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Phloem Sap and Wood Carbon Isotope Abundance ($\delta^{13}C$) Varies with Growth and Wood Density of *Eucalyptus globulus* under Nutrient Deficit and Inform Supplemental Nutrient Application

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Abstract: *Eucalyptus globulus*, commonly known as blue gum or southern blue gum, is a tall, evergreen tree endemic to southeastern *Australia*. *E. globulus* is grown extensively in plantations to improve the sustainability of timber and fibre production across Australia. Sustainable forest management practices necessitate the consideration of 'off-site' carbon and ecological footprints. Pursuing optimal supplemental nutrient application and maximum growth rates is therefore critical to the establishment of a sustainable timber and fibre production industry. Biological indicators that can predict growth responses are therefore of extreme value. We investigated the carbon isotope abundance of wood cellulose ($\delta^{13}C_{cel}$) in *E. globulus* to determine potential relationships with the carbon isotope abundance of phloem sap ($\delta^{13}C_{phl}$) where the trees were subjected to different level of nutrient availability. This study also sought to determine the effect of nutrient additions on the growth of the *E. globulus* and to quantify the relationship between the volumetric growth of wood and $\delta^{13}C_{cel}$. Phloem sap and wood cores were collected from trees within study plots which were subjected to seven nutrient treatments over a two-year period in a monoculture *E. globulus* plantation in South Australia. Phloem sap was collected using the razor blade technique and wood cores were collected using a stem borer. The carbon isotope abundance ($\delta^{13}C$) of phloem sap and wood grown in the radial direction of the stem were determined. The basic and dry densities of wood were determined, and their relationships with phloem and wood $\delta^{13}C$ were established. The $\delta^{13}C_{phl}$ was significantly correlated with $\delta^{13}C_{cel}$. The relationship between $\delta^{13}C_{cel}$ and the wood density of the respective wood sections was significant but did not consistently show the same pattern. There was no significant variation in basic density observed along the radial direction of the stem wood of the short-rotation *E. globulus* trees. A positive correlation was observed between $\delta^{13}C_{cel}$ and the wood basic density, but the relationship was not consistent along the radial direction of the stem. However, positive correlations were observed between $\delta^{13}C_{cel}$ and the air-dry density of respective wood sections. The relationship between phloem and wood $\delta^{13}C$ and the relationship between $\delta^{13}C$ and wood density along the radial direction of the stem needs to be considered while monitoring forest growth under nutrient- and water-limited conditions.

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1. Introduction

Trees maintain the long-distance transport of metabolites and inorganic compounds from source (leaves) to sink (stem) through specialised tissues such as sieve elements and companion cells of the phloem [1,2]. While the carbon isotope abundance of phloem sap ($\delta^{13}C_{phl}$) is correlated with tree water status [3], the use of phloem sap as an indicator of plant health and nutrition is incomplete due to a limited understanding of phloem contents under varying environmental conditions. The relationships among sugar composition and $\delta^{13}C_{phl}$ with tree growth and physiological performance have been investigated, with site-specific responses found to be reflected in the phloem sap [4]. The relationship between $\delta^{13}C_{phl}$ and wood cellulose carbon isotope abundance ($\delta^{13}C_{cel}$) varies in accordance with the inter-specific location of the cellulose within the tree growth ring [5]. For *Eucalyptus globulus*—a tall, evergreen tree endemic to southeastern Australia, Pate and Arthur [3]—seasonal fluctuations predicted in bulk phloem $\delta^{13}C$ would be reproduced in xylem laid down approximately one month later, supported by empirical evidence detailing seasonal fluctuations in wood $\delta^{13}C$ sampled throughout the growth rings. Remobilisation of previously fixed carbon may interfere with this transfer of the $\delta^{13}C$ patterns from phloem to cellulose, as would heterotrophic fractionation [2]. Patterns in phloem sap $\delta^{13}C$ may offer additional surrogate measures of plant nutritional status, as well as for the processes of carbon allocation in plant tissues.

