Monitoring forest growth and carbon sequestration using phloem sap and dendrochronological properties

Nirmol Kumar Halder

A thesis submitted in fulfilment of the requirements for the degree of

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Declaration of Originality

I do hereby certify that this thesis is entirely my own work and all the materials written by others have been acknowledged in the text. To the best of my knowledge and belief, it does not have any material previously submitted, either in full or in part, for a degree at this, or any other institution.

(Nirmol Kumar Halder)

29 April 2020
Statement of Authorship

Chapters II, III, IV, V, and VI are written as the style of manuscripts for publication. Consequently, there is some repeated information in the methods section of each chapter. I intend to be the primary author of each of the manuscripts.

Chapter II of this thesis has been submitted for publications as “Halder, N. K., Fuentes, D., Possell, M., Ingram, L., Bradshaw, B., Merchant, A. (2020). Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions. Status: under review, Name of the journal: “Tree Physiology”.

I co-designed the study with the co-authors, analysed the data and wrote the MS.

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Chapter IV of this thesis will be submitted for publications as “Halder, N. K., Fuentes, D., Possell, M., Merchant, A. (2020). Relationships between phloem sap and leaf δ^{13}C are resilient to changes in nutritional status”. This manuscript will be submitted to the journal Physiological Plantarum”.

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I co-designed the study with the co-authors, analysed the data and wrote the MS.

*(Nirmol Kumar Halder)*

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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

*(Ass. Prof. Andrew Merchant)*

29 April 2020
Abstract

Global climate is perturbed by anthropogenic activities such as deforestation, a significant source of greenhouse gas emissions. Forests play an integral role in offsetting greenhouse gas emissions through the assimilation of atmospheric CO₂ and subsequent storage. Forest growth and development is influenced by a complex interaction of temperature, light, water, nutrient availability, and biotic interactions. An improved understanding and ability to predict growth is useful for both local productivity and forest carbon budgets. Therefore, monitoring forest growth and the development of tools to assess both time and spatially integrated properties of carbon sequestration are vital, particularly for forests under significant resource limitation. In this thesis, I have sought to develop monitoring tools to determine forest growth and carbon sequestration, specifically based upon the integrative properties of phloem sap and dendrochronological patterns in chemical and carbon isotopic abundance.

This thesis reviews and identifies gaps in the capacity of existing tools to determine forest growth limited by nutrient and water availability and models to determine the carbon content of forests. Assessment of forest and tree nutritional status is traditionally obtained via foliar analysis, which is time consuming and not spatially representative of the tree canopy. To address this, I propose phloem sap analysis shown here in use for blue gum (*Eucalyptus globulus*) grown in both field (South Australia) and controlled environmental conditions to characterise growth under contrasting nutrient supply.

Analysis of metabolites obtained from phloem sap show changes in amino acid concentrations in response to nutrient supply, but also across seasonal cycles. Added to this, patterns in metabolite abundance show both the interactive effects of nutrient availability (specifically N and P), and counterintuitive patterns in resource availability (specifically N) illustrating that
the interpretation of chemical and isotopic patterns obtained from phloem need to be interpreted in the context of whole plant physiology and resource distribution or remobilisation.

Relationships between phloem sap and dendrochronological properties are also investigated, specifically the relationships between carbon isotope abundance ($\delta^{13}C$) of carbon obtained from isolated cellulose and phloem borne soluble carbon. These properties, combined with a systematic determination of carbon concentration among growth rings are used to generate predictions of forest carbon sequestration in production forests of both Australia and Bangladesh. I show that current modelling approaches for the determination of forest carbon sequestration systematically underestimate the realised carbon gain in the absence of seasonal variation in carbon density.

Combined, these studies employ a diverse range of techniques, locations and disciplines with the ultimate objective of improving our ability to predict and monitor the carbon sequestration potential. Spanning the chemical, physiological, isotopic and ecological sciences, this work represents significant improvements in the methodologies employed to measure the health and productivity of forest ecosystems for both production and environmental purposes. Improved predictions of forest carbon storage and sequestration will provide more informed management decisions and an empirical framework for the development of forest management and policy.
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# Table of Contents

Publications........................................................................................................................................ 1

Status of manuscripts to be submitted to peer review journals:......................................................... 1

Conference papers (Oral or poster presentation) .............................................................................. 2

General Introduction............................................................................................................................. 3

Chapter 1: Current challenges to determining forest growth and carbon sequestration under water and nutrient limitations ................................................................. 4

Chapter II: Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions........................................................................................................ 4

Chapter III: Phloem and leaf amino acids reflect physiology, growth, and nutritional status of blue gum (*Eucalyptus globulus*) seedlings .............................................................................. 5

Chapter IV: Relationships between phloem sap and leaf δ¹³C are resilient to changes in nutritional status ........................................................................................................................................ 5

Chapter V: Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown *Eucalyptus globulus* .. 5

Chapter VI: Effects of drought on carbon sequestration potential of tropical trees in Bangladesh .................................................................................................................................................... 6

References........................................................................................................................................ 6

Chapter I: Current challenges to determining forest growth and carbon sequestration under water and nutrient limitations ........................................................................................................ 7

Abstract........................................................................................................................................... 7

1.1. Introduction................................................................................................................................. 8
Chapter II: Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions

Abstract

2.1. Introduction

2.2. Materials and methods

2.2.1. The study site

2.2.2. Experimental design

2.2.3. Collection of phloem sap

2.2.4. Phloem sap extraction for amino acid analysis

2.2.5. Phloem sap extraction for carbohydrate analysis

2.2.6. Phloem sap preparation for $\delta^{13}C$ analysis

2.2.7. Growth data collection
2.2.8. Analysis of amino acids, carbohydrates and $\delta^{13}C$ .................................................. 43
2.2.9. Statistical analysis ............................................................................................................. 44

2.3. Results ....................................................................................................................................... 44

2.3.1. Seasonal and treatment effects on phloem sap amino acid concentration ................. 44
2.3.2. Seasonal and treatment effects on phloem carbohydrates ........................................... 46
2.3.3. Relationships between phloem carbohydrates and $\delta^{13}C$ ........................................... 47
2.3.4. Relationship between phloem metabolites and tree growth ....................................... 48
2.3.5. Influence of fertiliser rate on DBH growth ................................................................. 50

2.4. Discussion ................................................................................................................................ 50

2.4.1. Phloem sap amino acids vary with nutrient supply and season ................................. 51
2.4.2. Phloem sap carbohydrates and $\delta^{13}C$ vary with season but not nutrient supply ... 52
2.4.3. Relationship between sugar concentration and phloem $\delta^{13}C$ ................................ 53
2.4.4. Relationships with tree growth ...................................................................................... 54

2.5. Conclusion ............................................................................................................................. 55

References ...................................................................................................................................... 56

Chapter III: Phloem and leaf amino acid reflect physiology, growth, and nutritional status of
blue gum (Eucalyptus globulus) seedlings .................................................................................. 62

Abstract ......................................................................................................................................... 62

3.1. Introduction ............................................................................................................................. 63

3.2. Material and methods ........................................................................................................... 64

3.2.1. Experimental design ......................................................................................................... 64
3.2.2. Growth and physiological data collection .............................................................. 65

3.2.3. Leaf tissue and phloem sap collection ............................................................... 66

3.2.4. Extraction of leaf and phloem materials ........................................................... 66

3.2.5. Analysis of leaf and phloem amino acids ......................................................... 67

3.2.6. Statistical analysis ............................................................................................ 68

3.3. Results .................................................................................................................... 68

3.3.1. Nutrient effect on net CO₂ assimilation rate and growth ............................... 68

3.3.2. Nutrient effect on leaf and phloem amino acid concentrations .................. 69

3.3.3. Relationships between leaf and phloem amino acid concentrations .......... 72

3.3.4. Relationships between leaf and phloem amino acids with growth .......... 72

3.4. Discussion .......................................................................................................... 75

3.4.1. Nutrient effect on plant physiology and growth ........................................... 75

3.4.2. Nutrient effect on leaf and phloem amino acids .......................................... 76

3.4.3. Relationships between leaf and phloem amino acid concentrations .......... 77

3.4.4. Leaf and phloem amino acids are tightly correlated with plant growth ...... 78

3.5. Conclusion ........................................................................................................... 79

References ................................................................................................................ 80

Chapter IV: Relationships between phloem sap and leaf δ¹³C are resilient to changes in nutritional status ................................................................. 87

Abstract ..................................................................................................................... 87

4.1. Introduction ........................................................................................................ 88
4.2. Material and methods ........................................................................................................ 89

4.2.1. Experimental design ............................................................................................... 89

4.2.2. Growth data collection .......................................................................................... 90

4.2.3. Leaf tissue and phloem sap collection ................................................................... 91

4.2.4. Extraction of leaf & phloem materials ................................................................... 91

4.2.5. Analysis of carbohydrates and $\delta^{13}C$ .................................................................. 92

4.2.6. Statistical analysis .................................................................................................. 93

4.3. Results ......................................................................................................................... 93

4.3.1. Fertiliser effect on plant growth ............................................................................. 93

4.3.2. Relationships between leaf and phloem $\delta^{3}C$ .................................................. 94

4.3.3. Fertiliser effect on leaf and phloem $\delta^{3}C$ ........................................................ 95

4.3.4. Relationships between phloem $\delta^{3}C$ and carbohydrates .................................... 96

4.3.5. Relationships between leaf and phloem carbohydrates ........................................... 97

4.3.6. Fertiliser effect on leaf and phloem carbohydrate concentrations ......................... 98

4.3.7. Relationships between phloem carbohydrates $\delta^{3}C$ and growth ......................... 101

4.4. Discussion .................................................................................................................... 102

4.4.1. Fertilizer effect on plant growth ............................................................................. 102

4.4.2. Nutrient effect on leaf and phloem $\delta^{3}C$ ........................................................ 103

4.4.3. Nutrient effect on leaf and phloem carbohydrates ................................................... 104

4.4.4. Phloem $\delta^{13}C$ and plant growth .......................................................................... 106

4.5. Conclusion ................................................................................................................... 106
Chapter V: Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown *Eucalyptus globulus* ................................................................................................................................................ 113

Abstract .................................................................................................................................. 113

5.1. Introduction ..................................................................................................................... 114

5.2. Material and methods ...................................................................................................... 117

  5.2.1. The study site ........................................................................................................ 117

  5.2.2. Experimental design ............................................................................................ 117

  5.2.3. Collection of phloem sap ...................................................................................... 118

  5.2.4. Collection of wood core ........................................................................................ 118

  5.2.5. Phloem sap preparation and $\delta^{13}C$ analysis ................................................ 119

  5.2.6. Growth and wood density .................................................................................... 119

  5.2.7. Sample preparation for cellulose $\delta^{13}C$ analysis ........................................... 120

  5.2.8. Extraction of cellulose .......................................................................................... 120

  5.2.9. Analysis of isotope ratios in tree rings ................................................................. 121

  5.2.10. Carbon sequestration rate .................................................................................. 121

  5.2.11. Statistical analysis .............................................................................................. 122

5.3. Results ............................................................................................................................. 122

  5.3.1. Relationship between phloem and wood cellulose $\delta^{13}C$. ................................ 122

  5.3.2. Basic wood density and cellulose $\delta^{13}C$ .......................................................... 123
5.3.3. Intra-specific growth ring density and cellulose $\delta^{13}C$ ............................................ 124
5.3.4. Nutrient effect on growth and cellulose $\delta^{13}C$ ..................................................... 126
5.3.5. Nutrient effect on carbon sequestration rate .............................................................. 128

5.4. Discussion .......................................................................................................................... 129
5.4.1. Phloem and wood cellulose $\delta^{13}C$ ........................................................................ 129
5.4.2. Wood density and cellulose $\delta^{13}C$ ....................................................................... 131
5.4.3. Nutrient effect on wood growth and its cellulose $\delta^{13}C$ .......................................... 132
5.4.4. Nutrient effect on carbon sequestration rate ............................................................ 133

5.5. Conclusions ...................................................................................................................... 134
References ............................................................................................................................... 135

Chapter VI: Effects of drought on carbon sequestration potential of tropical trees in Bangladesh
................................................................................................................................................ 143

Abstract .................................................................................................................................. 143

6.1. Introduction ...................................................................................................................... 144

6.2. Material and methods .................................................................................................... 147
6.2.1. Study area .................................................................................................................. 147
6.2.2. Climate ...................................................................................................................... 148
6.2.3. Soil properties .......................................................................................................... 148
6.2.4. Silviculture and management .................................................................................... 149
6.2.5. Sample collection and preparation ............................................................................ 150
6.2.6. Radial growth and wood density measurements ....................................................... 150
List of Tables

Chapter II: Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions

Table 2.1. Soil Properties of the study site, Mount Gambier, South Australia.....................40
Table 2.2. Composition of fertiliser and associated element(s) added to each treatment........41

Chapter III: Phloem and leaf amino acids reflect physiology, growth, and nutritional status of blue gum (Eucalyptus globulus) seedlings

Table 3.1. Experimental design for finding growth performance of E. globulus ....................65
Table 3.2. Nutrient effect on leaf and phloem amino acid concentrations (µg mL⁻¹) of E. globulus seedlings and their variations between the groups (control vs 50% fertilised and control vs 0% fertilised seedlings)..................................................................................................................71
Table 3.3. Relation between leaf and phloem amino acid concentrations (µg mL⁻¹) with relative collar diameter growth (cm yr⁻¹) of E. globulus seedlings.................................................................74

Chapter IV: Relationships between phloem sap and leaf δ¹³C are resilient to changes in nutritional status

Table 4.1. Experimental design for finding growth performance of E. globulus .......................90
Table 4.2. Fertiliser effect on leaf and phloem carbohydrates concentration (µg mL⁻¹) and their variation between the groups (control vs 50% and control vs 0% seedlings) .........................100
Table 4.3. Relation between phloem carbohydrates conc. (µg mL⁻¹) and dry biomass (g) ...101
Chapter V: Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown *Eucalyptus globulus*

Table 5.1. Composition of fertiliser and associated element(s) added to each treatment with the plantation of *Eucalyptus globulus* ........................................................................................................................................118

Chapter VI: Effects of drought on carbon sequestration potential of tropical trees in Bangladesh

Table 6.1. Mean parameters of the species in both study sites (Rajshahi and Sylhet) ........155
List of Figures

Chapter II: Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions

Figure 2.1. (A-K) Amino acids variations between seasons and among fertiliser treatments. The dark bar shows growing season concentration of amino acid and grey bar shows non-growing season concentration of amino acid; * denotes significant differences between the season, lowercases letter denotes significant differences among treatments in the growing season (November) only. No significant differences were detected among treatments in the non-growing season (February). Post-hoc test was carried out with Bonferroni test ($p \leq 0.05$); error bars show ± s.e., n=6.................................................................45

Figure 2.2. Seasonal changes of sucrose concentration due to fertiliser treatment. Dark bars represent growing season concentration of amino acids and grey bars show non-growing season concentration, * denotes significant variation between the season, Post-hoc test was carried out with Bonferroni test ($p \leq 0.05$) and represent between treatment and within season differences only; error bars show ± s.e., n=6.................................................................46

Figure 2.3. A, relationship between sucrose concentration and $\delta^{13}C$ in growing season and B, relationship between sucrose concentration and $\delta^{13}C$ in non-growing season. The solid line shows regression line the symbol, • denotes nil control plots, ▼ denotes 250 kg ha$^{-1}$ N, o denotes 350 kg ha$^{-1}$ N, ● denotes 450 kg ha$^{-1}$ N, □ = 250 kg ha$^{-1}$ NP, ■ denotes 350 kg ha$^{-1}$ NP, ▲ denotes 450 kg ha$^{-1}$ NP. ......................................................................................................47

Figure 2.4. A & B, relationship between total amino acid concentration and tree growth of in different fertiliser treatment plot; C & D, relationship between sucrose concentration and tree growth; E & F, relationship between carbon isotope abundance ($\delta^{13}C$) and tree growth. The left hand side figures A, C, and E are the relationships in growing season and the right hand side
figures B, D, and F are the relationships of non-growing season. The solid line shows regression line and the symbol, ◆ denotes nil control plots, ▼ denotes 250 kg ha\(^{-1}\) N, ◆ denotes 350 kg ha\(^{-1}\) N, ● denotes 450 kg ha\(^{-1}\) N, □ = 250 kg ha\(^{-1}\) NP, ■ denotes 350 kg ha\(^{-1}\) NP, ▲ denotes 450 kg ha\(^{-1}\) NP. 

Figure 2.5. Fertiliser effect on annual DBH growth. No significant variation (denoted by small letter) of DBH change observed due to the treatments effect. Post-hoc test was carried out with Bonferroni test (\(p \leq 0.05\)) and represent between treatment and within season differences only; error bars show ± s.e., n=6.

Chapter III: Phloem and leaf amino acids reflect physiology, growth, and nutritional status of blue gum (Eucalyptus globulus) seedlings

Figure 3.1. Net CO\(_2\) assimilation rate (µmol m\(^{-2}\) s\(^{-1}\)) of E. globulus seedlings in response to different fertiliser treatments, such as control, 50% and 0%, small letters indicate significant differences (\(p \leq 0.05\)) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., n=10.

