 2019) after biochar application for the treatment of 10 t ha^{-1} compared with those of the 5 t ha^{-1} and the control, while the lowest foliar total C was found at month 9 (March 2020) (48.0%) in the control (Table 3). Meanwhile, foliar total C was significantly higher (50.96%) in the winter sampling (August 2019), while the lowest foliar total C was found in the autumn sampling (March 2020) (48.2%) (Table 4).

Foliar $\delta^{13}\text{C}$ was significantly higher ($P < 0.05$) (-31.04‰) for *A. disparimma* after 6 months of biochar application (Fig. 4a), while foliar $\delta^{13}\text{C}$ was significantly

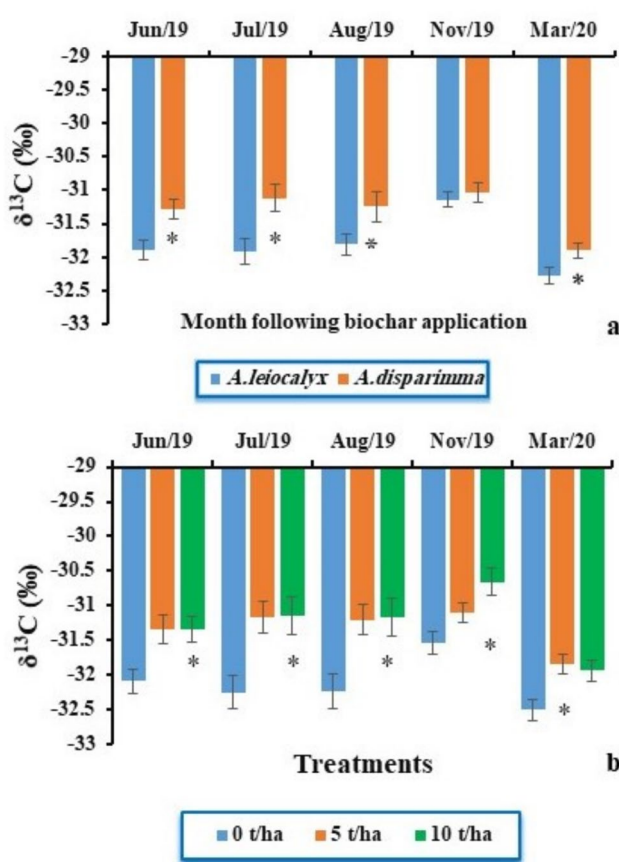
lower (-32.28‰) at month 9 (March 2020) for *A. leiocalyx* (Fig. 4a). The present study indicated that foliar $\delta^{13}\text{C}$ was significantly higher for the treatment of biochar applied at 10 t ha^{-1} for all sampling months (1, 2, 3 and 6 months) after biochar application than those of 5 t ha^{-1} and control (Fig. 4b), but not in the autumn season (March 2020).

Our study indicated that foliar $\delta^{13}\text{C}$ was significantly higher (-30.66‰) after 6 months of biochar application for the treatment 10 t ha^{-1} , while foliar $\delta^{13}\text{C}$ was significantly lower (-32.50‰) after 9 months of biochar application for the control (Fig. 4b). Meanwhile, foliar $\delta^{13}\text{C}$ was significantly higher ($P < 0.05$) in the spring sampling period (November 2019) (-31.09‰), while the lowest foliar $\delta^{13}\text{C}$ was found in the autumn sampling (March 2020) (-32.09‰) (Table 4).

Our results showed that WUE was significantly higher ($P < 0.05$) for *A. disparimma* ($45.07 \mu\text{mol mmol}^{-1}$) after 6 months of biochar application in November 2019, while WUE was significantly lower at month 9 (March 2020) ($32.18 \mu\text{mol mmol}^{-1}$) for *A. leiocalyx* (Table 2). The current

Table 4 Impact of foliar sampling periods shortly before (April 2019) and 1, 2, 3, 6 and 9 months after biochar application (June, July, August and November 2019 and March 2020 respectively) on foliar total C (TC), total N (TN), C isotope composition ($\delta^{13}\text{C}$), N isotopecomposition ($\delta^{15}\text{N}$), biological N fixation (BNF) and water use efficiency (WUE) within the Toohey Forest in southeast Queensland. Different letters indicate significant differences among biochar application periods at $P < 0.05$