Stem core analysis has great capacity to characterise tree ring width and wood density along the radial direction of the stem. These measures can be correlated with stem diameter at breast height (dbh) and/or tree height to estimate aboveground carbon sequestration without harvesting the tree (i.e., using a non-destructive sampling approach). The $\delta^{13}C$ in the stem core along the radial direction varies and can be used as a surrogate measure of water use efficiency [6]. The $\delta^{13}C$ values in the CO_2 of well-mixed atmospheric air is approximately −8‰ but leaves and the wood of trees produce lower values ranging from −20‰ to −30‰, representing a depletion in ^{13}C as CO_2 diffuses into leaves and wood is fixed into plant components—known as fractionation [7]. Fractionation due to diffusional or biochemical processes can be influenced by environmental factors. Consequently, the abundance of carbon isotopes can allow for the inference of environmental factors at the time of C deposition in plant tissues [7]. Dominating fractionation events such as diffusion of CO_2 through the stomatal aperture and carboxylation reactions of photosynthesis are well characterised [8] but there has been relatively little focus on the subsequent heterotrophic events. Whilst the mechanistic origin of fractionation events post-photosynthesis may be significant, investigations into the use of $\delta^{13}C$ obtained from heterotrophic tissues remain promising [9]. $\delta^{13}C$ from purified cellulose is now widely used to avoid overwhelming fractionation events associated with lignin formation [7,10].

The present study seeks to determine the interactive influences of a key environmental factor (i.e., nutrient availability) on phloem sap carbon isotope abundance ($\delta^{13}C_{phl}$) and wood cellulose carbon isotope abundance ($\delta^{13}C_{cel}$), and to investigate the relationships between these parameters and wood density as a means of developing surrogate measures for tree growth. Thus, the hypotheses of this study are as follows: (1) $\delta^{13}C_{phl}$ and $\delta^{13}C_{cel}$ are correlated, (2) $\delta^{13}C_{cel}$ varies in accordance with wood density, (3) $\delta^{13}C_{cel}$ is correlated with the volumetric growth of a forest stand, and (4) volumetric growth of wood and $\delta^{13}C_{cel}$ vary with nutrient availability. These hypotheses were tested in an economically important monoculture plantation of *E. globulus* grown under different nutritional treatments in South Australia.

2. Materials and Methods

2.1. Site Selection and Experimental Design

This study was conducted in a commercial 4-year-old plantation of *E. globulus* located in the Mount Gambier region of South Australia. Geographically, the area is located between 37.75° S latitude and 140.77° E longitude with an elevation of 63 m above sea level. Six different nutrient treatments were applied on the plantation (see Table 1). The region is characterised by a Mediterranean climate, with most of the rainfall occurring in winter and spring (June to November). From 2014 to 2018, the mean maximum and minimum air temperatures at the Mount Gambier region were 27.72 °C and 13.34 °C, respectively. The mean annual rainfall was 739.12 mm and mean daily evaporation was 3.5 mm [11].

Table 1. Composition of fertiliser and associated element(s) added to each treatment within the *Eucalyptus globulus* plantation.

Treatment	Fertiliser Product	Element Amounts (kg ha^{-1})		
		N	P	S
250 kg N	Urea	115		
350 kg N	Urea	161		
450 kg N	Urea	207		
250 kg NP	Urea + Mono-ammonium phosphate	81	23	2
350 kg NP	Urea + Mono-ammonium phosphate	113	32.2	2.8
450 kg NP	Urea + Mono-ammonium phosphate	146	41.4	3.6
Nil/Control	No addition of exogenous nutrient	-	-	-

A factorial design was established for fertiliser doses consisting of 54 trees per plot, each with 9 trees per row. The row spacing was 4 × 2.5 m and the total size of each plot was 0.06 ha (24 × 25 m). The experiment included 42 plots, comprising seven treatments in six replicated plots. The total trial area was 2.52 ha (168 m wide and 150 m long). Treatments were randomly applied in each plot. The fertiliser treatments consisted of three rates of N fertiliser application three rates of urea, three rates of ammonium + phosphorus application as superphosphate, and a control plot that had no fertiliser application (Table 1). Fertiliser was applied in late September 2016.