Figure 3.2. Relative collar diameter (CD) growth (cm yr\(^{-1}\)) of E. globulus seedlings in response to different fertiliser treatments, such as control, 50% and 0%, small letters indicate significant differences (\(p \leq 0.05\)) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., n=25.

Figure 3.3. A, leaf total amino acid, and B, phloem total amino acid concentration (µg mL\(^{-1}\)) of E. globulus seedlings in response to different fertiliser treatments, such as control, 50% and 0%, small letters indicate significant differences (\(p \leq 0.05\)) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., n=12.
Figure 3.4. Relation between leaf and phloem amino acids of *E. globulus* seedlings. The solid black line shows regression line and the symbols ● denotes control seedlings, ○ denotes 50% fertilised seedlings, and ▼ denotes 0% fertilised seedlings. ................................................................. 72

Figure 3.5. Leaf and phloem relation with growth. A, leaf total amino acid concentration showed positive linear relation with relative collar diameter growth (cm yr⁻¹), B, phloem total amino acid concentration also showed a positive relation with relative collar diameter growth (cm yr⁻¹) of *E. globulus* seedlings. The solid black line shows regression line and the symbols ● denotes control seedlings, ○ denotes 50% fertilised seedlings ▼ denotes 0% fertilised seedlings................................................................. 73

**Chapter IV: Relationships between phloem sap and leaf δ¹³C are resilient to changes in nutritional status**

Figure 4.1. Leaf and phloem δ¹³C abundance with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, ○ denotes 50%, and ▼ denotes 0% fertilised seedlings. ........................................................................................................ 95

Figure 4.2. Nutrient effect on leaf and phloem δ¹³C, (a) leaf δ¹³C and (b) phloem δ¹³C of *E. globulus* seedlings in response to different fertiliser treatments (control, 50% and 0%), data shown are means ± s.e., n= 12. ......................................................................................................................... 96

Figure 4.3. Phloem δ¹³C and carbohydrate relation in response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, ○ denotes 50%, and ▼ denotes 0% fertilised seedlings................................................................. 97

Figure 4.4. Leaf and phloem carbohydrate concentration with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, ○ denotes 50%, and ▼ denotes 0% fertilised seedlings................................................................. 98
Figure 4.5. Nutrient effect on leaf and phloem carbohydrate conc. A, leaf total carbohydrates, and B, phloem total carbohydrates conc. (µg mL⁻¹) of *E. globulus* seedlings in response to different fertiliser treatments, such as control, 50% 0%, small letters indicate significant differences (*p*≤0.05) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., n= 12. ................................................................................................................................ 99

Figure 4.6. Total biomass and phloem carbohydrate concentration with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control group seedlings (100% fertilised), ○ denotes group A seedlings (50% fertilised), ▼ denotes group B seedlings (0% fertilised). ............................................................................................................ 101

Figure 4.7. Total dry biomass and phloem δ¹³C with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, ○ denotes 50%, and ▼ denotes 0% fertilised seedlings. ........................................................................ 102

Chapter V: Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown *Eucalyptus globulus*

Figure 5.1. Relation between phloem δ¹³C with wood cellulose δ¹³C across the radial direction, A, B, and C showed positive correlation between phloem δ¹³C and first, second and third section of 5 mm wood cellulose respectively, D and E showed negative correlation between phloem δ¹³C and fourth and fifth section of 5 mm wood cellulose respectively. The solid line shows regression line. ............................................................................................................ 123

Figure 5.2. Basic density of wood and cellulose δ¹³C of corresponding outer (1st section of wood) to inner part of the tree trunk (5th section of wood) across the radial direction. Small letter shows significance difference (Bonferroni test, *p*≤0.05), the data shown as means ± s.e., n = 6. ...................................................................................................................................... 124
Figure 5.3. Relationships between cellulose $\delta^{13}$C with basic density of wood across the radial direction, A and E showed positive correlation between cellulose $\delta^{13}$C and basic density of first and fourth sections of wood, B, C and D showed negative correlation between cellulose $\delta^{13}$C and basic density of second, third and fourth sections of wood. The solid line shows regression line.

Figure 5.4. Relation between cellulose $\delta^{13}$C with dry density of wood across the radial direction of the growth ring, A, B, C, D, and E showed positive correlation between cellulose $\delta^{13}$C and dry density of first, second, third, fourth and fifth sections of wood respectively. The solid line shows regression line.

Figure 5.5. Nutrient effect on average dry volume growth (black bar) and corresponding cellulose $\delta^{13}$C (dark grey line graph) of 5 sections of 1cm wood across the radial direction of the growth ring. The data shown as means ± s.e., n = 6.

Figure 5.6. Nutrient effect on average estimated volume under bark (ESVUB) and corresponding wood cellulose $\delta^{13}$C of trees grown in treatment plots. The solid line shows regression. Plots with different nutrient treatments are denoted by the symbols $\bullet$ = Nil control, $\blacktriangleleft$ = 250 kg ha$^{-1}$ N, $\circ$ = 350 kg ha$^{-1}$ N, $\bullet$ = 450 kg ha$^{-1}$ N, $\square$ = 250 kg ha$^{-1}$ NP, $\blacksquare$ = 350 kg ha$^{-1}$ NP, $\blacktriangle$ =450 kg ha$^{-1}$ NP.

Figure 5.7. Nutrient effect on carbon sequestration rate of 2.5 years old *E. globulus*. Data shown as means ± s.e., n = 6.

Chapter VI: Effects of drought on carbon sequestration potential of tropical trees in Bangladesh

Figure 6.1. Study areas are marked by rectangle: (i) the left side arrow head shows the dry site located at Tanore upazilla in Rajshahi, and ii) the right side arrow head shows the wet site located at Sylhet Sadar upazilla in the district of Sylhet).
Figure 6.2. Mean annual precipitation (mm) and temperature (°C) of the study sites: (a) mean annual precipitation and mean temperature at the dry site (b) mean annual precipitation and mean temperature at the wet site since 1997-2017). The bar graph represents the precipitation and the line graph represents temperature. ................................................................. 148

Figure 6.3. A, wood cellulose $\delta^{13}$C of $T.\ grandis$, $A.\ mangium$ and $E.\ camaldulensis$ showed variation between the study sites, B, radial growth variation between the study sites, C, density variation between the study sites., small letters shows the significant variation ($p \leq 0.05$) between the study sites and among the species, significance level was tested by t test for site difference and repeated ANOVA with post-hoc (Bonferroni) for inter-specific variation. Values are means ± s.e., n = 10................................................................. 154

Figure 6.4. Relationship between cellulose $\delta^{13}$C and wood density. A, B show dry and wet site relations between cellulose $\delta^{13}$C and wood density for $T.\ grandis$; C, D show dry and wet site relations between cellulose $\delta^{13}$C and wood density for $A.\ mangium$ and E, F show dry and wet site relations between cellulose $\delta^{13}$C and wood density for $E.\ camaldulensis$. The solid line shows regression and the symbol ● denotes for dry site and ■ denotes for wet site relation between the parameter. ................................................................. 156

Figure 6.5. Intra and inter-species specific variation carbon sequestration rate of $T.\ grandis$, $A.\ mangium$ and $E.\ camaldulensis$. (A) Carbon sequestration rate calculated with the calculated wood density in this study and (B) Carbon sequestration rate calculated with the Global Wood Density (GWD) published wood density (https://datadryad.org/handle/10255/dryad.235), mean wood density of $T.\ grandis$ 0.61, $A.\ mangium$ 0.51 and $E.\ camaldulensis$ 0.72, small letters shows the significant variation ($p \leq 0.05$) of CSR between the sites and among the species, significance level was tested by t test for the site difference and repeated ANOVA with post-hoc (Bonferroni) for inter-specific variation. Values are means ± s.e., n = 10............................................. 158
### Publications

#### Status of manuscripts to be submitted to peer review journals:

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Name of the writers</th>
<th>Title of the article</th>
<th>PhD Thesis Chapter</th>
<th>Name of the journal</th>
<th>Planned date of submission/present status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Halder, N. K., Fuentes, D., Possell, M., Ingram, L., Bradshaw, B., Merchant, A.</td>
<td>Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions</td>
<td>Chapter II</td>
<td>Tree Physiology</td>
<td>Under Review</td>
</tr>
<tr>
<td>2</td>
<td>Halder, N. K., Fuentes, D., Possell, M., Merchant, A.</td>
<td>Phloem and leaf amino acids to assess physiology, growth, and nutritional status of blue gum (Eucalyptus globulus) seedlings</td>
<td>Chapter III</td>
<td>Physiological Plantarum</td>
<td>15/05/2020</td>
</tr>
<tr>
<td>3</td>
<td>Halder, N. K., Fuentes, D., Possell, M., Merchant, A.</td>
<td>Relationships between phloem sap and leaf δ¹³C are resilient to changes in nutritional status</td>
<td>Chapter IV</td>
<td>Physiological Plantarum</td>
<td>30/05/2020</td>
</tr>
<tr>
<td>4</td>
<td>Halder, N. K., Chowdhury, M.Q, Fuentes, D., Possell, M., Bradshaw, B., Merchant, A.</td>
<td>Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown Eucalyptus globulus</td>
<td>Chapter V</td>
<td>Journal of Forestry</td>
<td>30/04/2020</td>
</tr>
<tr>
<td>5</td>
<td>Halder, N. K., Chowdhury, M.Q., Fuentes, D., Possell, M., Merchant, A.</td>
<td>Effects of drought on carbon sequestration potential of tropical trees in Bangladesh</td>
<td>Chapter VI</td>
<td>Annals of Forest Science</td>
<td>Under Review</td>
</tr>
<tr>
<td>Sl no.</td>
<td>Name of the writers</td>
<td>Title of the presentation/poster</td>
<td>Name of the seminar/symposium/conference</td>
<td>Place &amp; date</td>
<td></td>
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<tr>
<td>1</td>
<td>Halder, N.K., Fuentes, D., Possell, M., Bradshaw, B., Merchant, A.</td>
<td>Using phloem sap derived assessments of nutrient status to improve forest carbon sequestration (Oral presentation)</td>
<td>International Union of Forest Research Organizations (IUFRO) World Congress</td>
<td>Curitiba, Brazil, 29 September - 5 October 2019</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Halder, N.K., Fuentes, D., Possell, M., Merchant, A.</td>
<td>Phloem sap as a diagnostic assessment of plant water and nutritional status (Oral Presentation)</td>
<td>4th International Congress on Planted Forests</td>
<td>Beijing, China, 23-27 October 2018</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Halder, N.K., Fuentes, D., Possell, M., Merchant, A.</td>
<td>Phloem sap as a diagnostic assessment of plant water and nutritional status (Poster presentation)</td>
<td>Sydney Institute of Agriculture (SIA), Poster competition 2018 (positioned 2nd place)</td>
<td>SIA, The University of Sydney, 06-07-2018</td>
<td></td>
</tr>
</tbody>
</table>
General Introduction

Forests play a vital role in the exchange of greenhouse gases from the atmosphere to the biosphere. To reduce and mitigate the effects of a changing climate, enhancement of CO$_2$ sequestration from the atmosphere into forest ecosystems is essential (see for example, Canadell and Schulze 2014). This enhancement can be achieved by either expanding the forest estate or increasing the carbon carrying capacity of a forest ecosystem. Major processes that increase greenhouse gas emissions at the global scale include fossil fuel burning, agricultural practices and land use changes (e.g. deforestation). Emission of CH$_4$ and N$_2$O are caused by the direct or indirect effect of agricultural practices, for example the increased use of fertiliser increases N$_2$O emissions into the atmosphere in addition to the ‘carbon cost’ of synthesising fertiliser products. Addition of nutrients in forestry practices is a common silvicultural activity to boost the growth of trees. This supplementary addition of nutrients contributes to the storage of carbon in forest ecosystem through enhancing volumetric growth of trees. Therefore, efficient use of fertiliser application is vital for both sustainable and profitable forest management.

Whilst the growth of any forest is limited by a range of resource availabilities, nitrogen and water are among the most limiting resources across a range of forest environments. In a N-limited ecosystem, its availability is tightly coupled to growth and carbon sequestration through various mechanisms including that of photosynthesis (see for example, Magnani et al. 2007). Equally, water availability limits plant growth through the dynamics of leaf gas exchange. The development of reliable, cost effective tools to assess tree nutritional and water status is therefore of great interest. Proposed techniques, such as phloem sap analysis and
dendrochronological properties can be used as integrative tools that reflect physiological properties of plants to cope with resource limitations and to monitor forest growth.

This thesis identifies research priorities to establish improved forest measurement techniques that provide increased precision over measurements of forest properties and predictive capacity for how forests will respond to future climatic and edaphic conditions. These objectives are addressed through six chapters, which are written in a form that is readily convertible to manuscripts for journal publication. The thesis is comprised of the following chapters:

**Chapter 1: Current challenges to determining forest growth and carbon sequestration under water and nutrient limitations**

A critical literature review is presented on the effect of nutrient and water availability on tree growth and carbon carrying capacity of a forest ecosystem. A distinctive review is presented related to forest growth limited by nutrient and water availability. This chapter highlights existing tools to determine plant water and nutrition status and identifies deficiencies in those methods. Several tools are proposed to assess time and spatially integrated plant growth and carbon storage in a rapid and cost effective way.

**Chapter II: Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions**

The efficient use of fertiliser application is paramount to both the sustainability and profitability of forest plantations. Developing reliable, cost effective tools to assess tree nutritional status is therefore of great interest. This chapter sought to assess the use of phloem sap derived metabolites as an indicator of nutritional status on a background of seasonal (hence water) availability of *Eucalyptus globulus* trees grown under field conditions. This chapter has been submitted to the peer review journal “*Tree Physiology*”.

4
Chapter III: Phloem and leaf amino acids reflect physiology, growth, and nutritional status of blue gum (*Eucalyptus globulus*) seedlings

Under glasshouse conditions, this chapter focuses on the effect of nutrient availability, on the abundance of phloem sap amino acids obtained from *Eucalyptus globulus* seedlings. The availability of nutrients influences the abundance of phloem metabolites however, results suggest that chemical patterns need to be interpreted on a background of resource remobilisation and whole plant resource distribution.

Chapter IV: Relationships between phloem sap and leaf $\delta^{13}C$ are resilient to changes in nutritional status

This chapter investigates the relationships between phloem carbohydrate concentration and phloem carbon isotope abundance ($\delta^{13}C$) with plant growth to see if established relationships between these parameters are resilient to changes in nutrient supply. For this, *Eucalyptus globulus* seedlings were grown under controlled environmental conditions (in a glass house and with regular water supply) and differing nutrient supply.

Chapter V: Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown *Eucalyptus globulus*

This chapter investigates the relationship between phloem and cellulose $\delta^{13}C$ to establish if systematic offsets in $\delta^{13}C$ occur during transfer of carbon from phloem sap to wood formation. The relationship between wood cellulose $\delta^{13}C$ and corresponding basic and dry density was also explored to determine relationships with carbon sequestration in field grown *E. globulus*. 
Chapter VI: Effects of drought on carbon sequestration potential of tropical trees in Bangladesh

This chapter applies tools developed in previous chapters to assess forest carbon storage and carbon sequestration of production forests in Bangladesh. To do this, a tropical forest ecosystem of Bangladesh was considered to widen the applicability of the output of this thesis. Two study sites having a different annual precipitation were selected and three tree species (Tectona grandis, Acacia mangium, and Eucalyptus camaldulensis) grown in both of the sites were chosen. The inter-specific and intra-specific variation of carbon sequestration rate were calculated using the basic density calculated from the stem of wood cores. Calculations of forest carbon were compared to that of currently used models indicating significant underestimates of forest carbon. The impact of climatic variables (annual precipitation difference) on wood properties was also investigated, incorporating wood density in the annual growth rings. This chapter has been submitted to the journal “Annals of Forest Science”.