Sampling time	TC (%)	TN (%)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	BNF (%)	WUE ($\mu\text{mol mmol}^{-1}$)
April 2019	48.85 (0.20)cd	2.37 (0.05)a	−31.17 (0.18)ab	−1.57 (0.09)de	52.29 (3.65)c	44.04 (2.11)a
June 2019	48.48 (0.10)de	2.28 (0.03)a	−31.59 (0.11)b	−1.31 (0.08)cd	64.69 (2.72)bc	38.39 (1.35)ab
July 2019	49.03 (0.20)c	2.30 (0.04)a	−31.52 (0.15)ab	−0.74 (0.11)a	87.08 (3.18)a	39.29 (1.82)ab
August 2019	50.96 (0.13)a	2.31 (0.05)a	−31.53 (0.14)ab	−1.17 (0.08)bc	61.94 (3.64)bc	39.06 (1.75)ab
November 2019	49.83 (0.14)b	2.00 (0.03)a	−31.09 (0.10)a	−1.67 (0.08)e	58.88 (2.48)bc	44.14 (1.17)a
March 2020	48.18 (0.21)e	2.38 (0.05)a	−32.09 (0.09)c	−0.92 (0.05)ab	65.84 (2.96)b	33.77 (1.06)b

**Fig. 4** Carbon isotope compositions ($\delta^{13}\text{C}$) for all sampling times since biochar application (months): (a) Foliar $\delta^{13}\text{C}$ of *A. leiocalyx* and *A. disparimma*, and (b) Impact of biochar treatments on foliar $\delta^{13}\text{C}$ for each sampling period at 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between *A. leiocalyx* and *A. disparimma*, as well as among biochar treatments at $P < 0.05$

study indicated that WUE was significantly higher ($P < 0.05$) for the treatment of biochar applied at 10 t ha^{-1} than those of the 5 t ha^{-1} and the control ($49.63 \mu\text{mol mmol}^{-1}$) in the spring sampling period of November 2019, while the lowest WUE was found in the autumn sampling of March 2020

for the control ($29.73 \mu\text{mol mmol}^{-1}$) (Table 3). The WUE was significantly higher ($44.14 \mu\text{mol mmol}^{-1}$) in November 2019 (spring-wet season), while the lower WUE was found in the autumn sampling ($33.77 \mu\text{mol mmol}^{-1}$) (March 2020) (Table 4).

3.3 Relationship between plant physiological variables

Multiple regression analyses were performed for both acacia species (*A. leiocalyx* and *A. disparimma*) (3 years after burning) to find out the relationships among foliar $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and total N, or between foliar total N and $\delta^{13}\text{C}$, between foliar $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, and between total N and $\delta^{15}\text{N}$.

For the autumn sampling in April 2019, 36.7% of the variation in foliar $\delta^{13}\text{C}$ (Table 5, Row 1) of both acacia species was explained by foliar $\delta^{15}\text{N}$ (6.1%) (Fig. 5a) and total N (24.6%) (Table 5, Row 1). For the winter sampling of June 2019, 15.2% of the variation in foliar $\delta^{13}\text{C}$ (Table 5, Row 1) of both acacia species at 1 month after biochar application was explained by the corresponding foliar $\delta^{15}\text{N}$ (10.1%) (Fig. 5b) and total N (2.1%) (Table 5, Row 1), while 22.8% and 19.9% of the variation in foliar $\delta^{13}\text{C}$ of both winter sampling (July and August 2019) were explained by the corresponding foliar $\delta^{15}\text{N}$ and total N (Table 5, Rows 1 and 2). For the winter and spring sampling of July and November 2019, there were 19.6% and 10.5% of the variation in foliar total N of both acacia species at month 2 and 6 respectively explained by the corresponding foliar $\delta^{15}\text{N}$ (Fig. 6a, b). For spring sampling, 13.3% of the variation in foliar $\delta^{13}\text{C}$ of acacia species at month 6 was explained by $\delta^{15}\text{N}$ and total N (Table 5, Row 2).