2.2. Phloem Sap and Wood Core Collection

Phloem sap was collected from *E. globulus* trunks in February 2017 using a razor blade technique as described in Merchant et al.'s work [12]. The sap droplets were progressively collected using a glass disposable pipette from 1000 to 1400 h and kept in a single microtube for each tree, with an addition of 200 µL of methanol into the microtube for sample preservation. The samples were immediately transferred to a −20 °C freezer and stored in a −80 °C freezer within 48 h. The samples collected from individual trees were bulked into one sample per plot. The sampled trees were randomly selected within the plot.

The mean dbh of the trees of all the studied plot was calculated as 13.60 cm and the mean height of them was 11.40 m. Wood cores were collected at breast height (i.e., 1.3 m above the ground level) of the trunks of the same *E. globulus* trees sampled for phloem sap within the experimental plots. The wood cores were collected using a 40.64 cm two-threaded increment borer of 5.15 mm core diameter (Haglof, Sweden) from where phloem sap was collected. The extracted cores were immediately placed into a plastic drinking straw and wrapped with masking tape to reduce evaporation from the wood to determine the green volume and to calculate the wood (basic) density.

2.3. Tree Growth and Wood Density Measurement

The estimated stand volume under bark (ESVUB) over the two successive growing periods (2016 and 2017) was collected from data held by the company responsible for the

treatment plots, i.e., Australian Bluegum Plantations Pty. Ltd (Hamilton, Australia). The following equation was used to calculate the ESVUB:

$$\text{ESVUB} = (G \times \text{MDH} \times \text{FF}) \times \text{BT2} = (G \times \text{MDH} \times 0.344) \times 0.09446 \quad (1)$$

where G is the dbh, MDH is the mean height of the largest 200 stems per hectare, FF is the form factor of the site, and BT2 is the region-specific bark thickness factor.

Growth in the stand volume during the treatment period was calculated by subtraction (i.e., ESVUB at 2017 − ESVUB at 2016). Fresh wood sections were weighed, and the green volume of the samples was also determined. Core samples were put in an oven at 60 °C for 48 h, and then the oven dry weights were determined. The samples were kept in an oven for another 12 h at 60 °C and their dry weights were determined. This process was repeated 3 times until a constant dry weight was achieved. Green volumes of all wood sections and their basic densities were calculated via the following formulas:

$$v_g = \pi r_g{}^2 h \text{ and basic density of wood} = dw/v_g \text{ (g cm}^{-3}\text{)} \quad (2)$$

where r_g = radius of core (cm), h = length of core (cm), dw = oven dry weight (g), and v_g = green volume (cm^3).

The air-dry volume of wood was calculated using the following formula:

$$v_d = \pi r_d{}^2 h \quad (3)$$

where v_d = air-dry volume (cm^3), r_d = the dry radius of the core (cm), h = length of the core, and air-dry wood density = dw/v_d (g cm^{-3}).

2.4. $\delta^{13}C_{phl}$ and $\delta^{13}C_{cel}$ Analysis

A 5 µL aliquot of phloem sap solution was placed into an aluminium capsule (dimensions: 2.88/16 mm, IVA Analysentechnike. K. Meerbusch, Germany) and dried in an oven at 60 °C for 48 h. The $\delta^{13}C_{phl}$ was determined by a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The oxidation reactor was set to 1000 °C. Carbon isotope abundance (ratio) is expressed as delta notation, $\delta^{13}C = R_{sample}/R_{standard}$-1, R is the ratio of ^{13}C and ^{12}C in a sample and standard VPDB. The standard material precision is 0.06 ‰ to 0.11‰.

Annual dbh growth was around 3.5 cm with an average bark width of 0.5 cm. Annual wood growth at dbh was therefore calculated as 2.5 [3.5 − (0.5 + 0.5)] cm. As phloem sap was collected in 2016, a 3 cm section of the wood core was removed before five sections (each having 0.5 mm) were separated from the stem core to encapsulate wood formed during this time. These five sections encompassing 2.5 cm of wood were separated and extracted. For the cellulose extraction, the total sample size (n) was, therefore, 210 (1 stem × 5 sections of wood core × 42 sample plots). Each wood section (5 mm) was manually ground with a mortar and pestle. Ethanol was used to clean the mortar and pestle between each sample grinding. The ethanol was allowed to evaporate between each sample grinding.