References


Chapter I: Current challenges to determining forest growth and carbon sequestration under water and nutrient limitations

Abstract

Forest are an active source of carbon storage and play a pivotal role in climate change mitigation. Soil nutrient and water availability are two factors which influence the growth of a forest. Interactions between soil nutrient content, water availability and the growth response need to be properly considered and monitored to inform silvicultural treatments. Traditional techniques for monitoring forest nutritional status and growth are based on foliar analyses which are time consuming, laborious and costly. Hence, development of cost effective, reliable and informative methods of monitoring tree growth is required. To predict carbon storage and carbon sequestration of a forest ecosystem, the estimation of above and below ground biomass is carried out via allometric equations based upon empirically collected tree growth parameters (DBH, height, density etc.). Such approaches are usually obtained by destructive sampling. Developing non-destructive measurement protocols based upon chemical and physical properties of trees that infer physiological processes governing carbon sequestration offers significant advantages for measures and predictions of both carbon storage and sequestration capacity. Here we propose several time and spatially integrated tools with great potential to cost-effectively monitor forest water and nutritional status to support growth. Areas requiring further research to attain these are highlighted.
1.1. Introduction

Forests are an integral part of global carbon cycle as it removes nearly 3 billion tons of anthropogenic carbon every year (3 Pg C yr$^{-1}$), absorbing 30% of all CO$_2$ emissions, largely from fossil fuel burning and net deforestation (Carnicer et al. 2011). Globally, almost 4 billion hectares of forest ecosystem (about 30% of global land area) together hold more than twice the amount of carbon currently contained in the atmosphere (FAO 2015; Field and Raupach 2004). Among different means of climate change mitigation strategies there are four major strategies, as identified by Canadell and Raupach (2008), with regard to forests, which are: (i) increasing forest land through afforestation and reforestation, (ii) increasing carbon density of existing forests at stand and landscape level, (iii) extending use of forest products to replace fossil fuel CO$_2$ emissions; and (iv) reduce emissions from deforestation and degradation. Deforestation accounts for nearly 20% of all greenhouse gas emissions — more than the world’s entire transport sector (CIFOR 2016). Maintaining and appropriately managing the global forest estate is a critical component of mitigating the likely effects of a changing global climate. For example, forests in the tropics could be an active carbon sink amounting to 1.2 ± 0.4 Pg C per year in established forests (not affected by land use changes) during 2000-2007 (Pan et al. 2011). The combined effect of past and future land use change may lead to an additional 95 Pg C increase in atmospheric CO$_2$ by 2100. This is equivalent to a 45 ppm (parts per million) increase in atmospheric CO$_2$, an increase of more than 10% of present levels (Gitz and Ciais 2003). In addition, temporary carbon sinks of 1.7 ± 0.5 Pg C per year are estimated to come from regrowing tropical forests after abandonment of agricultural land for 2000-2007 (Pan et al. 2011).

Carbon carrying capacity (CCC) is defined as ‘the mass of carbon stored in an ecosystem in a dynamic equilibrium state under prevailing environmental conditions and natural disturbance regimes, excluding anthropological disturbance (Gupta and Rao 1994; Keith et al. 2009). The
difference between CCC and current carbon stock (CCS) gives the carbon sequestration potential (CSP) (Keith et al. 2010). To increase above- and below-ground forest biomass and carbon carrying capacity (CCC) at the landscape level, silvicultural management plays an important role. Water availability is the driving factor of forest growth across many Australian ecosystems and often not under the control of silvicultural management. However, management prescriptions governing fertiliser application and indirect governance of stand water use (through thinning) are important tools to enhance the growth and productivity of managed forest ecosystems. Fertiliser manufacture and application has a significant impact on greenhouse gas emissions which is of great concern for both economic and environmental targets. Therefore, optimum use of fertiliser to attain maximum production can significantly contribute to offsetting greenhouse gas (GHGs) emission to the atmosphere. Equipping forest managers with tools to monitor the water and nutritional status of trees greatly enhances their power in applying interventions. Traditionally, only a few selected techniques are available for this purpose, mainly targeted at foliar analyses which are laborious, costly, time consuming and relatively narrow in their spatial and temporal application. Hence, the necessity to develop tools which can be usable in the field with less cost has emerged.

Tools to measure forest growth and carbon sequestration are mainly based on different allometric equations, such as above ground biomass (AGB) and are linearly related to DBH, height and density of wood (Brown 1997; Chave et al. 2005; Chave et al. 2014; MacDicken 1997). The estimation of forest biomass production with destructive sampling (such as tree felling) may give misleading information if we consider it for all types of forest, particularly toward claiming carbon credits because environmental variation can influence on the relationships between growth parameters of tree. Therefore, improved techniques to estimate biomass production from a forest is necessary.
1.2. Nutrient limited forest growth and carbon sequestration

1.2.1. Forest growth and carbon carrying capacity when limited by nutrients

Carbon (C) sequestration is influenced by the availability of essential nutrients like nitrogen (N), and phosphorous (P), and their availability is influenced by microbial growth (Macdonald et al. 2011). Improved understanding of these interactions will greatly improve our ability to predict changes in global C balance (Macdonald et al. 2011). C is supplied to microbes by plants through rhizodeposition and litterfall whilst microbes replenish N by N-fixation and mineralization processes (Lukac et al. 2010). Plant growth (hence C sequestration) can be stimulated by elevated atmospheric CO₂ (Drake et al. 1997; Pendall et al. 2004), but this stimulation is also dependent on a continuous and increasing supply of N, especially in an N-deficit system (Luo et al. 2004). Nitrogen fertilisation has been shown to promote growth under elevated CO₂ (Lu et al. 2011). Under such conditions, a strong correlation is observed between leaf N and photosynthetic rate (Ellsworth et al. 2004), which indicates that N availability has the important role on CCC in forest ecosystems.

The demand for additional N by trees growing under elevated CO₂ can be modified by plant physiological adjustment, relocation (Reich et al. 2006), or enhanced microbial N mineralization and N-fixation (Hungate et al. 1999; Phillips et al. 2011). Plants balance this the N demand with increasing N uptake or increasing N use efficiency (NUE) (Calfapietra et al. 2007) although the preferred mechanism is not clear (Finzi et al. 2007). Consequently, the capacity for plant growth may be evaluated via assessments of nutritional status.

Traditional methods of foliar analysis (e.g. leaves of different strata of the crown) are time consuming, expensive and are not necessarily integrative of time or spatial patterns in nutrient status. Alternative measures, such as phloem sap analysis can give a quick assessment that is more integrative of tree nutritional status. The cost of grinding leaf material and extracting can
also be avoided when phloem sap analysis is used to analyse nutritional status of plants. Phloem sap analysis has been conducted by several authors (Merchant et al. 2010b; Merchant et al. 2011; Pate and Arthur 1998; Pate et al. 1998; Peuke and Merchant 2019; Tausz et al. 2008) to investigate the growth status of plants. Whilst preliminary work has been done on small seedlings (Peuke and Merchant 2019) to date, no study has investigated the influence of nutrient additions on the components of phloem sap under field conditions.

N supply is greatly influential on photosynthetic performance and the growth rate of trees (Magnani et al. 2007), thus has the great capacity to enhance carbon sequestration. Meta-analysis has shown that N addition also enhances soil C storage in forest (Janssens et al. 2010; Norby 2007), likely due to enhanced C exudation from the rhizosphere. Microbial communities such as mycorrhizal growth are also influenced by N addition in soil under elevated CO₂ conditions (Alberton et al. 2005; Hu et al. 2006), as plants supply increase C availability (Diaz et al. 1993; Drake et al. 2011; Phillips et al. 2011). Feedback loops within the soil-plant interface are complex with the amount of N also controlled by microbial depolymerisation, mineralization of soil organic carbon (SOC) and microbial immobilization (Macdonald et al. 2011). At the ecosystem level, increased C storage will only occur when net C input is higher than the rate of SOC mineralization (De Graaff et al. 2006). Hence, ecosystem carbon budgets need to account for both the soil carbon sequestration process and above-ground biomass carbon content as this is one of many interactive processes among different components of the ecosystem that influence the C balance.

1.2.2. Forest growth and carbon carrying capacity when limited by water

The water requirement of forests varies due to their variable structures, types, ages, height and the size of the tree trunk (Nepstad et al. 2007; Zhang et al. 2009). Forest water use also varies with a range of factors including season, ecotype and degree of succession. These attributes
need to be considered to appropriately understand the relationship between water availability and forest growth (Xu et al. 2018). Under the circumstances of exceeding a biologically relevant threshold of water stress that is forest-type specific, forest growth is greatly affected (see for example, Huang et al. 2015). Increased tree ring width reflecting increased volumetric growth is commonly observed as a result of increased water availability (Graumlich et al. 1989b; Salzer et al. 2009). Conversely, tree growth and the width of tree rings are reduced with the reduced availability of water often combined with stress of heat and drought (see for example, Allen et al. 2010).

The climatic drivers of gas exchange between forest canopies and the atmosphere has been characterised by many authors (see, Jung et al. 2017), which stated that Gross Primary Productivity (GPP) and ecosystem respiration are dependent on water availability at the site level. Canopy dynamics and CO₂ exchange of forests can be predicted at the global scale based on knowledge of leaf level process, however, spatial manifestation of climatic drivers of plant growth are uncertain due to extensive geography and a paucity of long-term investigations. This highlights the importance of in situ observations of forest growth (Babst et al. 2019). The predicted global warming (Ciais et al. 2014) can accelerate redistribution of climatic drivers, especially atmospheric water demand - a primary determinant of tree growth at the landscape scale (Charney et al. 2016; Novick et al. 2016) through reduced gas exchange and consequently, reduced transport of sugars from leaves to stem (Adams et al. 2017; Sack et al. 2016; Sevanto et al. 2014). Elevated atmospheric CO₂ concentrations may mitigate some of these effects via increases in water use efficiency (WUE), but the magnitude and consistency of this effect remain questionable (Girardin et al. 2016; Rollinson et al. 2017; Tei et al. 2017).
1.2.3. Existing tools to determine forest growth and carbon carrying capacity

Measurement protocols for terrestrial carbon pools have been described by earlier authors (Post et al. 2001; Brown and Masera 2003; Pearson et al. 2005; IPCC 2006). Many efforts have been made to develop tools and models that can ‘scale up’ or extrapolate destructive harvest techniques to larger scales. These efforts have been traditionally based on proxies measured in the field or from remote sensing instruments (Brown et al. 1989; Waring et al. 1995; Brown 1997; Chave et al. 2005; Saatchi et al. 2007). A significant amount of work has been carried out at both the landscape and single site approach (MacDicken 1997; Brown and Masera 2003; Pearson et al. 2005). At the national level, the IPCC set guidelines outlining different tiers of quality in scientific data (Penman et al. 2003; IPCC 2006; Gibbs et al. 2007) to estimate biomass. Many methods exist to determine aboveground biomass estimation via destructive sampling, or allometric calculations based upon optical, radar or laser remote sensors. All of them have benefits and limitations (for details, see Gibbs et al. 2007). The forest carbon stock is estimated by applying the standardized biome averages to a widely accepted forest classification scheme of Global Land Cover 2000 (GLC 2000), termed as an FAO ecological zone map, and then overlying country boundaries in a geographic information system. This analysis does not delineate for forest dynamics such as logging, fire cycles or secondary forest regeneration which can indicate lower carbon stocks. The same biome-average carbon value is used for all forests in a broad range of classifications. Several methods are used to estimate carbon content by providing a breakdown by forest type and continent (Houghton 1999; Achard et al. 2002; IPCC 2006). Gibbs and Brown (2007) estimate carbon stock within forest classes from human disturbance and ecological conditions. Brown et al. (1989) suggests a model for the tropics through developing the allometric relationships with diameter at breast height, total height of tree and wood density whereas root biomass may be estimated for a tree at 15% of the above ground biomass (MacDicken 1997). Combined, an array of allometric equations can
be used to estimate the carbon stock of a given forest, yet all depend on the quality of ground-based reconnaissance to calculate forest carbon.

Country specific estimations of biomass estimation has been completed for Asia (Brown et al. 1993), Africa (Brown and Gaston 1995) and America (S. Brown pers. Comm. 1995). For forest type, estimations of temperate forest carbon has been completed by Houghton et al. (1983) whilst boreal forests have been estimated by Bazilevich (1993) (for details, see UNEP/OECD/IEA/IPCC by Houghton et al. 1997). These estimations were set through developing the default value for large scale regions, which may not be useful where growth is variable along the successional stages of a forest. In addition, soil nutrient status and rainfall may be inconsistent among the regions also affecting biomass accumulation. Site specific data is also important, for example, trees may be grown on slopes with shallower soil profiles (hence lower productivity) and soil properties may vary in water retention capacity. Equally, water use efficiency of different species may not be similar in nature (Sands and Mulligan 1990) and the default value of wood density to calculate stem biomass and expansion factor for leaf and branches may also differ (Dixon et al. 1991; Brown et al. 1989; ECA/FAO 1992). Variation in one or any of these parameters can lead to significant misrepresentations of forest biomass.

The carbon content of woody biomass averages about 0.5 t C t⁻¹ dry matter (dm), while the leafy biomass and grasses averages about 0.45 t C t⁻¹ dm (World Bank 1998). Carbon content on a mass basis is commonly calculated as 50% of the dry biomass of wood. However, to estimate biomass weight, the wood density is an important factor to be considered as it is likely to differ among species. Developing allometric equations to encompass such variation is time consuming and destructive particularly among multispecies forests (De Oliveira and Mori 1999). Generalised allometric equations have a major advantage of being based on larger numbers of trees that span wider range of diameters (Brown 1997; Brown 2002; Chave et al.
2005), however, these are still prone to systematic errors in biomass calculation (Chave et al. 2004). Some authors (Chambers et al. 2001; Keller et al. 2001; Chave et al. 2005) developed allometric equations based on geographic locations of forests. Gibbs et al. (2007) highlights that developing species-or location specific equations will not typically improve accuracy at the forest scale but occasionally the generalised equations may not adequately represent all forest types in all areas. National level forest biomass carbon stocks varied based on source data derived from inventory conducted by different organisation (Gibbs et al. 2007). Chave et al. (2005) and Chave et al. (2014) present methods, using total tree height (H), diameter at breast height (DBH), wood specific gravity and climatic stress ($E$) of the site. For different forest types for broad leaved trees Brown et al. (1989), Nelson et al. (1999), and Chave et al. (2003) have suggested that if detailed floristic information is unavailable species level average wood densities may be used. However, ignoring variation of wood densities resulted in poor overall estimation of the stand AGB (Baker et al. 2004) likely, perhaps, to differences in ecotype variation within species and even within individuals.

1.2.4. Developing tools to assess forest growth and its carbon carrying capacity

Methods for estimating carbon stocks are mostly based on allometric approaches. The use of allometric regression models includes several steps (e.g. measurement of dbh, height, density) to estimate above ground biomass (AGB), yet it is seldom directly tested (Houghton et al. 2001; Chave et al. 2001). One contributing factor is that one hectare of tropical forest might have about 300 different tree species (De Oliveira and Mori 1999), therefore, it is not wise to use the same species-specific regression model (Ter-Mikaelian and Korzukhin 1997; Shepashenko et al. 1998; Brown and Schroeder 1999). Instead, mixed species models have been devised (Chave et al. 2005) but most regression models remain based on small number of directly harvested tress and include very few large diameter specimens, thus under-representing older age classes. When using a similar model for two different sites, AGB estimation may
differ and be exacerbated for large trees, thus giving an uncertainty on stand level biomass estimations (Brown 1997; Nelson et al. 1999; Clark and Clark 2000; Houghton et al. 2001; Chave et al. 2004). It has traditionally been assumed that carbon content of dry biomass of a tree is 50% (Brown and Lugo 1982; Roy et al. 2001; Malhi et al. 2004), however wood carbon fraction may exhibit some small across species variation. Elias and Potvin (2003) observed an inter- and intra-specific variation of tree trunk wood carbon amongst 32 species where the carbon content ranges from 44.4 to 49.4%. Large scale biomass estimation is mostly carried out by analysing aerial photography or remote sensing data, but quantitative, non-destructive tools to estimate tree biomass are rare. Despite this shortcoming, opportunities exist to improve biomass predictions at the tree and stand level whilst adopting low cost, non-destructive empirical data.

1.2.5. Phloem sap analysis for determining tree growth

Plant growth depends upon the co-dependence of organs specialised to extract water and mineral ions from the soil and aerial organs for capturing sunlight to produce photosynthetic products by reducing CO₂ from the atmosphere. The nutritional interdependence of soil and aerial organs is through the exchange of required nutrients between them (Lalonde et al. 2003). In vascular plants, this adjustment is performed by conductive tissues like xylem and phloem (Van Bel 1993), that transport nutrients over long distances. Xylem facilitates upward movement of sap driven by evaporative loss of water. In contrast, phloem flow is thought to be driven by osmotically generated pressure differentials generated by the movement of photosynthetic products to heterotrophic organs (sink tissue) (Lalonde et al. 2003). Phloem is the central conduit for the distribution of photoassimilates and for remobilization of plant nutrients among plant organs. As a result, the amounts and chemical composition of carbon rich solutes loaded into phloem have a significant influence on carbon distribution among plant organs and finally on overall growth (Komor 2000; Lalonde et al. 2003; Turgeon 2000;
Consequently, the composition of phloem sap can be used for diagnosis plant health (Merchant et al. 2010a). The critical information e.g. elucidation of seasonal, developmental and species specific patterns of carbon and nutritional translocation in woody tree species can be obtained through phloem sap sampling and analysis (Pate and Jeschke 1995; Simpson 1986). It is strongly argued by Pate et al. (1998) that the same potential exists for study of tree species, like *E. globulus*, (a phloem bleeding tree) and hence, has the utility for analysing exudates to assess nutritional status. Studies related to phloem sap analysis are limited, with characterisation of phloem contents occurring mainly with herbaceous species (Turgeon and Wolf 2009). Lalonde et al. (2003) described that photosynthetically reduced carbon contributes to some 90% of plant biomass and is transported from photosynthetic source leaves to heterotrophic sinks principally as sugars and amino nitrogen (N) compounds. Sucrose is a ubiquitous constituent of phloem sap; in some families it is supplemented by raffinose family oligosaccharides (RFOs) and/or sugar alcohols (Zimmermann and Milburn 2012). Commonly aspartate and glutamate and their corresponding amides are the principal forms of amino N compounds transported in the phloem (Delrot et al. 2001). Together with potassium (Patrick and Offler 2001), sugars and amino N compounds are the principal osmotic components of phloem saps and thus impact on rates of phloem transport and assimilate partitioning patterns. Despite this potential for use in diagnostic assessments of tree water and nutritional status, little information exists regarding the influence of environmental conditions on carbon isotope composition of phloem loaded compounds and amino acids (Merchant et al. 2010a). Evidence exists for the effect of environmental conditions on fractionation of carbon isotopes as they are incorporated into heterotrophic tissues indicating the importance of plant scale research. Badeck et al. (2005) found consistent inter-organ differences in abundance of carbon isotopes and highlight many uncertainties regarding fractionations of carbon isotopes during phloem loading and transport. Similarly, Cernusak et al. (2009) provided a series of
physiochemical processes that might lead to this fractionation. For nutritional status, the quantification of mineral nutrients and nitrogen which is transported in the form of amino acids in the phloem stream may be a good predictor to assess the whole plant nutritional status (Tegeder and Hammes 2018; White 2012). Phloem analysis may be a viable replacement for foliage analysis as it is easier to collect and potentially to avoid intra-canopy variation (Tausz et al. 2008; Merchant et al. 2010b). Finally, as phloem sap has little contamination of molecules involved in cellular metabolism that reside in leaf (Merchant et al. 2012), it may be a more suitable metabolic pool on which to investigate the impact of short term environmental fluctuations (Smith and Merchant 2018). Despite this, little information on the impact of changes in nutrient supply or water supply and demand on phloem composition is available.