3.4 Plant H and DGL

The H of *A. leiocalyx* was significantly higher than that of *A. disparimma* ($P < 0.05$; Table 6). Our study indicated that the highest H (12.55 cm) was found in *A. leiocalyx*, while the lowest H (10.07 cm) was found in *A. disparimma*

Table 5 Relationships among foliar C isotope composition ($\delta^{13}\text{C}$), total N (TN) and N isotope composition ($\delta^{15}\text{N}$), and between foliar $\delta^{13}\text{C}$ and TN of both acacia species for the autumn sampling before biochar applied in April 2019; and for the winter samplings in June and July 2019 (1 and 2 months after biochar application) in Row 1, and relationships among foliar $\delta^{13}\text{C}$, TN and $\delta^{15}\text{N}$, and between foliar

$\delta^{13}\text{C}$ and TN of both acacia species for the winter sampling 3 months after biochar applied in August 2019; the spring sampling in November 2019 at 6 months after biochar applied, and the autumn sampling in March 2020 at 9 months after biochar application in Row 2 in Toohey Forest of southeast Queensland

Row 1	Month 0 April 2019	Month 1 June 2019	Month 2 July 2019
	$\delta^{13}\text{C} (\text{‰}) = -25.81 + 0.634 (\delta^{15}\text{N}) - 1.78 (\text{TN})$ ($R^2 = 0.367$, $n = 48$, $P < 0.05$) $\delta^{13}\text{C} (\text{‰}) = -27.30 - 1.58 (\text{TN})$ ($R^2 = 0.246$, $n = 48$, $P < 0.05$)	$\delta^{13}\text{C} (\text{‰}) = -29.07 + 0.547 (\delta^{15}\text{N}) - 0.791 (\text{TN})$ ($R^2 = 0.152$, $N = 48$, $P < 0.05$) $\delta^{13}\text{C} (\text{‰}) = -30.54 - 0.459 (\text{TN})$ ($R^2 = 0.021$, $n = 48$, $P > 0.05$)	$\delta^{13}\text{C} (\text{‰}) = -26.82 + 0.627 (\delta^{15}\text{N}) - 1.84 (\text{TN})$ ($R^2 = 0.228$, $N = 48$, $P < 0.05$) ns
Row 2	Month 3 August 2019	Month 6 November 2019	Month 9 March 2020
	$\delta^{13}\text{C} (\text{‰}) = -28.30 + 0.357 (\delta^{15}\text{N}) - 1.22 (\text{TN})$ ($R^2 = 0.199$, $n = 48$, $P < 0.05$) $\delta^{13}\text{C} (\text{‰}) = -28.87 - 1.15 (\text{TN})$ ($R^2 = 0.157$, $n = 48$, $P < 0.05$)	$\delta^{13}\text{C} (\text{‰}) = -28.95 + 0.401 (\delta^{15}\text{N}) - 0.734 (\text{TN})$ ($R^2 = 0.133$, $n = 48$, $P < 0.05$) ns	$\delta^{13}\text{C} (\text{‰}) = -30.857 - 0.518 (\text{TN})$ ($R^2 = 0.083$, $n = 48$, $P < 0.05$)

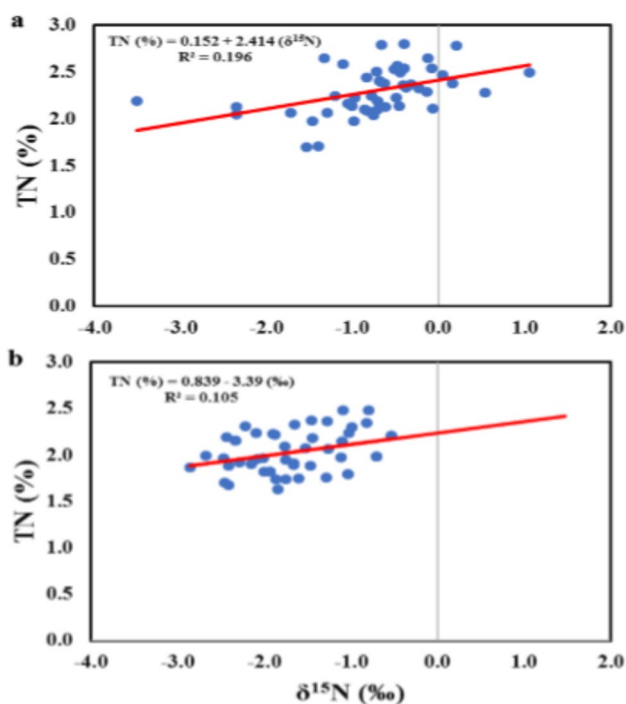


Fig. 5 Relationships between foliar total N (TN) and $\delta^{15}\text{N}$ of both acacia species (a) for the winter sampling in June 2019; and (b) for the spring sampling in November 2019 in Toohey Forest of south-east Queensland