Cellulose extraction from the ground wood was carried out following the work of Brendel et al. [13]. Wood core samples (5 mm) were finely ground, and 2–3 mg was weighed into 1.5 mL polypropylene tubes. Then, 120 µL acetic acid (80% acetic acid, reagent grade), followed by 12 µL nitric acid (69% nitric acid, reagent grade), was added. The tubes were capped and inserted into heating blocks at 120 °C for 30 min, and were agitated every 5 min, and then allowed to cool, after which 400 µL of ethanol was added, recapped, agitated, and then centrifuged for 5 min at 10,000 rpm (Eppendorf, Centrifuge 5424, Hamburg, Germany). The resulting supernatant was then carefully removed and discarded. Then, 300 µL of distilled deionised water (DDW) was mixed with the remaining pellet and again capped, agitated, and centrifuged for 5 min at 10,000 rpm. Upon removal of the supernatant, 150 µL of ethanol was added to the extract and capped, tapped firmly 2–3 times without inverting, and centrifuged for 5 min at 10,000 rpm. Again, the supernatant was carefully removed

and discarded. To obtain clean cellulose, steps using DDW and ethanol were repeated 2 times and 150 µL acetone was used to separate the cellulose from the supernatant. The supernatant was carefully removed, and these samples were placed at 45 °C for 24 h. The final product appeared as white, loosely packed pellet of cellulose.

A weight of approximately 0.35 mg of cellulose was placed into a tin capsule for analysis via Isotope Ratio Mass Spectrometry (IRMS). The $\delta^{13}C$ were determined by a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The oxidation reactor was set to 1000 °C.

2.5. Statistical Analysis

We analysed the effects of treatments using analysis of variance (ANOVA) followed by post hoc (Bonferroni) tests. To identify the relationships between parameters, linear regression analysis was performed. We used Sigmaplot software (version 12.5, Systat Software, Inc., San Jose, CA, USA) for all statistical analyses.

3. Results

3.1. Relationship between $\delta^{13}C_{phl}$ and $\delta^{13}C_{cel}$

The $\delta^{13}C_{phl}$ was correlated with $\delta^{13}C_{cel}$ but the relationship varied substantially along the radial direction of the stem (Figure 1). The $\delta^{13}C_{cel}$ of the first, second and third sections of the wood core showed a positive linear correlation with the $\delta^{13}C_{phl}$. In contrast, $\delta^{13}C_{cel}$ in the fourth and fifth sections of the wood core showed negative relationships with $\delta^{13}C_{phl}$ (see Figure 1).