1.2.6. Tree ring analysis for estimating forest growth and carbon carrying capacity

Sampling growth rings can be achieved with little to no impact on the health and productivity of the tree. Tree rings are a long-term record of seasonal changes in growth and C sequestration in a forest stand. Today, stem cores are commonly used as an environmental record to indicate tree growth under variable environmental conditions e.g. Water use efficiency reflected in wood of tree ring (see details, Baker et al. 2008; Heinrich and Allen 2013; Macfarlane and Adams 1998). Tree ring width provides an estimate of biomass accumulation (Bascietto et al. 2004). Tree rings are commonly used to assess above-ground net primary-productivity trends at the stand level (Graumlich et al. 1989a), but only recently have they been used to assess carbon-sequestration trends (young to mature forest differ in carbon accumulation, see details, Acker et al. 2002; Yanai et al. 2000). In an example of site-specific characterisation of forest dynamics, Bascietto et al. (2004) studied tree ring width where harvests and thinning are carried out regularly to fulfil a management plan and concluded that forest-management treatments strongly influence tree growth in diameter and height and canopy architecture. A management
prescription e.g. fertilisation rather than silvicultural treatments might have an effect on tree growth as well as biomass accumulation both above-ground and below ground. Similarly, interactions between the application of fertiliser and tree ring development are likely to be substantial but rarely are they studies at appropriate time and spatial scales.

1.3. Conclusion

Forests are limited in reaching the notional maximum carbon carrying capacity (CCC) due to both nutrient and water limitations. Site specific variation in carbon content has significant implications for generalised calculations of landscape scale carbon stocks. Estimating the carbon content of standing forests is limited by cost and time efficient tools that can encompass this site-specific variation. New quantitative tools are required to collect empirical data upon which site-specific allometric calculations can be made. Similarly, rapid and reliable tools to assess the nutrient status of forests are crucial to both intervention strategies (fertiliser application in production forests) and assessing the interactions between nutrient status and forest growth in natural systems. The recent development of phloem sap composition analysis combined with a more comprehensive consideration of information contained within tree rings may offer considerable advantages to the incorporation of site-specific variation into existing models through the collection of empirical data.

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Chapter II: Phloem sap metabolites vary according to the interactive effects of nutrient supply and seasonal conditions

Abstract

Improving the efficiency of fertiliser application is paramount to both the sustainability and profitability of forest plantations. Therefore, developing reliable, cost effective tools to assess tree nutritional status is of great interest. This investigation sought to assess the use of phloem sap derived metabolites as an indicator of nutritional status on a background of seasonal water availability of blue gum (E. globulus) trees grown under field conditions. Phloem are the central conduit for long distance transport and signalling in plants and offers great promise in reflecting plant scale resource limitations. Changes in the abundance of solutes and isotopes in phloem sap are sensitive to environmental cues. With a focus on both water and nutrient availability, we characterise patterns in phloem sugars, amino acids and the abundance of carbon isotopes in phloem sap obtained from E. globulus among different seasons and fertiliser treatments. Phloem derived amino acids were found to vary in accordance with nitrogen supply, however, this response was lost with the concurrent addition of phosphorus. Significant seasonal variation in all measured parameters was also detected highlighting the need for caution in making quantitative relationships with growth. Broader implications of the interactive effects of both water supply and multi-nutrient additions, and relationships with growth are discussed.

2.1. Introduction

Fertilisers are used widely in plantation forests to ameliorate nutrient deficiencies and increase tree growth. Fertiliser management now forms a crucial component of most plantation
management systems and plays a key role in determining plantation forest profitability. Major factors determining the quantity and timing of fertiliser use include the need to ameliorate nutritional deficiencies, maximise profit, increase wood production, and the cost of fertiliser (May et al. 2009). Many studies have shown linkages between nutrient cycling and uptake (Raison et al. 1992a), foliage production (Raison et al. 1992b) and stem and branch growth (Snowdon 2002) and highlight the critical nature of interactions between water and nitrogen supply on foliar use efficiency (Thompson and Wheeler 1992), tree growth and responses to fertiliser application (Crane and Banks 1992). Therefore, developing reliable, cost effective tools to assess tree nutritional status is of great interest.

Phloem are the central conduit for the distribution of photoassimilates and remobilization of nutrients among plant organs. Consequently, the quantity and composition of carbon rich solutes loaded into phloem have a significant influence on carbon distribution among plant organs and on overall growth (Komor 2000; Lalonde et al. 2003; Turgeon 2000; Turgeon and Medville 2004). Information such as the characterisation of seasonal, developmental and species specific patterns of carbon and nutrient translocation in woody tree species can be obtained through phloem sap sampling and analysis (Pate and Jeschke 1995; Simpson 1986) with implications for plant health (Merchant et al. 2010a). Pate and Jeschke (1995) described phloem sap composition changes during seasonal growth, which illustrate that phloem translocation is the major route to distribute photoassimilates from leaves to shoot apex and to mobilize nutrient from older leaves to the shoot extension. Despite considerable promise, the composition and dynamics of phloem sap contents remain subject to considerable debate due largely to both the difficulty of phloem collection arising from its susceptibility to invasive collection (see Dinant and Kehr 2013) and variation in phloem sap composition over seasonal and developmental cycles. To date, no study has sought to investigate the potential for phloem sap to reflect nutritional status on a background of seasonal (water) variability.
Assimilated carbohydrates are transported from source (photosynthesis) to active growth areas and sink tissues (Savage et al. 2015). Notwithstanding the importance of understanding patterns of carbon allocation in plants, there are very few studies linking the implications of phloem transport on carbon allocation (see Nikinmaa et al. 2013; Schiestl-Aalto et al. 2015; Woodruff and Meinzer 2011). Changes in environmental conditions have been shown to influence the chemical and isotopic constituents of phloem sap (Cernusak et al. 2003; Claudia et al. 2006; Marion et al. 2009). The naturally occurring ratio of $^{13}$C to $^{12}$C (expressed as $\delta^{13}$C ‰) of plant components can be used as a surrogate estimation of water use efficiency (Seibt et al. 2008) and in phloem sap it has shown both positive and negative relationships throughout the seasonal cycles (Merchant et al. 2010a; Merchant et al. 2010b). Phloem sap $\delta^{13}$C has also been shown to be positively related to tree growth (Cernusak et al. 2003; Tausz et al. 2008) and both $\delta^{13}$C and phloem sap derived sucrose are excellent predictors of growth across sites of contrasting water availability (Merchant et al. 2010a; Merchant et al. 2010b). Despite this, little is known regarding how the composition of other metabolites found in phloem sap change according to nutritional status or availability despite its role in nutrient uptake/transport and remobilisation (Yeo and Flowers 2007). Phloem sap contents may provide insight into the nutritional status of the plant. Peuke and Merchant (2019) recently identified that total amino acid concentration correlates strongly with leaf amino acid concentration in pot grown seedlings of *E. globulus* grown under well-watered conditions, however, little is known regarding the interaction of nutrient status with water uptake. Plant water and nutritional status are tightly linked (discussed in chapter V, paragraph 5.4.3 and 5.4.4), thus under field conditions, water limitation may interact with nutrient uptake inhibiting the use of phloem derived metabolites as indicators of plant nutritional status. It is also well known that specific amino acids are utilised in nitrogen transport in the phloem stream in plants (Santiago and Tegeder 2017). To date, no study has
sought to systematically determine the ability of phloem sap derived amino acids to reflect the nutritional status of the plant, under water limited or field conditions.

Here we use field grown *E. globulus* trees subjected to a replicated factorial experimental design incorporating quantitative and qualitative differences in fertiliser (N or NP) supply and monitor the composition of phloem sap across a seasonal cycle. By determining changes in phloem metabolites (amino acids, carbohydrates) and $\delta^{13}$C of phloem sap obtained from *E. globulus* subjected to differing fertiliser applications over a seasonal cycle, we investigate the ability of these parameters to reflect nutritional status. Our primary hypotheses are: (1) the relative abundance of phloem metabolites and $\delta^{13}$C changes with supplementary addition of nutrients under field grown conditions; (2) changes in phloem metabolites and $\delta^{13}$C as a result of fertiliser additions are consistent across seasons under field grown conditions; (3) quantitative changes in phloem metabolites (amino acids and carbohydrates) and $\delta^{13}$C vary in accordance with tree growth; and (4) relationships between growth, metabolite composition and $\delta^{13}$C can be used as a diagnostic tool to determine limiting factors to growth (e.g. nutrients and water).

### 2.2. Materials and methods

#### 2.2.1. The study site

Nutritional treatments were applied to a commercial plantation stand of *E. globulus* located in the Mount Gambier district of South Australia located at $37.75^0$ S latitude, and $140.77^0$ E longitude and an elevation of 63 m above sea level. This region is characterised as having a Mediterranean type climate with the majority of rainfall occurring in the winter and spring months (June to November). Soils are sandy loam and their characteristics are presented in Table 2.1. The soil test data was collected from the owner of the trial plot, Australian Bluegum Plantation Pty. Ltd, Mount Gambier, South Australia.
Table 2.1. Soil Properties of the study site, Mount Gambier, South Australia

<table>
<thead>
<tr>
<th>Soil Properties</th>
<th>Pre Treatment Value (Mean ± s.e.)</th>
<th>Post Treatment Value (Mean ± s.e.)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravel (%)</td>
<td>5.00 ± 0.00</td>
<td>5.00 ± 0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Texture (mg/kg)</td>
<td>1.50 ± 0.00</td>
<td>1.50 ± 0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium Nitrogen (mg/kg)</td>
<td>15.29 ± 1.47</td>
<td>37.14 ± 9.64</td>
<td>0.04$^a$</td>
</tr>
<tr>
<td>Nitrate Nitrogen (mg/kg)</td>
<td>4.14 ± 0.40</td>
<td>23.85 ± 6.84</td>
<td>0.01$^a$</td>
</tr>
<tr>
<td>Phosphorus Colwell (mg/kg)</td>
<td>11.71 ± 0.64</td>
<td>15.28 ± 1.64</td>
<td>0.06$^a$</td>
</tr>
<tr>
<td>Potassium Colwell (mg/kg)</td>
<td>67.85 ± 2.75</td>
<td>71.42 ± 3.19</td>
<td>0.41</td>
</tr>
<tr>
<td>Sulphur (mg/kg)</td>
<td>6.744 ± 0.44</td>
<td>5.65 ± 0.46</td>
<td>0.08$^a$</td>
</tr>
<tr>
<td>Organic Carbon (%)</td>
<td>3.73 ± 0.05</td>
<td>3.97 ± 0.19</td>
<td>0.26</td>
</tr>
<tr>
<td>Conductivity (ds/m)</td>
<td>0.08 ± 0.00</td>
<td>0.13 ± 0.01</td>
<td>&lt;0.001$^a$</td>
</tr>
<tr>
<td>pH Level (CaCl$_2$)</td>
<td>5.00 ± 0.07</td>
<td>5.24 ± 0.17</td>
<td>0.20</td>
</tr>
<tr>
<td>pH Level (H$_2$O)</td>
<td>5.86 ± 0.06</td>
<td>5.96 ± 0.12</td>
<td>0.36</td>
</tr>
<tr>
<td>Phosphorus Retention Index (PRI)</td>
<td>9.26 ± 2.04</td>
<td>10.53 ± 2.41</td>
<td>0.69</td>
</tr>
<tr>
<td>Phosphorus Buffering Index (PBI)</td>
<td>35.27 ± 3.97</td>
<td>38.17 ± 4.41</td>
<td>0.63</td>
</tr>
<tr>
<td>Total Nitrogen (%)</td>
<td>0.28 ± 0.00</td>
<td>0.31 ± 0.00</td>
<td>0.01$^a$</td>
</tr>
<tr>
<td>Total Phosphorus (mg/kg)</td>
<td>144.24 ± 7.19</td>
<td>149.67 ± 6.27</td>
<td>0.58</td>
</tr>
<tr>
<td>Potassium (Nitric) (mg/kg)</td>
<td>118.19 ± 2.00</td>
<td>126.32 ± 4.12</td>
<td>0.10</td>
</tr>
</tbody>
</table>

One way ANOVA was carried out to test the variation between the soil properties of pre-treatment and post-treatment. Small letter denotes the significant variation ($p \leq 0.05$) of the soil property between the two periods, s.e., standard error, n = 6.

In the period 2014 to 2018, mean maximum and minimum air temperatures of Mount Gambier, SA were 27.72 °C and 13.34 °C, respectively and mean annual rainfall was recorded as 739.12 mm and mean annual daily evaporation was 3.5 mm (ABOM 2018).

2.2.2. Experimental design

A factorial treatment design was established for fertiliser doses consisting of 54 trees per plot, each with 9 trees per row. Row spacing is maintained at 4 m × 2.5 m. The total size of each plot was 0.06 ha. (24 m × 25 m). There are 42 plots consisting of seven treatments in six replicated plots. The total trial area was 2.52 ha (168 m wide and 150 m length). Treatments were randomly applied to each individual plot. The fertiliser treatments consisted of three rates of N fertiliser applied as ammonium and three rates of ammonium + phosphorus applied as superphosphate (Table 2.2), and the nil control plot had no fertiliser application.
Table 2.2. Composition of fertiliser and associated element(s) added to each treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fertiliser application (ha⁻¹)</th>
<th>Elemental amounts ha⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>250N</td>
<td>250 kg anhydrous ammonia</td>
<td>206 kg N</td>
</tr>
<tr>
<td>350N</td>
<td>350 kg anhydrous ammonia</td>
<td>289 kg N</td>
</tr>
<tr>
<td>450N</td>
<td>450 kg anhydrous ammonia</td>
<td>371 kg N</td>
</tr>
<tr>
<td>250NP</td>
<td>250 kg anhydrous ammonia + 250 kg superphosphate</td>
<td>206 kg N + 22 kg P + 28 kg S</td>
</tr>
<tr>
<td>350NP</td>
<td>350 kg anhydrous ammonia + 350 kg superphosphate</td>
<td>289 kg N + 31 kg P + 39 kg S</td>
</tr>
<tr>
<td>450NP</td>
<td>450 kg anhydrous ammonia + 450 kg superphosphate</td>
<td>371 kg N + 40 kg P + 50 kg S</td>
</tr>
</tbody>
</table>

2.2.3. Collection of phloem sap

Phloem sap was collected from randomly selected single tree of six replicated plots of seven treatments in November, 2016 (the growing or spring season) and February, 2017 (the summer or non-growing season). In South Australia the mean maximum temperature and rainfall of November 2016 were recorded 26.4 °C and 76.1 mm and in February 2017 were recorded 32.2 °C and 67.6 mm (ABOM 2018). Phloem sap was collected from the main stem of trees using the “razor blade technique” following the methods described by Merchant et al. (2011). Phloem sap droplets were progressively collected using a glass disposable pipette from 1000 h to 1400 h and kept in a single micro tube for each tree, with an addition of 200 µl of methanol into the micro tube for sample preservation. Samples were immediately transferred to a -20 °C freezer and within 48 h were stored in a -80 °C freezer. Samples collected from individual trees were bulked into one sample per plot. The sampled trees were randomly selected within the plot. Considering the intra-specific interaction on sharing nutrient and water among the trees between treatments plots, boundary trees are avoided while sampling.

2.2.4. Phloem sap extraction for amino acid analysis

Samples were allowed to equilibrate to room temperature before mixing for 1 min (Vortex machine, 3000 rpm) to get a homogeneous mixture. To 100 µL of phloem sap sample, 220 µL of acetonitrile solution was added for protein precipitation. The sample was centrifuged for 3
min at 1000 rpm. 280 µL of the upper layer of solution was taken without disturbing protein precipitate. The samples were dried to dryness and reconstituted with 300 µL of Milli Q water. The samples were filtered using a 25 mm syringe filter (0.2 µm nylon membrane). A further 300 µL of water was added and the solution was approximately 600 µL. The solution was dried in a Speed vac until dry. The sample was reconstituted to 300 µL with Milli-Q water and vortexed for the Liquid Chromatography–Mass Spectrometry (LC-MS) analysis. For the glutamine analysis after complete drying of 100 µL phloem sap we added 1000 µL deionised water. 200 µL of that solution was removed and added to 200 µL deionised water awaiting. LC-MS analysis.