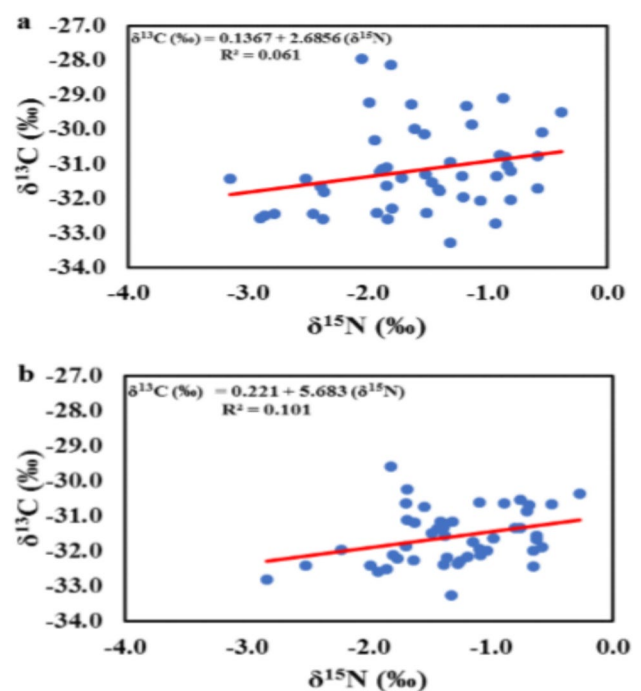


Fig. 6 Relationships between foliar $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of both acacia species (a) for the autumn sampling in April 2019; and (b) for the winter sampling in June 2019 in Toohey Forest of southeast Queensland

(Table 6). No significant difference was found in DGL of *A. leiocalyx* and *A. disparimma*. The current study indicated that the highest DGL (3.30 cm) was found in *A. leiocalyx*, while the lowest DGL (3.15 cm) was found in *A. disparimma* (Table 6).

Our study indicated that the highest H (12.73 cm) was observed in the treatment 10 t ha⁻¹, but there was no significant difference in H between the treatments of 10 t ha⁻¹ and the 5 t ha⁻¹. The lowest H (8.50 cm) was found in the control (Table 7). Meanwhile, DGL (3.65 cm) in the treatment 10 t ha⁻¹ was significantly higher than that (2.93 cm) in the control.

Table 6 Height and diameter at ground level (DGL) of *A. leiocalyx* and *A. disparimma* 3 and 6 months after biochar application in Toohey Forest south-east Queensland. Different letters indicate significant differences in heights and DGL between the acacia species at $P < 0.05$

Species	Height (cm)	DGL (cm)
<i>A. leiocalyx</i>	12.55 (1.7)a	3.30 (0.2)a
<i>A. disparimma</i>	10.07 (0.7)b	3.15 (0.1)a

Table 7 Height and diameter at ground level (DGL) among biochar treatments. Different letters indicate significant differences in Heights and DGL among the biochar treatments at $P < 0.05$

Treatment	Height (cm)	DGL (cm)
0 t/ha ⁻¹	8.50 (0.8)b	2.93 (0.2)b
5 t/ha ⁻¹	12.70 (1.5)a	3.11 (0.1)b
10 t/ha ⁻¹	12.73 (2.1)a	3.65 (0.2)a

Overall, our hypothesis that short-term effect after biochar application may alter soil C and N pools, thus it could influence plant growth. This study showed that short-term (1–9 months) of biochar application increased foliar total N and N availability as reflected in foliar $\delta^{15}\text{N}$ which plays a vital role in improving plant growth.

4 Discussion

4.1 Foliar total N, BNF, and $\delta^{15}\text{N}$

Our study showed that foliar total N was increased for the treatment of 10 t ha⁻¹ at months 1, 2 and 3 for the winter of June, July and August 2019, followed by a decrease in foliar total N in November 2019, 6 months after biochar was applied for both treatments of 5 t ha⁻¹ and control in the spring of November 2019. This would mean that N availability would be higher under the biochar application since there would be lower N losses in this case. We also found that biochar applied at 10 t ha⁻¹ had higher total N than 5 t ha⁻¹ and 0 t ha⁻¹ in the autumn of March 2020 after 9 months of biochar application, which is similar to the findings from Zhang et al. (2017a, b). This is consistent with the findings of Fan et al. (2020), who found that foliar total N significantly increased after 6 years of biochar application.