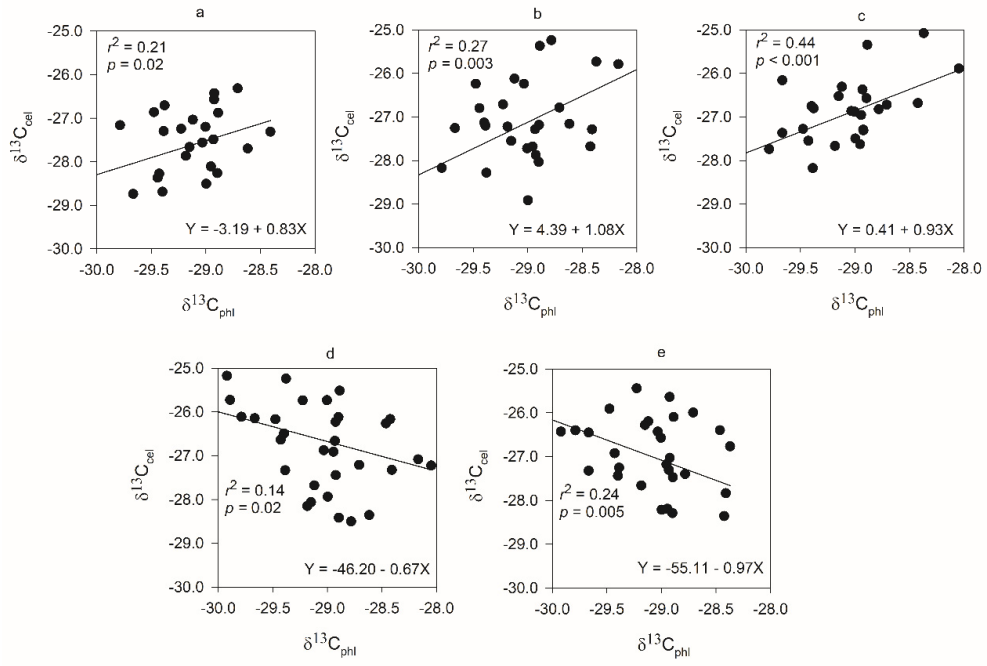

Figure 1. Relationship between cellulose carbon isotope abundance ($\delta^{13}C_{cel}$) and phloem carbon isotope abundance ($\delta^{13}C_{phl}$) along the radial direction of the stem wood. Here, (**a**–**c**) showed positive correlations between $\delta^{13}C_{cel}$ and $\delta^{13}C_{phl}$ of the first, second and third sections of 5 mm wood, and (**d**,**e**) showed negative correlations.

3.2. Relationship between Wood Density and $\delta^{13}C_{cel}$

We observed no significant variation in basic density along the radial direction of the stem wood (Figure 2); however, a gradual increase in the mean value of $\delta^{13}C_{cel}$ across the first to fifth section of stem wood was found. The $\delta^{13}C_{cel}$ of the first section of wood significantly differed from that of the fourth and fifth sections of wood ($p \leq 0.05$).

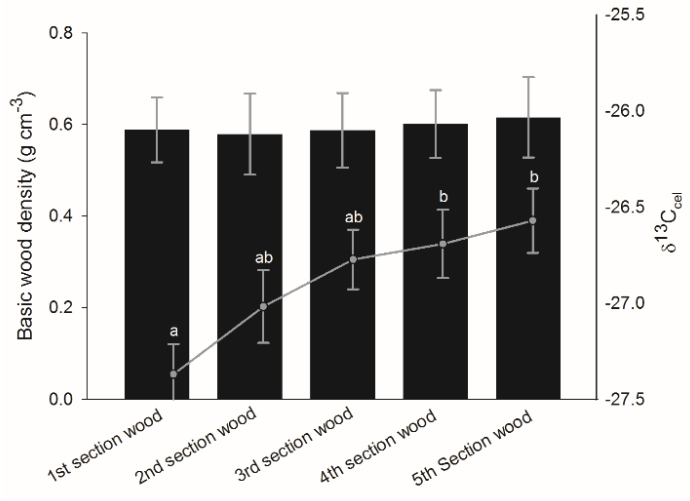

Figure 2. Basic density of wood and cellulose carbon isotope abundance ($\delta^{13}C_{cel}$) of corresponding outer (first section of wood) to inner parts of the tree trunk (fifth section of wood) along the radial direction of the stem. Here, small letters show significant differences (Bonferroni test), the data shown are means ± s.e., n = 7, the bar graph shows the basic density, and the line graph shows the $\delta^{13}C_{cel}$.

There were positive correlations between $\delta^{13}C_{cel}$ and the basic density of wood in the first and fifth sections along the radial direction, whereas negative correlations were observed in the second, third, and fourth sections of wood (Figure 3).