2.2.5. Phloem sap extraction for carbohydrate analysis

Samples were allowed to equilibrate to room temperature before mixing for one min (Vortex machine, 3000 rpm) to get a homogeneous mixture. One µL of solution was placed into a glass vial, diluted with 500 µL of deionised water. From this solution, 50 µL was then transferred into a glass GC vial and dried under vacuum. Samples were then resuspended in 450 µl anhydrous pyridine and immediately sealed to prevent water absorption. Then 50 µl of a 10:1 mixture of bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchloroacetamide (TMCS) were added. Samples were heated at 75 °C for one hour immediately prior to injection (modified from Merchant et al. 2006).

2.2.6. Phloem sap preparation for δ13C analysis

5 µL of phloem sap solution was placed into an aluminium capsule (dimensions: 2.88/16mm, IVA Analysentechnik e. K. Meerbusch, Germany) and dried in an oven at 60 °C for 48 h.
2.2.7. Growth data collection

Stem diameter at breast height (DBH) of individual trees in each study plot was collected in both the growing and non-growing season to determine the relative DBH growth rate of the study plot. The relative DBH growth rate was calculated as (DBH of the previous year – DBH of the Current year)/DBH of the previous year.

2.2.8. Analysis of amino acids, carbohydrates and δ¹³C

LC-MS analysis was completed on a 1290 Infinity LC system (Agilent Technologies, USA) coupled to a 6520 QTOF Mass selective detector (Agilent Technologies, USA). A 3.5 µL sample was injected into a Zorbax SB-C18 column (2.1 x 150 mm, 3.5 µm) and separation achieved by a flow rate of 0.5 mL min⁻¹ with a gradient elution of 0-100% water and methanol over 16 min. The QTOF was tuned to operate at the low mass range <1700 AMU and data acquisition completed in scan (60-1000 m/z) positive ion mode. LC-MS results were identified based on their retention times relative to standards as well as formula mass. Peaks were integrated and their relative quantities calculated by MassHunter™ software (Agilent Technologies, US).

For carbohydrates, analysis was carried out on an Agilent 7890B Gas Chromatograph with QQQ 7000 Mass selective detector. Samples were injected with a 20:1 split injection onto a HP-5 column (30 m, 0.25 mm ID, 0.25 µm film thickness) with helium carrier gas at 1 mL min⁻¹ constant flow. The temperature programme had an initial oven temperature set at 60 ⁰C for 2 min ramping to 300 ⁰C at 10 ⁰C min⁻¹ for 10 min. Mass Hunter software was used for peak integration.

For δ¹³C analysis, an Isochrome Mass Spectrometer was used. The instrument was a Delta V with Conflo IV and FlashHT peripherals in a dual-reactor setup (Thermo Fisher Scientific,
Bremen, Germany). The samples were combusted by Dumas combustion. The oxidation reactor is set to 1000 °C. The standard material precision was 0.11‰.

2.2.9. Statistical analysis

Analysis of variance and post-hoc comparisons was made using Bonferroni test ($p \leq 0.05$). Linear regression analysis was performed to identify relationships between parameters. All these statistical analysis were carried out by Sigmaplot (version 12.5, Systat Software, Inc. San Jose, CA, USA).

2.3. Results

2.3.1. Seasonal and treatment effects on phloem sap amino acid concentration

Average concentrations of 10 amino acids (leucine, methionine, valine, serine, isoleucine, threonine, tyrosine, tryptophan, phenylalanine, and glutamine) were found to vary with season and fertiliser application (Figure 2.1, A-K). Three amino acids (aspartic, glycine and histidine) were quantified in the growing season but were not consistently above detection limits for all the fertilised plots in the non-growing season. All amino acids concentrations differed significantly between season except for serine and methionine.

Post-hoc groupings for treatment differences reveal increases in total amino acid concentrations in the N only treatments corresponding to a decrease in the fertiliser dosage with the exception of the 450 kg treatment which did not differ from the control (Figure 2.1K). This difference is largely influenced by the relative abundance of glutamine (Figure 2.1J).
Figure 2.1. (A-K) Amino acids variations between seasons and among fertiliser treatments. The dark bar shows growing season concentration of amino acid and grey bar shows non-growing season concentration of amino acid; * denotes significant differences between the season, lowercases letter denotes significant differences among treatments in the growing season (November) only. No significant differences were detected among treatments in the non-growing season (February). Post-hoc test was carried out with Bonferroni test ($p \leq 0.05$); error bars show ± s.e., n=6.
During the growing season, an increased availability of nitrogen leads to a decrease in amino acid concentration in phloem sap across many of the amino acids quantified as well as the sum total. However, with the addition of phosphorus, this response was not observed. Glutamine was the most abundant amino acid, and varied significantly among the fertiliser treatments (Figure 2.1J). Therefore, the contribution of glutamine led to the sum total of amino acids also varying significantly among fertiliser treatments (Figure 2.1K).

2.3.2. Seasonal and treatment effects on phloem carbohydrates

Sucrose was the most abundant carbohydrate in the phloem sap of *E. globulus* in all nutrient treatments. All other carbohydrate contents were orders of magnitude lesser in concentration. Phloem sucrose concentration was significantly different between the growing and non-growing season (Figure 2.2).

![Figure 2.2](image-url)

**Figure 2.2.** Seasonal changes of sucrose concentration due to fertiliser treatment. Dark bars represent growing season concentration of amino acids and grey bars show non-growing season concentration, * denotes significant variation between the season, Post-hoc test was carried out with Bonferroni test (*p*≤0.05) and represent between treatment and within season differences only; error bars show ± s.e., *n*=6.

No significant differences in phloem sap sucrose concentration were detected among fertiliser treatments, however no quantitative patterns were observed (Figure 2.2).
2.3.3. Relationships between phloem carbohydrates and $\delta^{13}C$

Phloem sap sucrose concentration and carbon isotope abundance ($\delta^{13}C$) were positively correlated (Figure 2.3) with a stronger correlation detected in the growing season ($r^2 = 0.33, p = 0.002$) than non-growing season ($r^2 = 0.24, p = 0.01$).

**Figure 2.3.** A, relationship between sucrose concentration and $\delta^{13}C$ in growing season and B, relationship between sucrose concentration and $\delta^{13}C$ in non-growing season. The solid line shows regression line the symbol, ◆ denotes nil control plots, ▼ denotes 250 kg ha$^{-1}$ N, o denotes 350 kg ha$^{-1}$ N, ● denotes 450 kg ha$^{-1}$ N, □ = 250 kg ha$^{-1}$ NP, ■ denotes 350 kg ha$^{-1}$ NP, ▲ denotes 450 kg ha$^{-1}$ NP.
Fertiliser treatment does not show any influence over the quantitative relationship between parameters in either season (Figure 2.3).

2.3.4. Relationship between phloem metabolites and tree growth

Total amino acid concentration in phloem sap was positively correlated with relative DBH increment in both seasons (Figure 2.4A and B). The largest DBH change over the measurement period was observed with increased availability of both N and NP Fertiliser (Figure 2.4A). A positive correlation between DBH and sucrose was observed only in the growing season (Figure 2.4C and D) whilst the strongest correlation of all parameters was that of DBH and δ^{13}C in the growing season (Figure 2.4E).
Figure 2.4. A & B, relationship between total amino acid concentration and tree growth of in different fertiliser treatment plot; C & D, relationship between sucrose concentration and tree growth; E & F, relationship between carbon isotope abundance (\( \delta^{13}C \)) and tree growth. The left hand side figures A, C, and E are the relationships in growing season and the right hand side figures B, D, and F are the relationships of non-growing season. The solid line shows regression line and the symbol, ♦ denotes nil control plots, ▼ denotes 250 kg ha\(^{-1} \) N, □ denotes 350 kg ha\(^{-1} \) N, ● denotes 450 kg ha\(^{-1} \) N, ▲ denotes 250 kg ha\(^{-1} \) NP, ■ denotes 350 kg ha\(^{-1} \) NP, ▲ denotes 450 kg ha\(^{-1} \) NP.
2.3.5. Influence of fertiliser rate on DBH growth

There was no significant variation of tree DBH growth observed due to the different fertiliser treatment. It was observed that comparing to other fertiliser treatments 350 kg ha\(^{-1}\) N and 350 kg ha\(^{-1}\) NP showed highest growth rates among the groups (see Figure 2.5).

![Figure 2.5. Fertiliser effect on annual DBH growth. No significant variation (denoted by small letter) of DBH change observed due to the treatments effect. Post-hoc test was carried out with Bonferroni test \((p \leq 0.05)\) and represent between treatment and within season differences only; error bars show ± s.e., \(n=6\).]

2.4. Discussion

Methods to determine the nutritional status of trees are valuable to assist in developing informed management decisions and improved growth predictions. Here we show for the first time, variation in phloem sap amino acid concentrations in response to likely changes in nutrient supply under field grown conditions. Further, significant seasonal variation in phloem sap amino acid content is observed and that interactive effects on phloem composition are elicited by the availability of N and P. Combined, our results suggest that phloem sap amino acid content is a useful indicator of growth and nutritional supply. However, interactive effects
among nutrients and seasonal variation may mask straightforward dose-response relationships between nutrient supply and amino acid abundance.

2.4.1. Phloem sap amino acids vary with nutrient supply and season

Of the ten phloem sap amino acids that were consistently above detection limits, eight were significantly higher in abundance during the growing season, often by up to ten fold concentration (see Figure 2.1). Of all the amino acids, glutamine accumulates in the highest concentrations in phloem sap among a range of tree and plant species (Pate et al. 1984; Näsholm and Ericsson 1990; Amiard et al. 2004; Palmer et al. 2014; Yeo and Flowers 2007). In the present study, glutamine was the highest concentration amino acid in agreement with previous studies of phloem sap obtained from *Eucalyptus globulus* (Pate et al. 1998; Merchant et al. 2010a). Our study extends upon these observations by investigating multilevel, controlled dosage of independent N and P availability. Higher levels of phloem sap amino acids during the growth seasons are likely due to a combination of increased uptake and/or increased mobilisation of nutrients within the canopy. Consequently, interpretations of tree nutritional status must acknowledge variation in phloem sap composition attributable to seasonal, and perhaps even developmental status (such as age, and reproductive cycles), and cannot be necessarily extrapolated across either location or time. However, comparative nutritional status appears possible if the influence of these factors can be mitigated through a point-in-time comparison of similar aged trees at the same site. Further work on quantifying these relationships is required to expand the application of this technique.

Interactive effects of N and P supply were observed on the composition of phloem sap in our study. Increasing N supply increased amino acid concentrations in the phloem sap but concurrent supply of additional P did not lead to significant increases in phloem amino acids. Increases in amino acid abundance has been shown to occur under growth restriction imparted by water in *E. globulus* under controlled conditions (Merchant et al. 2010a) but such increases
could be attributed mainly to the accumulation of phenylalanine, presumably in response to restriction of lignin synthesis. In the present study, increases in glutamine were the major influence on phloem amino acid abundance – an amino acid commonly implicated in transport mechanisms. Additionally, increased amino acid concentrations in phloem sap may be attributable to remobilisation of N to provide materials for the construction of photosynthetic infrastructure to optimise light harvesting at the canopy scale. Emerging evidence for the stimulation of N uptake by trees through the addition of phosphorus has been shown suggesting that P deficiency in soils promotes microbial immobilisation of N (Mori et al. 2013; Baral et al. 2014; Zhang et al. 2014) enhancing its availability for uptake by plants through mineralisation. In the present study, the presence of P in the treatment has subdued N build-up in the phloem. Further studies should seek to determine the relative roles of xylem and phloem in the redistribution of tree resources, and the relative portions of N obtained from uptake and remobilisation to sustain new canopy growth. An interactive relationship between N and P supply was detected as phloem sap amino acid concentrations did not decline with increasing N supply if P supply was increased too. Understanding these relationships, in both native and managed systems would be highly beneficial to assist in the ecosystem scale management of N supply and N loss and further emphasises the key role of appropriate soil management in silvicultural practice.

2.4.2. Phloem sap carbohydrates and $\delta^{13}C$ vary with season but not nutrient supply

Phloem carbohydrate content and $\delta^{13}C$ has been shown previously to have strong relationships with tree water status (Geßler et al. 2001; Cernusak et al. 2003; Seibt et al. 2008). In the present study, both phloem sap sucrose (see figure 2.2) and $\delta^{13}C$ varied with season (see figure 2.3) in accordance with established patterns observed in *E. globulus* across a range of field based
seasonal studies (Pate and Arthur 1998; Merchant et al. 2010b). Similarly, seasonal changes in the total sugar content of phloem sap were observed in maritime pine trees (Pinus pinaster) by Marion et al. (2009). For the present study, we analysed phloem sap carbohydrate and $\delta^{13}C$ on a background of replicated, controlled and independent differences in nutrient availability to establish if patterns in carbohydrates and $\delta^{13}C$ withstand changes in the nutritional status of trees. We did not find such a relationship; however, Pate and Arthur (1998) noted that for the relationship between sugar concentration and $\delta^{13}C$, seasonal variation in both parameters exists between and within sites of contrasting nutrition, indicating the robustness of the technique.

2.4.3. Relationship between sugar concentration and phloem $\delta^{13}C$

Sucrose was the most abundant carbohydrate obtained from phloem sap, in agreement with previous literature (Dinant 2008; Merchant et al. 2010b). A positive correlation between phloem sugar concentration and $\delta^{13}C$ in both growing and non-growing season was observed. Total concentrations of sugar in phloem and $\delta^{13}C$ were more closely correlated in the growing E. globulus trees than those in the non-growing season (presumably more water limited). Variation in the relationship between phloem sucrose concentration and $\delta^{13}C$ is well characterised (Pate and Arthur 1998; Tausz et al. 2008; Merchant et al. 2010a) often showing improved relationships under conditions of water limitation. At times in which water is perhaps not the most limiting factor, alternative limitations have the capacity to influence this relationship, particularly that of $\delta^{13}C$ through light, temperature and/or nutrient supply. The relationship between $\delta^{13}C$ and phloem sap sugar has an influence on instantaneous water use efficiency (see detail Cernusak et al. 2003). Several authors status (Geßler et al. 2001; Cernusak et al. 2003) have reached in a good agreement that phloem sap $\delta^{13}C$ and phloem sugar concentration can be widely used for the assessment of tree water status. The $\delta^{13}C$ of phloem
sap can also be an integrative to assess the physiological performance of tree (Gessler et al. 2004; Claudia et al. 2006).

2.4.4. Relationships with tree growth

A positive relationship between total amino acid concentrations of phloem sap with annual DBH growth of trees was observed. Similarly, phloem sap $\delta^{13}$C was also positively related to tree growth in agreement with previous studies (Tausz et al. 2008; Merchant et al. 2010b). Under water-limited conditions, phloem $\delta^{13}$C has been shown to be strongly correlated with growth (Merchant et al. 2010b; Scartazza et al. 2015), likely due to the influence of stomatal limitations to carbon assimilation. Our study was conducted in an environment that is likely water limited throughout the summer periods because of the prevalence of sandy soils with low water retention. Nevertheless, correlations of growth with total amino acid concentrations in the phloem (despite likely complications of the interaction with N and P supply identified earlier) appear to reflect processes imparting some influence on the growth response. This relationship is likely driven at least in part by the NP interaction, and a significant growth response is nevertheless observed for both N and NP fertilizer treatment. Despite this, overall growth did not significantly differ between treatments. Combined, these observations illustrate the potential for phloem contents to assist in determining nutritional status of trees, but the exact information gleaned from these properties requires careful interpretation.

Growth responses to the addition of fertilisers are well characterised throughout the forest plantation industry. Previous investigations within the industry have also shown a reduction in growth response to higher levels of N addition. Although variation was observed, the addition of N and P did not significantly enhance the growth response, perhaps as a consequence of the sandy soils and low nutrient retention used in this investigation. May et al. (2009) found that there is no evidence for significant basal area increase in response to N application rates higher
than 200 kg ha\(^{-1}\) N across stands in the region of the present investigation. It was assumed that the response to 400 kg ha\(^{-1}\) N was 7\% greater production than that of 200 kg ha\(^{-1}\) N for all N forms fertiliser applied to sandy soils. However, based on evidence of N applied to fine textured soils, 300 kg ha\(^{-1}\) N showed 25\% greater production than that of 200 kg ha\(^{-1}\) N (FWPA 2017). McGrath et al. (2003) showed that the primary deficiency in a softwood plantation on an ex-native forest site in southern WA (a region similar to that for the present investigation) was phosphorus, with no response to nitrogen alone, but a significant N \(\times\) P interaction when both nutrients were applied leading to an 86\% increase in growth to the application of 270 kg N ha\(^{-1}\) plus 90 kg P ha\(^{-1}\). In this study a variation of DBH growth response was observed for 350 kg ha\(^{-1}\) N and 350 kg ha\(^{-1}\) NP treated plots compared to other treated plots, though they were not significant (see Figure 2.5).