This study demonstrates that *A. leiocalyx* had relative higher total N than *A. disparimma* in the autumn of March 2020. This is similar to the findings of Bai et al. (2012) and Ma et al. (2015) who found that foliar total N was significantly greater in *A. leiocalyx* than that of *A. disparimma*. This field work indicated that *A. leiocalyx* had significantly higher total N than *A. disparimma*, partly due to the higher BNF by *A.*

leiocalyx, and also partly due to more N derived from the soil. Although BNF was higher for *A. disparimma*, it had less demand for N due to the slow growth, with more N left in the soil. The temporal and spatial variations in foliar total N concentrations might account for these differences in response to the prescribed burning (Bai et al. 2017). Therefore, *A. leiocalyx* may recover more quickly after 9 months of biochar application and may also make a more significant contribution to the N recovery because N is the main component of Rubisco, the enzyme facilitating C fixation at the carboxylation site (Wilson et al. 2000), and our foliar total N values were within the range of foliar total N for acacia species. There were no significant effects of biochar treatments and sampling time on foliar total N and no significant interaction between the treatment and sampling times (Bai et al. 2012).

We assessed the BNF among the biochar treatments and between two acacia species growing in suburban Toohey Forest. We found that even 3 years after the prescribed burning and biochar treatments, both acacia species still depended upon BNF for their N supply. This study demonstrated that BNF was higher with the biochar applied at 10 t ha⁻¹ compared with those of the 5 t ha⁻¹ and the control (0 t ha⁻¹) in the winter of July 2019 after 2 months of biochar application, but BNF was decreased in the winter and spring of August and November 2019 and autumn of 2020, followed with an increase in BNF in the autumn-wet season (March 2020), 9 months after biochar was applied for both treatments of 10 t ha⁻¹ and 5 t ha⁻¹. This is contradicting the finding by Hiama et al. (2019) who found that increasing biochar rate beyond 5 t ha⁻¹ decreased BNF and nutrients uptake in cowpea in the short term. The decrease in nodulation and BNF beyond the 5 t ha⁻¹ biochar was possibly a result of increased C: N ratio (Quilliam et al. 2012), which reduced the BNF. The BNF was significantly increased after biochar was applied at 10 t ha⁻¹, leading to the highest rate of BNF compared with those of the biochar application rate at 0, 50 and 120 t ha⁻¹ (Mia et al. 2014).

This study also demonstrated that *A. disparimma* had higher BNF in the winter of July 2019 than those of *A. leiocalyx*. This contradicts the findings from Ma et al. (2015) and Reverchon et al. (2020). *A. leiocalyx* could have higher TN, partly due to higher BNF, and also partly due to more N from the soil. The seasonal differences in BNF were found for both *A. disparimma* and *A. leiocalyx* (Reverchon et al. 2020). However, both Ma et al. (2015) and Reverchon et al. (2020) reported the BNF for the short- and medium- term impact of prescribed burning at the relevant sites.

The estimated BNF, based on ^{15}N natural abundance technique, for *A. leiocalyx* and *A. disparimma* agreed well with those for other acacia species reported in Australia. We found that BNF ranged from 52.4 to 78.7% for *A. leiocalyx* and from 65.0 to 84.2% for *A.*

disparimma for all foliar sampling times after biochar application during the winter and spring seasons of July, August and November 2019. Our results showed that BNF was higher in the winter of July 2019 than those in spring and autumn. The range in our study was much narrower for both species, and this variability may have been due to the within treatment variation caused by spatial variation of mutualistic organisms (Boddey et al. 2000) or non-homogenized N and water availability in the soil (Stevenson et al. 1995).

Overall, the current study was established to determine the effect of short-term biochar application on BNF of leguminous plants (N_2 -fixing species) mixed with non- N fixing trees, such as acacia species mixed with eucalypt species. Our study concluded that not all potential impacts are evident at 1–9 months after biochar application, and hence specific questions may require long-term experiments to attain answers. Whereby, an implication of this is the possibility that effects on the biochar-soil system in the long-term (above 3 years) might be different than those found in the short-term (Gul et al. 2015).