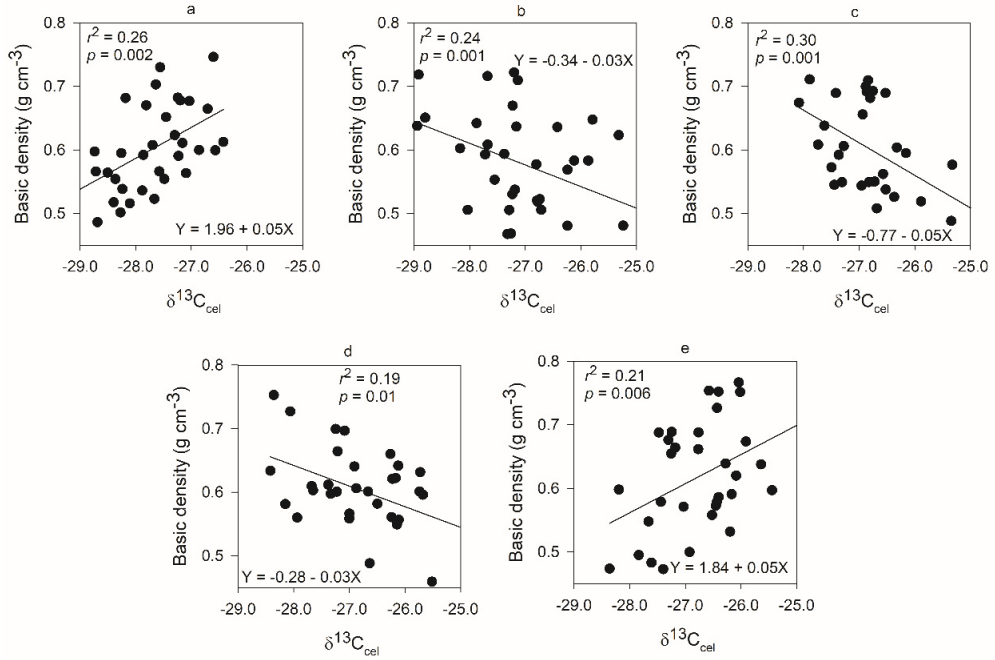

Figure 3. Relationship between wood basic density and cellulose carbon isotope abundance ($\delta^{13}C_{cel}$) along the radial direction of the stem wood. Here, (**a**,**e**) showed positive correlations between $\delta^{13}C_{cel}$ and basic density of the first and fifth sections of wood, and (**b**–**d**) showed negative correlations between $\delta^{13}C_{cel}$ and basic density of the second, third and fourth sections of wood.

For the air-dry wood density, positive correlations were observed between $\delta^{13}C_{cel}$ and the air-dry density of the respective wood sections. The $\delta^{13}C_{cel}$ of all five sections of the wood cores showed positive linear correlations with the air-dry density of wood (see Figure 4).

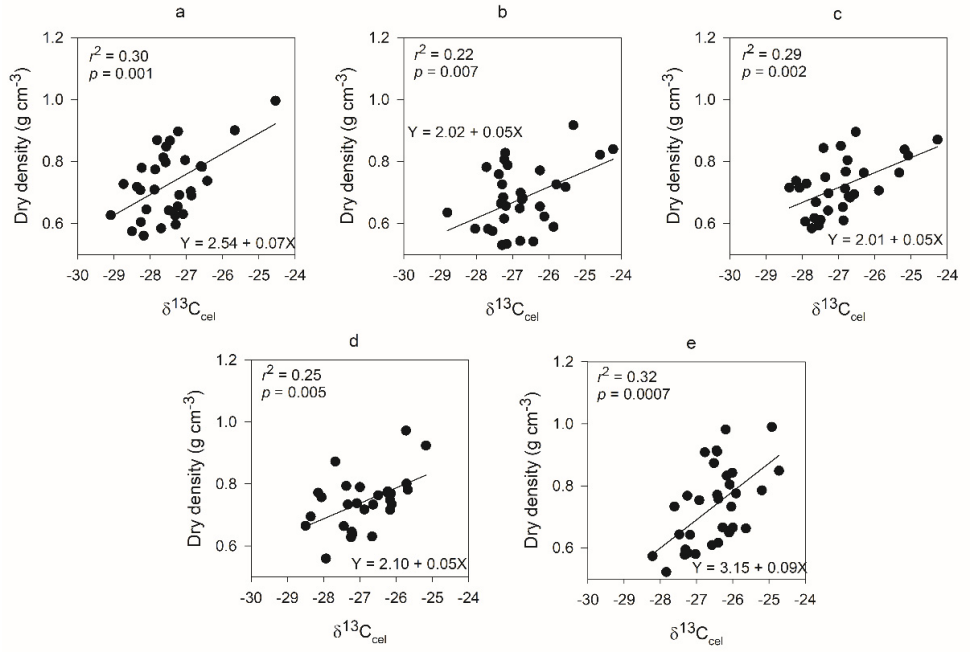

Figure 4. Relationship between wood dry density and its cellulose carbon isotope abundance ($\delta^{13}C_{cel}$) along the radial direction of the stem. Here, (**a**–**e**) showed positive correlations between $\delta^{13}C_{cel}$ and dry density of the first, second, third, fourth and fifth sections of wood, respectively.