**2.5. Conclusion**

The abundance of phloem sap metabolites and carbon isotope composition reflect both the availability of nutrients and water. Whilst increased N supply possibly increased free amino acid concentration in the phloem sap reflecting remobilisation, this pattern was not occurred with the concurrent supply of P. Such a pattern suggests an interactive effect potentially governed by below ground process. These results suggest great potential for the use of phloem sap in diagnosing water and nutrient status of trees. However, further work is required to investigate the interactive effects of both water and nutrient availability. Phloem sap metabolite and carbon isotope composition reflect nutritional status and growth performance of *E. globulus* which is of great use for plantation management in assessing water and nutritional status.
References


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Snowdon, P. 2002. Modeling Type 1 and Type 2 growth responses in plantations after application of fertiliser or other silvicultural treatments. Forest Ecology and Management. 163:229-244.


Chapter III: Phloem and leaf amino acid reflect physiology, growth, and nutritional status of blue gum (*Eucalyptus globulus*) seedlings

Abstract

Phloem are the central conduit for long distance transport and signalling in plants and offer great potential to reflect plant scale resource limitations. Specifically, the abundance of solutes and carbon isotopes in phloem sap are sensitive to environmental cues. With a focus on both water and nutrient availability, we characterise patterns in phloem sap and amino acids, obtained from *Eucalyptus globulus* seedlings grown under controlled environmental conditions (in a glass house and with regular water supply) and differing nutrient supply. Treatments consisted of three levels of a multi-element nutrient supply such as control (16 g fertiliser dissolved in 9 L of water), 50% (8 g fertiliser dissolved in 9 L of water), and 0% (no fertiliser). A significant variation among treatments are detected with implications for the development of spatially and temporally integrative tools for the rapid assessment of plant water and nutritional status. Average photosynthesis and collar diameter significantly differed among treatments indicating a growth restriction. Phloem amino acid concentration was found to positively correlate with the concentration of leaf amino acid concentration ($r^2 = 0.52$, $p<0.001$). Leaf and phloem amino acid concentration showed a linear regression relation with the seedlings collar diameter (CD) growth during the treatment period. For both plant materials (leaf and phloem), the correlations were significant ($r^2 = 0.63$, $p<0.001$ for leaf and $r^2 = 0.64$, $p<0.001$ for phloem). Correlations between leaf and phloem metabolites suggest phloem sap analysis can be used as an indicator of nutritional status and growth performance in *E. globulus*. 
3.1. Introduction

Nutrient management of production forests is an important component of overall productivity for both timber production and its profitability. In Australia, a total of approximately 173,000 ha of hardwood plantations and 84,000 ha of softwood plantations are fertilised each year (May et al. 2009). Fertilisers are widely used in plantations across Australia to ameliorate nutrient deficiencies and boost stand growth. In Australia, much of the research into fertiliser induced growth responses has focused on measuring site- and species-specific responses to different compositions or rates of fertiliser application. Plant growth responses to nutrition and water availability are largely based on analysing different plant organs such as foliar analysis. These analyses are time consuming for sample collection and are high cost. Phloem sap analysis avoids the difficulties of collecting leaf materials from different strata of the canopy, thus it offers a great potential of faster, straightforward and simple way of sample collection method than others, and it has the great potential of more accurate assessments of nutritional status (Peuke and Merchant 2018).

Translocation, allocation and distribution of assimilates and nutrients among different plant organs vary with nutrient supply (Maathuis 2009; Peuke 2010). Nutrient limitation elicits a range of growth responses including diversion of photoassimilates to root tissues to promote growth (Peuke 2010). Xylem sap has often been analysed to identify relationships among tree nutrient and soil nutrition status (Journet 1980; Walsh et al. 1984; Dambrine et al. 1995) however, these analysis typically result in poor indications of nutritional status due to complications arising from (1) transient and long-term storage of xylem-delivered nutrients in mature parts of shoots and roots (Jeschke and Pate 1995), (2) phloem-xylem transfer in leaves and stem, and (3) fluctuations of nutrient concentrations in xylem and variations in soil water status impacting transpiration (Dambrine et al. 1995). Determining the nutritional status of trees through leaf analysis is very often time consuming and costly due to spatial and temporal
heterogeneity (Peuke and Merchant 2018). Compared to xylem sap analysis, phloem sap composition and the highly complex role of phloem sap in the translocation of nutrients is well documented for some herbaceous species, but very little information exists for woody species. To date, no study has been conducted to observe the effect of fertiliser application on the composition of phloem and leaf metabolites in accordance with tree growth.

Concentrations of amino acids in the phloem can be utilised to assess the nitrogen status of *E. globulus* plantations (Pate and Arthur 1998). Therefore, it is, valuable to further our understanding of how phloem contents reflect tree nutrient status to improve plantation management decisions. Thus, the present study consists of the following hypotheses: (1) nutrient availability restricts photosynthesis and growth of blue gum seedlings; (2) leaf and phloem amino acids vary in accordance with nutrient availability; (3) leaf and phloem amino acid concentrations are proportional to seedling growth.

### 3.2. Material and methods

#### 3.2.1. Experimental design

Three month old *Eucalyptus globulus* seedlings were obtained from ERA Nurseries, Australia. Seedlings were transplanted into 9 L pots in February 2017 and grown in a commercial potting mixture (Landscape All Purpose Potting Mix; Bunnings, Hawthorn East, VIC, Australia) under glasshouse conditions. Seedlings were regularly watered by an automated dripper system twice a day for 10 min duration to field capacity of the soil matrix. As the plant size increased, the frequency of watering was adjusted to maintain water supply. After six months (January 2017) the fertiliser treatment was commenced. Treatment was at three levels of nutrient supply: control, 50% and 0% fertiliser (Table 3.1). Each treatment group consisted of 25 plants. An all-purpose soluble fertiliser was used (Thrive; Yates, Padstow, NSW, Australia). The ratio of nutrients such as nitrogen, phosphorous and potassium was 25:5:8. The standard application
was set by the manufacturer (8 g of fertiliser can be dissolved in 4.5 L water to use in 1 m² area).

Table 3.1. Experimental design for finding growth performance of E. globulus

<table>
<thead>
<tr>
<th>Level of treatments</th>
<th>Description of treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16 g fertiliser dissolved in 9 L water and used for 6 seedlings</td>
</tr>
<tr>
<td>50%</td>
<td>8 g fertiliser dissolved in 9 L water and used for 6 seedlings</td>
</tr>
<tr>
<td>0%</td>
<td>No fertiliser</td>
</tr>
</tbody>
</table>

Treatments were applied for two months with two weeks interval between applications. Growth and physiological data were recorded at two weeks intervals. Different plant materials (leaves, bark, and whole plant etc.) were harvested and weighed separately at the end of the experiment.

3.2.2. Growth and Physiological data collection

Initial height and collar diameter of each seedling was measured prior to the commencement of fertiliser treatments. The collar diameter (CD) and height of all individual seedlings were also recorded on the day of treatment and at two weeks intervals until the end of experiment (two months). Relative collar diameter (CD) growth is calculated as follows:

Relative CD growth (cm yr⁻¹) = \{(CD_e - CD_i)/CD_i \} × (12/2)………………………….. (Eqn 3.1)

Where CD_i is the collar diameter at the prior of the treatment and CD_e is the collar diameter at the end of the treatment.

Physiological data (CO₂ assimilation rate) were recorded using a Li-COR-6400 Infrared gas analyser (Li-Cor, Lincoln, USA) from 1000 h to 1500 h from a subset of sample size. The subset sample was a single fully expanded leave, randomly selected from 10 numbers of seedlings in each treatment group (having 25 number of seedlings) at two weeks intervals for the duration of the treatments. Light conditions within the Li-Cor chamber were set to 400
µmol m\(^{-2}\) s\(^{-1}\) PAR, and temperature and relative humidity in the measuring chamber were maintained within the Li-Cor chamber to approximate ambient conditions. Net CO\(_2\) assimilation rate (A, µmol m\(^{-2}\) s\(^{-1}\)), as well as stomatal conductance to water vapour (g\(_s\), µmol m\(^{-2}\) s\(^{-1}\)) were recorded.

### 3.2.3. Leaf tissue and phloem sap collection

At the end of the experiment, fully expanded leaf material and phloem sap from the same plant were collected from a subset of sample size. The subset sample were a single leaf and a piece (1 x 1 cm) of bark strip from 12 numbers of seedlings from each treatment group. A total of 36 leaf samples were collected and immediately transferred to the freezer at -10 °C. At the end of the day, samples were transferred to -80 °C until extraction. From the same plants of collected leaf material, a 1 cm length strip of bark (periderm) was peeled off the main stem 1 cm above the surface of the soil. Phloem contents were determined by a method approximating that of Gessler et al. (2004). Bark samples were weighed and transferred into 2 cm screw-cap vials. 1 mL deionised water was added to the vials and left to stand for 4 h. After this time, the bark was removed and transferred to an oven for 60 h at 60 °C and subsequently weighed. Therefore, moisture content of the bark was determined gravimetrically. Phloem samples were immediately capped and transferred to -80 °C until further analysis.

### 3.2.4. Extraction of leaf and phloem materials

For extraction of leaf material, samples were taken from the refrigerator and microwaved (650 Watt) for 10 sec then immediately transferred into an oven and dried at 60 °C for 24 h. Dried samples were ground to a powder and 40 mg of ground material was weighed into a 2 mL screwcap micro tube. 20 µL penta-erythritol (500 ppm) and then 0.1 mL of MeOH:CHCl\(_3\):H\(_2\)O (12:5:3) mixture were added to each sample. The samples were incubated at 75 °C for 30 min with periodic agitation. The samples were then centrifuged at 3000 rpm for 10 min and 800 µL
of supernatant removed and placed into a 2 ml microtube. 500 µL of MQ water and 200 µL chloroform were added, mixed thoroughly with a vortex mixer, centrifuged at 12,000 rpm (Eppendorf, Centrifuge 5424, Hamburg, Germany) for 20 min and stood for 10 min. Finally, 700 µL of the alcohol/aqueous phase (top) was removed and placed into a clean, round bottom microtube. Samples were kept at -80°C, awaiting LC-MS analysis. The concentration of leaf metabolites were calculated with respect to the weight of leaf material and then expressed by volume (µg mL⁻¹).

For phloem analysis, samples were thawed and vortexed for one minute. The mixture was then centrifuged at 12,000 rpm (Eppendorf, Centrifuge 5424, Hamburg, Germany) for ten min. 300 µL of extract was placed put into a glass vial for amino acid analysis. Final concentrations of phloem metabolites were calculated based upon the gravimetric difference in bark sample between fresh and dried material.

3.2.5. Analysis of leaf and phloem amino acids

For the amino acids, LC-MS analysis of the underivatized extract was carried out on a 1290 Infinity LC system (Agilent, USA) coupled to a 6520 QTOF Mass selective detector (Agilent, USA). A 3.5 µL sample was injected into a Zorbax SB-C18 column (2.1 x 150 mm, 3.5 µm) and a separation achieved by a flow rate of 0.5 mL min⁻¹ with a gradient elution of 0-100% water and methanol over 16 min. The QTOF was tuned to operate at the low mass range <1700 AMU and data acquisition completed in scan (60-1000 m/z) and positive ion mode. LC-MS results were identified based on their retention times relative to standards as well as their formula mass. Peaks were integrated and their relative quantities calculated by the Mass Hunter software (Agilent Technologies, Santa Clara, CA95051, United States of America).
3.2.6. Statistical analysis

Analysis of variance and post-hoc comparisons was made using Bonferroni test ($p \leq 0.05$). Linear regression analysis was performed to identify relationships between parameters. All statistical analysis were carried out in Sigmaplot (version 12.5; Systat Software, Inc., San Jose, CA, USA).

3.3. Results

3.3.1. Nutrient effect on net CO$_2$ assimilation rate and growth

Net CO$_2$ assimilation rate among treatments differed throughout the experimental period. The net CO$_2$ assimilation rate of control seedlings were significantly greater than that of 50% ($p = 0.01$) and 0% fertilised seedlings ($p < 0.01$). The CO$_2$ assimilation rate of 50% fertilised seedlings were also significantly greater than that of 0% fertilised seedlings ($p < 0.01$) (see Figure 3.1).

![Figure 3.1. Net CO$_2$ assimilation rate (µmol m$^{-2}$ s$^{-1}$) of E. globulus seedlings in response to different fertiliser treatments, such as control, 50% and 0%, small letters indicate significant differences ($p \leq 0.05$) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., n=10.](image)

Fertiliser application showed differing growth of seedlings among treatments during the experiment period. The relative collar diameter (CD) growth (cm yr$^{-1}$) of control seedlings was
significantly greater than that of 50% (p<0.01) and that of 0% fertilised seedlings (p<0.01).
The CD growth (cm yr⁻¹) of 50% fertilised seedlings also showed significant differences with
that of 0% fertilised seedlings (p = 0.01) (see Figure 3.2).

**Figure 3.2.** Relative collar diameter (CD) growth (cm yr⁻¹) of *E. globulus* seedlings in response to different
fertiliser treatments, such as control, 50% and 0%, small letters indicate significant differences (p≤0.05) among
the treatments (Bonferroni post hoc test), data shown are means ± s.e., n=25.

### 3.3.2 Nutrient effect on leaf and phloem amino acid concentrations

Seventeen amino acids were quantified in both the phloem and leaf extracts of *E. globulus*
under the conditions of this study. Concentrations significantly differed among treatment
groups. For the leaves, some of the amino acid concentrations were significantly different
between the controls with 50% and 0% fertilised seedlings (p values are detailed in Table 3.2).
Total amino acid concentrations in leaves of the control seedlings were significantly varied
with that of 50% fertilised seedlings (p = 0.09) and that of 0% fertilised seedlings (p = 0.09)
(see Table 3.2 & Figure 3.3A).

For the phloem, all of the quantified amino acids, except alanine and glycine, significantly
differed between the control with 50% and 0% fertilised seedlings (p values are detailed in
Table 3.2). Phloem total amino acid concentration of control seedlings were significantly varied that of 50% fertilised seedlings ($p<0.001$) and that of 0% fertilised seedlings ($p<0.001$) (see Table 3.2 & Figure 3.3B).

![Phloem amino acid concentrations](image)

**Figure 3.3.** A, leaf total amino acid, and B, phloem total amino acid concentration ($\mu$g mL$^{-1}$) of *E. globulus* seedlings in response to different fertiliser treatments, such as control, 50% and 0%, small letters indicate significant differences ($p \leq 0.05$) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., $n=12$. 

![Leaf amino acid concentrations](image)
Table 3.2. Nutrient effect on leaf and phloem amino acid concentrations (µg mL\(^{-1}\)) of *E. globulus* seedlings and their variations between the groups (control vs 50% fertilised and control vs 0% fertilised seedlings).