Our results showed significant differences in foliar $\delta^{15}N$ among the biochar treatments and between *A. leiocalyx* and *A. disparimma*. This might be attributed to the temporal and spatial variations in foliar $\delta^{15}N$ of understory acacia species in response to the prescribed burning. We found that foliar $\delta^{15}N$ was increased in the first two months for winter of July 2019 after biochar was applied, followed with a decrease in foliar $\delta^{15}N$ for all biochar treatments at months 3 and 6 for winter and spring of August and November 2019, due to biochar amendment which reduced the leaching of a ^{15}N depleted N (Menke 2017), which is consistent with the findings by Asadyar et al. (2021) and Menke (2017), who found that $\delta^{15}N$ was decreased after biochar was applied. Biochar application showed reduced foliar $\delta^{15}N$ values combined with increased plant total N values due to reduction in N losses (Menke 2017). The treatment of 10 t ha^{-1} had significantly higher foliar $\delta^{15}N$ in the winter of July 2019 after 2 months of biochar application compared with those of the control, but the 10 t ha^{-1} did not differ from that of 5 t ha^{-1} . Meanwhile, this study showed that *A. leiocalyx* had less N from BNF than *A. disparimma*. The findings by Bai et al. (2017) and Nguyen et al. (2017) showed that foliar $\delta^{15}N$ was linked up with plant uptake from N sources with depleted ^{15}N .

4.2 Foliar total C, $\delta^{13}C$ and WUE

Our study showed that foliar total C was higher in the winter of August 2019 after 3 months of biochar applied for the treatment of 10 t ha^{-1} compared with those of the control, but did not differ from that of 5 t ha^{-1} , which

is similar to the findings from Reverchon et al. (2015) and Fan et al. (2020). Conversely, this study highlighted that foliar total C decreased in the spring and autumn of November 2019 and March 2020 after 6 and 9 months of biochar application respectively. This contradicts the findings from Menke (2017) who reported that biochar treatments increased foliar total C than those of the control at months 6 and 11.

Our study showed that biochar applied at 10 t ha^{-1} had higher foliar $\delta^{13}C$ than 5 t ha^{-1} and control in the spring of November 2019, 6 months after biochar application. Thereafter, this study also demonstrated that foliar $\delta^{13}C$ was reduced in the autumn of March 2020 after 9 months of biochar application for all treatments, which is consistent with findings by Menke (2017) who found that $\delta^{13}C$ was reduced after biochar application. Furthermore, the current study showed that foliar $\delta^{13}C$ was increased in the spring of November 2019, 6 months of biochar application for *A. leiocalyx*. Thereafter, foliar $\delta^{13}C$ was decreased in the autumn of March 2020 for both *A. leiocalyx* and *A. disparimma*. Both *A. leiocalyx* and *A. disparimma* were different in WUE. The *A. leiocalyx* had higher WUE and hence higher photosynthesis under water limiting conditions.

The current study indicated that *A. disparimma* had higher WUE than that of *A. leiocalyx* for all sampling months, but not in the spring of November 2019. This contradicts the findings of Bai et al. (2012) and Ma et al. (2015). This might be due to the temporal and spatial variations in foliar $\delta^{13}C$ between the species, as foliar $\delta^{13}C$ has been reported to change with water and light availability (Bai et al. 2017). However, we found that WUE was gradually reduced in the autumn season of March 2020 for both *A. disparimma* and *A. leiocalyx*, because the biochar addition may increase the soil water holding capacity (Ajayi et al. 2016; Fischer et al. 2019).

5 Conclusion

Biochar application improved foliar total N, BNF and growth of understory acacia species in the first 9 months of biochar application in the Toohey Forest of subtropical Australia. Both acacia species were improved after biochar application in the first three months, with the increased foliar total N, total C, BNF and $\delta^{15}N$, compared with those of the control. The foliar $\delta^{13}C$ and WUE were increased after 6 months of biochar application. There were significant and positive relationship between foliar $\delta^{13}C$ and $\delta^{15}N$, highlighting the role of BNF in improving plant WUE and subsequently, tree growth of understory acacia species in the suburban native forest ecosystem subjected to the repeated prescribed burning.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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