3.3. Relationship between Stand Volumetric Growth and $\delta^{13}C_{cel}$

The $\delta^{13}C_{cel}$ of corresponding wood segments were plotted against the growth of the ESVUB. The $\delta^{13}C_{cel}$ was found to be positively correlated with an average ESVUB (r^2 = 0.30, p = 0.001, Figure 5).

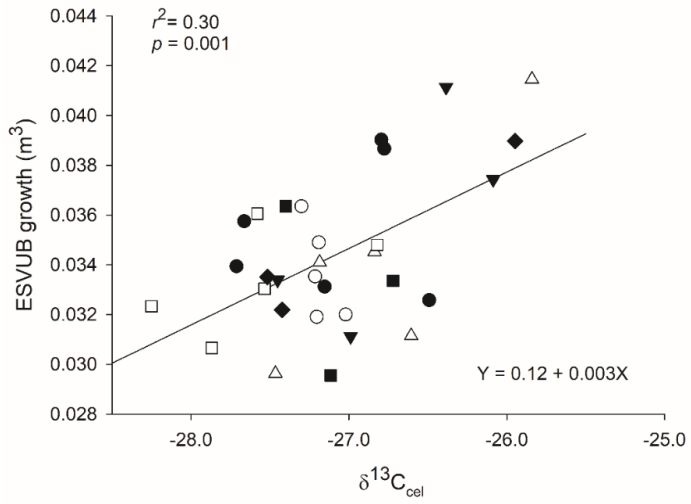

Figure 5. Nutrient effect on average estimated volume under bark (ESVUB) and its relationship with cellulose carbon isotope abundance ($\delta^{13}C_{cel}$). Plots with different nutrient treatments are denoted by the symbols: ♦ = Nil control, ▼ = 250 kg ha^{-1} N, o = 350 kg ha^{-1} N, ● = 450 kg ha^{-1} N, □ = 250 kg ha^{-1} NP, ■ = 350 kg ha^{-1} NP, Δ = 450 kg ha^{-1} NP.

4. Discussion

In the present study, wood cellulose $\delta^{13}C$ rather than bulk wood $\delta^{13}C$ was successfully used to determine relationships, similar to the work of Loader et al. [14]. Cellulose extraction and the use of $\delta^{13}C$ of purified cellulose is usually considered to result in more reliable trends in annually grown wood [15]. This is because it could be very close to that

of the primary products of photosynthesis with little post-photosynthetic discrimination and cellulose deposition occurring during the year of ring formation, while lignin can still be deposited several years later [10]. It was observed that phloem sap $\delta^{13}C$ was positively correlated with cellulose $\delta^{13}C$ in the first three sections of the wood core, and for the fourth and fifth sections, the relationship was negative (Figure 1). These contrasting relationships may indicate limitations on carboxylation, notably diffusional (i.e., stomatal and mesophyll conductance to CO_2) and biochemical (i.e., nutrient and light limitations) origins. Similarly, $\delta^{13}C$ variation along the radial direction of the stem in three types of *Eucalyptus* species (*E. diversicolor*, *E concinna*, and *E. phaenophylla*) was observed in South Australia [16]. A recent study of Halder et al. [5] showed intra-specific patterns of $\delta^{13}C$, with growth and wood density variation being observed along the radial direction of the stem. Similar to earlier studies, the current study also describes the $\delta^{13}C_{cel}$ variation along the radial direction of the stem, suggesting the transition of juvenile to mature wood in the studied tree species (Figure 2).

Wood density has important implications for estimations of biomass and the carbon content of a tree [17]. The observed trend of increasing basic density of wood correlating with an increasing $\delta^{13}C_{cel}$ value of different sections of wood samples along the radial direction of the stem wood was not consistent in this study. However, a positive correlation was detected in the first and fifth sections of wood, whilst negative correlations were observed in the inner segments, such as the second to fourth sections (Figure 3). However, for dry density of the wood, all relationships were positive (Figure 4). These results differ from those of Macfarlane and Adams [18], where they showed that wood density in drought-stressed *E. globulus* trees is not correlated with $\delta^{13}C_{cel}$.