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Control ((\bar{X} \pm se))</th>
<th>50% fertilised ((\bar{X} \pm se))</th>
<th>(p) value(^+) (control vs 50% fertilised)</th>
<th>0% fertilised ((\bar{X} \pm se))</th>
<th>(p) value(^++) (control vs 0% fertilised)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.012 ± 0.001</td>
<td>0.005 ± 0.001</td>
<td>&lt;0.001(^*)</td>
<td>0.005 ± 0.000</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.017 ± 0.007</td>
<td>0.009 ± 0.001</td>
<td>ns</td>
<td>0.009 ± 0.001</td>
<td>ns</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.002 ± 0.001</td>
<td>0.004 ± 0.002</td>
<td>ns</td>
<td>0.002 ± 0.001</td>
<td>ns</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.025 ± 0.005</td>
<td>0.019 ± 0.004</td>
<td>ns</td>
<td>0.004 ± 0.002</td>
<td>0.004(^b)</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.027 ± 0.013</td>
<td>0.013 ± 0.003</td>
<td>ns</td>
<td>0.009 ± 0.004</td>
<td>ns</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.024 ± 0.004</td>
<td>0.016 ± 0.002</td>
<td>ns</td>
<td>0.020 ± 0.003</td>
<td>ns</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.015 ± 0.003</td>
<td>0.014 ± 0.003</td>
<td>ns</td>
<td>0.012 ± 0.003</td>
<td>ns</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>0.145 ± 0.050</td>
<td>0.136 ± 0.015</td>
<td>ns</td>
<td>0.145 ± 0.030</td>
<td>ns</td>
</tr>
<tr>
<td>Proline</td>
<td>0.079 ± 0.015</td>
<td>0.049 ± 0.007</td>
<td>ns</td>
<td>0.046 ± 0.006</td>
<td>ns</td>
</tr>
<tr>
<td>Valine</td>
<td>0.087 ± 0.014</td>
<td>0.058 ± 0.003</td>
<td>0.06</td>
<td>0.028 ± 0.004</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.042 ± 0.007</td>
<td>0.024 ± 0.001</td>
<td>0.01(^a)</td>
<td>0.014 ± 0.002</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.079 ± 0.015</td>
<td>0.049 ± 0.005</td>
<td>ns</td>
<td>0.033 ± 0.005</td>
<td>0.005(^b)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.074 ± 0.011</td>
<td>0.043 ± 0.002</td>
<td>0.001(^a)</td>
<td>0.028 ± 0.012</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.134 ± 0.027</td>
<td>0.071 ± 0.004</td>
<td>0.03(^a)</td>
<td>0.047 ± 0.007</td>
<td>0.002(^b)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.029 ± 0.006</td>
<td>0.013 ± 0.001</td>
<td>0.001(^a)</td>
<td>0.011 ± 0.002</td>
<td>0.005(^b)</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.020 ± 0.006</td>
<td>0.018 ± 0.006</td>
<td>ns</td>
<td>0.045 ± 0.014</td>
<td>ns</td>
</tr>
<tr>
<td>Serine</td>
<td>0.278 ± 0.064</td>
<td>0.173 ± 0.042</td>
<td>ns</td>
<td>0.265 ± 0.057</td>
<td>ns</td>
</tr>
<tr>
<td>Total AA</td>
<td>1.0940 ± 0.157</td>
<td>0.719 ± 0.065</td>
<td>0.09</td>
<td>0.727 ± 0.010</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Phloem Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.164 ± 0.159</td>
<td>0.454 ± 0.080</td>
<td>&lt;0.001(^*)</td>
<td>0.270 ± 0.091</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.517 ± 0.329</td>
<td>0.409 ± 0.088</td>
<td>0.005(^a)</td>
<td>0.529 ± 0.098</td>
<td>0.002(^b)</td>
</tr>
<tr>
<td>Asparagine</td>
<td>3.875 ± 0.862</td>
<td>0.648 ± 0.205</td>
<td>&lt;0.001(^*)</td>
<td>0.000 ± 0.000</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Alanine</td>
<td>23.256 ± 7.189</td>
<td>15.934 ± 1.653</td>
<td>ns</td>
<td>12.091 ± 1.939</td>
<td>ns</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>11.827 ± 1.642</td>
<td>4.487 ± 0.544</td>
<td>&lt;0.001(^*)</td>
<td>2.018 ± 0.362</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.379 ± 0.503</td>
<td>0.973 ± 0.153</td>
<td>&lt;0.001(^*)</td>
<td>0.494 ± 0.103</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>15.625 ± 3.605</td>
<td>4.512 ± 1.283</td>
<td>0.005(^a)</td>
<td>3.782 ± 3.201</td>
<td>0.002(^b)</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>9.995 ± 0.846</td>
<td>5.149 ± 0.848</td>
<td>0.002(^a)</td>
<td>4.168 ± 1.003</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Proline</td>
<td>16.804 ± 2.420</td>
<td>6.212 ± 0.728</td>
<td>&lt;0.001(^*)</td>
<td>4.820 ± 0.507</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Valine</td>
<td>14.388 ± 2.159</td>
<td>4.818 ± 0.420</td>
<td>&lt;0.001(^*)</td>
<td>3.962 ± 0.275</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>11.732 ± 1.879</td>
<td>3.632 ± 0.464</td>
<td>&lt;0.001(^*)</td>
<td>2.792 ± 0.206</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Leucine</td>
<td>11.955 ± 0.924</td>
<td>4.395 ± 0.359</td>
<td>&lt;0.001(^*)</td>
<td>4.499 ± 0.344</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10.609 ± 1.914</td>
<td>5.000 ± 0.534</td>
<td>&lt;0.001(^*)</td>
<td>3.768 ± 0.309</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.370 ± 0.902</td>
<td>3.692 ± 0.324</td>
<td>&lt;0.001(^*)</td>
<td>3.515 ± 0.658</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>11.051 ± 2.129</td>
<td>4.035 ± 0.692</td>
<td>0.002(^a)</td>
<td>3.305 ± 0.343</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.770 ± 1.854</td>
<td>1.398 ± 0.786</td>
<td>ns</td>
<td>0.761 ± 0.761</td>
<td>ns</td>
</tr>
<tr>
<td>Serine</td>
<td>31.099 ± 0.621</td>
<td>14.231 ± 3.691</td>
<td>0.032(^a)</td>
<td>14.827 ± 4.698</td>
<td>0.025(^b)</td>
</tr>
<tr>
<td>Total AA</td>
<td>195.702 ± 15.763</td>
<td>80.851 ± 8.962</td>
<td>0.001(^*)</td>
<td>66.477 ± 8.887</td>
<td>&lt;0.001(^b)</td>
</tr>
</tbody>
</table>

Data shown as mean (\(\bar{X}\)) ± standard error (se), where n = 12, AA represents amino acid, \(^*\) denotes \(p\) value for the variance analysis between control and 50% fertilised seedlings, \(^++\) denotes \(p\) value for the variance analysis between control and 0% fertilised seedlings. Small letters denotes significant (\(p\leq0.05\)), and ns denotes non-significant (\(p\) value > 0.05) variation between the groups. Post hoc comparison was done by Bonferroni test.
3.3.3. Relationships between leaf and phloem amino acid concentrations

Total phloem amino acid concentration was found to be positively correlated with the total leaf amino acid concentration ($r^2 = 0.52, p<0.001$; Figure 3.4).

![Graph showing the correlation between total leaf and phloem amino acid concentrations.](image)

**Figure 3.4.** Relation between leaf and phloem amino acids of *E. globulus* seedlings. The solid black line shows regression line and the symbols ● denotes control seedlings, ○ denotes 50% fertilised seedlings, and ▼ denotes 0% fertilised seedlings.

3.3.4. Relationships between leaf and phloem amino acids with growth

Leaf amino acids concentrations showed a linear relationship with the seedlings relative CD growth. Most of the quantifiable amino acids showed significant positive linear relationships with seedlings relative CD growth (Table 3.3). Overall total amino acid in leaves showed a positive linear relationship with CD growth ($r^2 = 0.63, p<0.001$, Figure 3.5A). For the phloem, most of the quantifiable amino acids showed a significant positive linear regression relationship with the seedlings relative CD growth (Table 3.3). Overall, total amino acid concentration in phloem showed a positive linear relation with CD growth ($r^2 = 0.64, p<0.001$, Figure 3.5B).
Figure 3.5. Leaf and phloem relation with growth. A, leaf total amino acid concentration showed positive linear relation with relative collar diameter growth (cm yr$^{-1}$), B, phloem total amino acid concentration also showed a positive relation with relative collar diameter growth (cm yr$^{-1}$) of *E. globulus* seedlings. The solid black line shows regression line and the symbols ● denotes control seedlings, ○ denotes 50% fertilised seedlings ▼ denotes 0% fertilised seedlings.
Table 3.3. Relation between leaf and phloem amino acid concentrations (µg mL⁻¹) with relative collar diameter growth (cm yr⁻¹) of *E. globulus* seedlings

<table>
<thead>
<tr>
<th>Equation (Linear regression)</th>
<th>$r^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf Amino Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G = 1.76 + 109.39 \times \text{Lysine}$</td>
<td>0.56</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td>$G = 1.41 + 117.12 \times \text{Arginine}$</td>
<td>0.25</td>
<td>0.004$^a$</td>
</tr>
<tr>
<td>$G = 2.36 + 65.67 \times \text{Asparagine}$</td>
<td>0.13</td>
<td>0.053$^a$</td>
</tr>
<tr>
<td>$G = 2.20 + 25.02 \times \text{Alanine}$</td>
<td>0.33</td>
<td>$0.001^a$</td>
</tr>
<tr>
<td>$G = 2.58 + 5.05 \times \text{Aspartic acid}$</td>
<td>0.04</td>
<td>0.297</td>
</tr>
<tr>
<td>$G = 1.82 + 32.20 \times \text{Threonine}$</td>
<td>0.29</td>
<td>0.001$^a$</td>
</tr>
<tr>
<td>$G = 2.47 + 8.07 \times \text{Glutamine}$</td>
<td>0.02</td>
<td>0.548</td>
</tr>
<tr>
<td>$G = 2.10 + 2.35 \times \text{Glutamic acid}$</td>
<td>0.18</td>
<td>0.018$^a$</td>
</tr>
<tr>
<td>$G = 1.78 + 12.92 \times \text{Proline}$</td>
<td>0.32</td>
<td>0.001$^a$</td>
</tr>
<tr>
<td>$G = 1.82 + 11.48 \times \text{Valine}$</td>
<td>0.42</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td>$G = 1.83 + 24.57 \times \text{Isoleucine}$</td>
<td>0.45</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td>$G = 1.90 + 10.77 \times \text{Leucine}$</td>
<td>0.44</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td>$G = 1.71 + 15.28 \times \text{Tyrosine}$</td>
<td>0.53</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td>$G = 1.91 + 6.41 \times \text{Phenylalanine}$</td>
<td>0.45</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td>$G = 2.09 + 22.56 \times \text{Tryptophan}$</td>
<td>0.26</td>
<td>$0.002^a$</td>
</tr>
<tr>
<td>$G = 2.79 + 4.61 \times \text{Glycine}$</td>
<td>0.04</td>
<td>0.227</td>
</tr>
<tr>
<td>$G = 1.96 + 1.93 \times \text{Serine}$</td>
<td>0.31</td>
<td>0.001$^a$</td>
</tr>
<tr>
<td>$G = 1.47 + 1.20 \times \text{Total AA}$</td>
<td>0.63</td>
<td>$&lt;0.001^a$</td>
</tr>
<tr>
<td><strong>Phloem Amino Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G = 2.09 + 0.76 \times \text{Lysine}$</td>
<td>0.39</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.25 + 0.38 \times \text{Arginine}$</td>
<td>0.24</td>
<td>0.004$^b$</td>
</tr>
<tr>
<td>$G = 2.20 + 0.20 \times \text{Asparagine}$</td>
<td>0.59</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.23 + 0.02 \times \text{Alanine}$</td>
<td>0.19</td>
<td>0.014$^b$</td>
</tr>
<tr>
<td>$G = 2.05 + 0.08 \times \text{Aspartic acid}$</td>
<td>0.45</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.13 + 0.27 \times \text{Threonine}$</td>
<td>0.43</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.15 + 0.04 \times \text{Glutamine}$</td>
<td>0.42</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.00 + 0.08 \times \text{Glutamic acid}$</td>
<td>0.27</td>
<td>0.002$^b$</td>
</tr>
<tr>
<td>$G = 2.14 + 0.04 \times \text{Proline}$</td>
<td>0.26</td>
<td>0.002$^b$</td>
</tr>
<tr>
<td>$G = 2.01 + 0.07 \times \text{Valine}$</td>
<td>0.46</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.07 + 0.08 \times \text{Isoleucine}$</td>
<td>0.45</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 1.69 + 0.12 \times \text{Leucine}$</td>
<td>0.56</td>
<td>$&lt;0.001^b$</td>
</tr>
<tr>
<td>$G = 2.04 + 0.07 \times \text{Tyrosine}$</td>
<td>0.38</td>
<td>0.001$^b$</td>
</tr>
<tr>
<td>$G = 1.93 + 0.12 \times \text{Phenylalanine}$</td>
<td>0.34</td>
<td>0.001$^b$</td>
</tr>
<tr>
<td>$G = 2.19 + 0.06 \times \text{Tryptophan}$</td>
<td>0.26</td>
<td>0.002$^b$</td>
</tr>
<tr>
<td>$G = 2.49 - 0.06 \times \text{Glycine}$</td>
<td>0.14</td>
<td>0.032$^b$</td>
</tr>
<tr>
<td>$G = 2.23 + 0.02 \times \text{Serine}$</td>
<td>0.22</td>
<td>0.005$^b$</td>
</tr>
<tr>
<td>$G = 1.75 + 0.007 \times \text{Total AA}$</td>
<td>0.64</td>
<td>$&lt;0.001^b$</td>
</tr>
</tbody>
</table>

‘G’ represents collar diameter growth (cm yr⁻¹), AA represents amino acids, and the concentration of amino acids are expressed as µg mL⁻¹. The small letters denote significant relations ($p \leq 0.05$) between growth and amino acids concentration.
3.4. Discussion

3.4.1. Nutrient effect on plant physiology and growth

Nutrient limitation in general, decreases the photosynthetic rate (Wissuwa et al. 2005), thus restricting plant growth. The present study shows a positive effect of fertiliser (NPK) application on plant photosynthesis and growth of *E. globulus* seedlings under controlled conditions similar to that observed previously for *E. globulus* seedlings (Sheriff and Nambiar 1991).

Photosynthetic capacity was found to be significantly correlated with leaf organic nitrogen content as nitrogen is often correlated to photosynthetic capacity as it is a major component of both the light harvesting complexes and, more significantly the carboxylation enzyme Ribulose 1,5-biphosphate carboxylase/oxygenase, RuBiCo (Evans 1983; Field and Mooney 1983; Allison et al. 1997). Nitrogen rich RuBiCo and the chlorophyll or protein complexes are mainly responsible for CO₂ fixation and light harvesting, which occurred through photosynthesis process (Zubillaga et al. 2002; Wang et al. 2012). Phosphorous (P) is a component of energy metabolite ATP (Adenosine Tri-Phosphate) and NADP⁺ (Nicotinamide Adenine Dinucleotide Phosphate) which are produced in the light reaction of photosynthesis and provides energy in the Calvin cycle and regenerate Ribulose 1,5–bisphosphate (RuBP) (Farquhar et al. 1980). Potassium (K) was found to be tightly associated with photosynthesis and photosynthate translocation (Tränkner et al. 2018). K deficiency reduces Rubisco carboxylation activity in chloroplasts (Weng et al. 2007; Hu et al. 2015; Zahoor et al. 2017), thus influencing photosynthesis and CO₂ assimilation and plant growth. *Eucalyptus* responds to fertiliser application, particularly to nitrogen (N) through increasing leaf area and stem growth (Smethurst et al. 2003). In this study, a combination NPK fertiliser was used to determine its effect on the photosynthetic capacity and growth (collar diameter growth) of *E.*
seedlings and the results confirm that the major elements such as N, P, and K have a significant effect on the measured physiological processes of the plant. Several studies have related fertiliser application to growth responses of *E. globulus* and showed significant positive correlations between different rates and combinations of fertiliser application with their growth in terms of height, collar diameter, stem DBH, leaf and root area development (Kimanzu 1992; Pinkard et al. 2006; Fini et al. 2007). This current study determined not only the growth response but also the physiological process of plants to fertiliser (NPK) application.

### 3.4.2. Nutrient effect on leaf and phloem amino acids

This study provides for the first time a detailed analysis of *E. globulus* leaf and phloem metabolites concentration with response to nutritional availability. For plant tissues more generally, the effect of nitrogen fertilisation on amino acid concentrations in relation to different growing stages of plants such as flowering (Barnes and Bengtson 1968), seedling growth after germination (Pharis et al. 1964) and nitrogen metabolism (Durzan and Steward 1967), has been examined. For trees, jack pine (*Pinus banksiana*) and black spruce (*Picea mariana*), the effect of fertilisation on free amino acid concentrations revealed that it was more superior indicator of plant nutrition status than bulk elemental analysis (Kim et al. 1987).

Nitrogen fertilisation increased the concentration of total nitrogen in beech (*Fagus sylvatica*) leaves. Among individual amino acids concentrations of glutamic acid, aspartic acid, glutamine, asparagine and overall total free amino acids increased the most in response to N-fertilisation (see Påhlsson 1992), which is very much similar to the present study (see Table 3.2 & Figure 3.3). Although leaf glutamic acid, aspartic acid, glutamine, and asparagine concentrations of *E. globulus* seedlings showed no significant variation, those of phloem significantly varied with availability of fertiliser. Similar to this, the phloem exudates of bark flaps showed higher concentration of aspartic acid, alanine, and glutamine in high-N fertilised
almond trees (*Prunus dulcis*) compare to low-N fertilised trees (Youssefi et al. 2000). High N supply remarkably increased sugarcane leaf amino acid concentrations (e.g. tyrosine, threonine, alanine, serine, proline and asparagine etc.) which indicates the direct influence of N in protein biosynthesis (Bassi et al. 2018). The present study is consistent with those findings that leaf and phloem metabolite concentrations (amino acids) vary according the rate of fertiliser application. Bush (1999) explained that amino acids are a currency for N exchange via phloem to sink organs. These changes were observed among seasons and at different growing stages e.g. during shoot growth of scot pine (*Pinus sylvestris*) total soluble protein and Rubisco activity decreases in older needle (Gezelius et al. 1981), indicating that Rubisco may be a storage protein during this shoot development period (Näsholm and Ericsson 1990). Several environmental factors affect the concentration of free amino acids (Durzan and Steward 1983). In addition, the form and availability of nitrogen affect the amount and composition of amino acid pool (Kim et al. 1987). Our results are in agreement with this (see detail in Table 3.2) and support the notion that individual results must be interpreted in the context of both site and species-specific patterns in amino acid contents.