Macfarlane and Adams [18] speculated that the $\delta^{13}C$ of wood might be influenced by frequent relative mild water deficits, while cambial activity and wood density may be more influenced by less frequent but more severe water deficits, which reduces photosynthesis. Similarly, Halder et al. [5] reported that $\delta^{13}C$ correlates with basic density in *E. camaldulensis* at a dry site. Whilst not conclusive to determine the functional relationship, our results support the notion that under conditions through which water significantly limits growth, $\delta^{13}C$ may offer a suitable surrogate measure of forest growth.

In the present study, *E. globulus* tree growth was likely limited by a combination of water and nutrient deficiency and not by other factors, such as temperature. Also, under the conditions of our study, the relationship between wood density and its $\delta^{13}C_{cel}$ differed between the seasons, indicated by the radial direction of the studied wood samples collected from the stem (Figure 3). $\delta^{13}C_{cel}$ has previously been found to be negatively correlated with wood density in the wet season, while in the dry season, the relationship showed a positive correlation [19]. The influence of inter-annual changes in water availability in wood $\delta^{13}C$ was examined previously by several authors (see [20–25]). The positive correlation between those parameters (i.e., carbon isotope and density) suggested an accumulation of more carbon content reflected through enriched signals of $\delta^{13}C$ [26]. Combined, these results may assist in applications to spatially and temporally integrate stand $\delta^{13}C_{cel}$ or $\delta^{13}C_{phl}$ with carbon capture and volume growth. In this study, a good correlation was observed between ESVUB and $\delta^{13}C_{cel}$ (Figure 5).

In many cases, increased availability of macronutrients increases growth and improves the capacity of a crop to absorb and transfer water and nutrients from soil [27]. In the present study, water use efficiency was found to be higher with a higher N supply, which is consistent with previous studies [28], indicating that larger plants use more water resources, thus increasing competition for a limited supply. Livingston et al. [29] found a significant positive correlation between water use efficiency and needle $\delta^{13}C$ and biomass growth of *Picea glauca* seedlings in both fertilised and nitrogen-stressed conditions. Combined, our results indicate that relationships between nutrient effects and water use efficiency are not straightforward, and plants interactively control water use efficiency by physiological and morphological processes in a complex manner [30]. Our study was conducted in a very juvenile stand (nearly 3 years old) of *E. globulus*. Therefore, the nutrient and water use

efficiency relationship at this early stage of the growing cycle could differ in the long term, while nutrient applications may also induce substantial variations in water use efficiency that warrant further investigation. Again, the growth rates of trees at different periods may not be consistent. We chose the wood core length of 2.5 cm based on the annual dbh growth. This 'fixed length' approach was adopted to pragmatically overcome our inability to identify growth rings.

5. Conclusions

Among many nations across the world, contemporary forest management is transitioning away from native forest harvesting toward intensive, plantation-based timber and fibre production. Minimising the footprint and maximising the productivity of plantation forestry is therefore critical to a sustainable industry. Predictive, applicable tools to optimise nutrient supplementation are necessary to avoid off-site energy consumption during the manufacturing of synthetic fertilisers. Simultaneously, optimising growth per hectare reduces land pressures for nature conservation. Here, we demonstrate a biological, predictive tool for use at-scale in the forest industries to enhance sustainable forest practices.

Wood cellulose carbon isotope abundance ($\delta^{13}C_{cel}$) was analysed to identify potential relationships with phloem sap carbon isotope abundance ($\delta^{13}C_{phl}$) where the sampled *E. globulus* trees were subjected to different nutrient treatments. The $\delta^{13}C$ obtained from metabolites within the phloem sap correlated with $\delta^{13}C$ obtained from cellulose, with distinct intra-specific patterns observed along the radial direction of the stem. These parameters produced both positive and negative relationships, indicating contrasting limitations to the growth of trees under field conditions. Additionally, the $\delta^{13}C_{cel}$ was influenced by the nutrient regimes, and the relationship between $\delta^{13}C_{cel}$ and wood density varied considerably, reflecting the interactive effects of water and nutrient availability on a background of seasonal variation in climatic conditions. With some indicative results of the present study, it is suggested that stem cores can be used to assess physiological processes involved in the formation of wood through patterns in $\delta^{13}C$. Combined, these results illustrate that radial variation in wood density and $\delta^{13}C$ needs to be considered in the application of these tools in predicting historical patterns of forest productivity.

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