### 3.4.3. Relationships between leaf and phloem amino acid concentrations

Transport of amino acids is one of the major functions of the translocation system. The concentration and type of amino acids varies with species, environment and development (Pritchard 2007). In castor bean (*Ricinus communis*) mineral contents of leaf were found to be tightly correlated with phloem sap (Peuke 2010). In the sap, solute concentration is highly correlated with the concentrations in leaf tissue for mobile nutrients. Therefore, the composition of phloem saps is a good indicator for the nutritional conditions in leaves (Peuke 2010). Linear regression analysis revealed a high correlation between amino acid composition of exudate and phloem sap samples from the same leaf (Weibull et al. 1990). This study also shows for the woody species, *E. globulus* leaf and phloem amino acid concentrations were
tightly correlated. In a forest landscape collecting of leaves from different strata of the canopy will often also impart a cost of analysing plant nutritional content. As it was observed that there is a positive correlation between total amino acid concentration of leaf and phloem, phloem sap from the standing tree will be more economic for the plantation manager rather than total foliar analysis. Phloem sap analysis can be a replacement of foliage analysis of field grown trees condition as it is easier to collect and potential to avoid intra-canopy variation (Tausz et al. 2008; Merchant et al. 2010). As phloem sap has no soluble molecules involved in cellular metabolism such as found in leaves (Merchant et al. 2012), it is a more suitable metabolic pool on which predictions of whole-plant response to short-term environmental change can be made (Smith and Merchant 2018). The quantification of mineral nutrients and nitrogen which is transported in the form of amino acids in the phloem stream can be a good predictor to assess the whole plant nutritional status (White 2012; Tegeder and Hammes 2018).

3.4.4. Leaf and phloem amino acids are tightly correlated with plant growth

In this study, leaf and phloem amino acids were highly correlated with collar diameter growth of *E. globulus* seedlings, which is dependent on nutrient availability. Metabolic and developmental pathways draw on common resource pool and respond to environmental change and resource supply (Tonsor et al. 2004). Growth and the associated drain of metabolites to the cell needs to be adjusted for the metabolic capacity.

This type of regulation is elicited during growth depression with reduced primary metabolism (Chen et al. 2005; Fernie et al. 2002). The interaction between metabolism and the regulation of growth may operate in two ways: a high supply of metabolites triggers growth, or growth is restricted below minimum level metabolites. A large number of plant shoot and leaf metabolites showed a close correlation with growth, and variation in growth coincides with the characteristic of changes of metabolite levels. However, individual metabolites fluctuate
largely (Meyer et al. 2007), for example a seasonal variation of arginine, glutamine was observed by Näsholm and Ericsson (1990) for fertilised scot pine (*Pinus sylvestris*). In this study, changes in leaf and phloem metabolites are observed due to fertilisation, but variation in leaves was much less than that of the phloem. This might be because of an excess amount of those amino acids in leaves that can be passed through the phloem stream to the sink tissues. However, Pate (1980) stated that excess nitrogen content in leaves for protein synthesis is preferably drawn off into vacuoles rather than being cycled through the leaf via the outgoing phloem stream. Further investigation would be needed to examine amino acid partitioning among the plant organs due to nutrient supply. In Figure 3.5 and Table 3.3 it was observed that both leaf and phloem amino acids concentration were highly correlated to the collar diameter growth of seedlings and, as discussed, there was a positive correlation found between leaf and phloem amino acids; hence, phloem amino acid concentration can be postulated as a predictor of plant growth. Amino acids are treated as precursors and constituents of protein (Rai 2002) and stimulates cell growth (Sh Sadak et al. 2015). Several authors (Kowalczyk et al. 2008; Boras et al. 2011; El-Zohiri and Asfour 2009) have explained the role of amino acids in plant growth, yield, plant development. Similar to those studies, plant nutritional status in relation to the growth response can be predicted through analysing amino acid concentration of phloem sap. This can be both a quick and economical tool for the tree grower.

### 3.5. Conclusion

Plant metabolites (e.g. amino acid concentration in plant tissue) can be used as a tool to determine plant physiological performance and growth. Nutrient application causes changes in amino acid concentrations in leaf and phloem and these were observed to be correlated with photosynthetic rate, and growth rate of seedlings. The amino acids concentration of autotrophic organ (leaf) and heterotrophic tissue (phloem) varied proportionately with nutrient availability in *E. globulus* seedlings. This study, for the first time elucidated the relationship between leaf
and phloem amino acids concentration and demonstrated the use of phloem sap as a tool for studying nutritional status at the canopy level. These findings are a step towards the development of more economical, rapid and a reliable tools for the assessment of plant nutrition at large spatial and temporal scales.

References


82


Chapter IV: Relationships between phloem sap and leaf $\delta^{13}$C are resilient to changes in nutritional status

Abstract

The use of naturally occurring carbon isotope abundance ($\delta^{13}$C) obtained from the phloem has been used to infer plant physiological processes at a range of spatial scales. However, no study has evaluated the influence of nutritional supply on concurrent patterns in $\delta^{13}$C. Here, blue gum (Eucalyptus globulus) seedlings were grown under controlled environmental conditions (in a glass house and with regular water supply and differing nutrient supply). Treatments consisted of three levels of a multi-element nutrient supply: control (16 g fertiliser dissolved in 9 L water), 50% (8 g fertiliser dissolved in 9 L water), and 0% (no fertilizer). Variation in $\delta^{13}$C obtained from different plant tissues and metabolite concentrations were detected among treatments with implications for the development of spatially and temporally integrative tools for the rapid assessment of plant water status under contrasting nutrient supply. Leaf and phloem $\delta^{13}$C showed a positive linear relationship ($r^2 = 0.36, p = 0.0008$) indicating a consistent relationship independent of nutrient supply. Phloem $\delta^{13}$C and carbohydrate showed a positive correlation ($r^2 = 0.36, p = 0.0001$). Leaf and phloem carbohydrates were positively correlated to each other ($r^2 = 0.28, p = 0.006$). Phloem carbohydrate concentrations were significantly varied between control and 50% fertilised seedlings ($p = 0.001$). Sucrose was found to be the most concentrated metabolite in phloem among all the treatments. Phloem carbohydrate concentrations and $\delta^{13}$C showed positive correlations with total biomass ($r^2 = 0.45, p<0.001$ and $r^2 = 0.35, p = 0.001$, respectively) of seedlings. Observing a strong correlation between phloem carbohydrates and carbon isotopes ratio ($\delta^{13}$C) with plant growth under different
nutrient regime the phloem sap analysis can be used as a good predictor for determining plant growth.

4.1. Introduction

Carbon isotope abundance ($\delta^{13}C$) has been widely used to study plant function at the metabolic scale (Tcherkez 2006; Tcherkez et al. 2004), leaf level (Farquhar et al. 1989b; Farquhar et al. 1982), whole plant scale (Cernusak et al. 2009; Gessler et al. 2007), and ecosystem scale (Bowling et al. 2008; Seibt et al. 2008; West et al. 2006). The use of $\delta^{13}C$ to understand plant physiological processes has previously focused on leaf level and post photosynthetic carbon isotope fractionation at a range of scales and has been reviewed by several authors (Badeck et al. 2005; Cernusak et al. 2009). The $\delta^{13}C$ in leaf organic matter is controlled by stomatal resistance to CO$_2$ diffusion and biochemical processes such as the carboxylation of CO$_2$ by RuBisCo. The $\delta^{13}C$ is influenced by the ratio between intercellular and the ambient CO$_2$ concentration, where the fractionation constant for diffusion from air is -4.4‰ and carboxylation discrimination is -27‰ (Farquhar et al. 1989b; Farquhar et al. 1982). Phloem is the central pathway to move photosynthetic assimilates. Phloem level $\delta^{13}C$ have been recently investigated by several authors (Smith et al. 2016; Merchant et al. 2011; Merchant et al. 2012; Cernusak et al. 2003; Caludia et al. 2006; Gessler et al. 2004; Gessler et al. 2008). Most of these studies were based on linking $\delta^{13}C$ to understand physiology, water use efficiency and growth of plants where environmental impacts. The influence of nutrient availability on patterns of $\delta^{13}C$ has been considered with regard to variation in RuBisCo concentration in leaf tissues and its subsequent ‘upstream’ influence on photosynthesis and stomatal conductance (Farquhar et al. 1989b; O'Leary 1993; Vogel 1993). Increasing nitrogen supply generally leads to increased leaf area based photosynthesis and stomatal conductance (Schulze 1996), thus it reduces the value of $\delta^{13}C$, albeit Siegwolf et al. (2001) found no substantial differences of long term water use efficiency while plants were treated with high nitrogen supply.
In the past 15 years, studies among woody plants suggest the $\delta^{13}C$ of phloem sap can be correlated with water status (Cernusak et al. 2003; Pate and Arthur 1998; Pate et al. 1998), climatic conditions (Pate and Arthur 1998; Tausz et al. 2008), transpiration and stomatal conductance (Claudia et al. 2003), and drought related limitations of mixed rotation aged plantations (Tausz et al. 2008). The relationship among sugar composition and $\delta^{13}C$ of phloem sap to growth and physiological performance was investigated by Merchant et al. (2010), and site specific responses were reflected in phloem sap (Merchant et al. 2012). To date, no study has examined the relationship among phloem and leaf metabolites in accordance with tree growth when nutrients are a limiting factor. If such an impact exists, it reduces the utility of $\delta^{13}C$ as an integrated measure of plant water status. In this study, we examine the relationship of leaf and phloem $\delta^{13}C$ and carbohydrate concentration with the growth of *Eucalyptus globulus* seedlings over a range of nutrient availabilities. The hypotheses of the study were (1) relationships between leaf and phloem soluble $\delta^{13}C$ are tightly correlated; (2) relationships between leaf and phloem soluble $\delta^{13}C$ are not influenced by nutritional supply; (3) Phloem $\delta^{13}C$ is correlated with phloem carbohydrate concentration; (4) relationships between leaf and phloem carbohydrates are strongly correlated; (5) leaf and phloem carbohydrate concentrations vary in accordance with nutrient supply; (6) phloem carbohydrates and $\delta^{13}C$ are strongly correlated to plant growth; and (7) plant growth is restricted to nutrient availability.

4.2. Material and methods

4.2.1. Experimental design

Three month old *Eucalyptus globulus* seedlings were obtained from ERA Nurseries, Australia. Seedlings were transplanted into 9 L pots in February 2017 and grown in a commercial potting mixture (Landscape All Purpose Potting Mix; Bunnings, Hawthorn East, VIC, Australia) under glasshouse conditions. Seedlings were regularly watered by an automated dripper system twice
a day for 10 min duration to field capacity of the soil matrix. As the plant size increased, the frequency of watering was adjusted to maintain water supply. After six months (January 2017) the fertiliser treatment commenced. Treatment was at three levels of nutrient supply: standard amount of nutrient supply (100%), 50% of standard amount of nutrient supply and 0% nutrient supply (Table 4.1). Each treatment group consisted of 25 plants. An all-purpose soluble fertiliser was used (Thrive; Yates, Padstow, NSW, Australia). The ratio of nutrients such as nitrogen, phosphorous and potassium was 25:5:8. The standard application was set by the manufacturer (8 g of fertiliser can be dissolved in 4.5 L water to use in 1 m² area).

**Table 4.1.** Experimental design for finding growth performance of *E. globulus*.

<table>
<thead>
<tr>
<th>Level of treatments</th>
<th>Description of treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16 g fertiliser dissolved in 9 L water and used for 6 seedlings</td>
</tr>
<tr>
<td>50%</td>
<td>8 g fertiliser dissolved in 9 L water and used for 6 seedlings</td>
</tr>
<tr>
<td>0%</td>
<td>No fertiliser</td>
</tr>
</tbody>
</table>

Treatments were applied for two months with two weeks interval between applications. Growth and physiological data were recorded at two week intervals. Different plant materials (leaves, bark, and biomass etc.) were harvested and weighed separately at the end of the experiment.

**4.2.2. Growth data collection**

Initial height and collar diameter of each seedling was measured prior to the commencement of fertiliser treatments. The height of all individual seedlings were also recorded on the day of treatment and at two weeks intervals until the end of experiment (two months). Relative height growth is calculated as follows:

Relative height growth \( (\text{cm yr}^{-1}) \) = \( \{(H_e - H_i)/H_i\} \times (12/2) \) ……………………………… (Eqn 4.1)
Where \( H_i \) is the height of seedlings before the treatment and \( H_e \) is the height of seedlings at the end of the treatment.

The whole plants part (stem, leaves, and root) were dried at 60 °C for 48 h and subsequently weighed to get the dry weight of total biomass (in gram, g).

4.2.3. Leaf tissue and phloem sap collection

At the end of the experiment, fully expanded leaf material and phloem sap from the same plant were collected from a subset of sample size. The subset sample were a single leaf and a piece (1 x 1 cm) of bark strip from 12 numbers of seedlings from each treatment group. A total of 36 leaf samples were collected and immediately transferred to the freezer at -10 °C. At the end of the day, samples were transferred to -80 °C until extraction. From the same plants of collected leaf material, a 1 cm length strip of bark (periderm) was peeled off the main stem 1 cm above the surface of the soil. Phloem contents were determined by a method approximating that of Gessler et al. (2004). Bark samples were weighed and transferred into 2 cm screw-cap vials. 1 mL deionised water was added to the vials and left to stand for 4 h. After this time, the bark was removed and transferred to oven for 60 h at 60 °C and subsequently weighed. Therefore, moisture content of the bark was therefore gravimetrically. Phloem samples were immediately capped and transferred to -80 °C until further analysis.

4.2.4. Extraction of leaf & phloem materials

For extraction of leaf and phloem metabolites, samples were taken from the refrigerator and microwaved (650 Watt) for 10 sec in high then immediately transferred into an oven and dried at 60 °C for 24 h. Dried samples were ground to a powder and we took 40 mg of ground samples into a 2 ml Eppendorf screwcap micro tube. 20 µL penta-erythritol (500 ppm) and then 0.1 mL MeOH:CHCl₃: H₂O (12:5:3) were added to each sample. The samples were incubated at 75 °C for 30 min agitating them from time to time. Then the samples were centrifuged at
3000 rpm for 10 min and 800 µL of supernatant was removed into a clean Eppendorf tube. Again 500 µL of MQ water and 200 µL chloroform were added, mixed thoroughly with a vortex mixer, centrifuged at 12000 rpm for 20 min (Eppendorf, Centrifuge 5424, Hamburg, Germany) and let stand for 10 min. Finally, 700 µL of the alcohol/aqueous phase (top) was removed and place into a clean, round bottom Eppendorf tube. Samples were kept at -80 °C, awaiting GC-MS analysis.

For the phloem extraction, the phloem extracts were removed from the refrigerator to be normalized to room temperature. With the use of vortex machine the samples were homogenised and then centrifuged 12000 rpm for 10 min (Eppendorf, Centrifuge 5424, Hamburg, Germany). Fifty (50) µL of phloem extracts sample were taken from upper part of the solution and dried in speed vacuum concentrator (Scanvac, Scan Speed 40, Korea) with the help of double stage high vacuum pump (Javac Pty Ltd., VIC, Australia). After drying, 450 µL anhydrous pyridine was added and immediately sealed. A mixture solution of bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchloroacetamide (TMCS) with the ratio of 10:1 was added to the sample with existing pyridine. Samples were kept at -80 °C, awaiting for GC-MS.

For leaf δ<sup>13</sup>C analysis, dried leaves were ground and approximately 1.3 (± 0.025) mg of them were placed into a tin cup (5 × 9 mm) and analysed by Isotope Ratio Mass Spectrometry (IRMS) (see below).

4.2.5. Analysis of carbohydrates and δ<sup>13</sup>C

For carbohydrate analysis, leaf and phloem samples were incubated for 35 min at 75 °C and analysed by GC-MS within 24h. Analysis was carried out on an Agilent 7890b Gas Chromatograph with QQQ 7000 Mass selective detector (Agilent Technologies, Santa Clara, CA, United States). Samples were injected with a 20:1 split injection onto a HP-5 column (30
m, 0.25 mm ID, 0.25 µm film thickness) with helium carrier gas at 1 mL/min constant flow. The temperature programme had an initial oven temperature set of 60 °C for 2 min ramping to 300 °C at 10 °C min\(^{-1}\) for 10 min. Peaks were integrated and their relative quantities calculated by the Mass Hunter software (Agilent Technologies, Santa Clara, CA, United States). Phloem carbohydrate concentration was calculated with respect to volume of bark water equivalent.

For δ\(^{13}\)C analysis, an Isochrome Mass Spectrometer was used. The instrument was a Delta V with Conflo IV and FlashHT peripherals in a dual-reactor setup (Thermo Fisher Scientific, Bremen, Germany). The samples were combusted by Dumas combustion. The oxidation reactor is set to 1000 °C.

4.2.6. Statistical analysis

Analysis of variance and Post-hoc comparisons was made using Bonferroni test (\(p\leq0.05\)). Linear regression analysis was performed to identify relationships between parameters. Statistical analyses were carried out by Sigmaplot (version 12.5, Systat Software, Inc. San Jose, CA, USA).

4.3. Results

4.3.1. Fertiliser effect on plant growth

Different rates of fertiliser application elicited a variable growth response during the experiment period. The control seedlings height significantly increased compared to 50% (\(p<0.001\)) and 0% fertilised seedlings (\(p<0.001\)) (Figure 4.8A). It was also observed that the biomass of the control group seedlings were significantly increased compared to that of 50% (\(p = 0.01\)) and 0% fertilised seedlings (\(p = 0.02\)) (see Figure 4.8B).
Figure 4.8. Fertiliser effect on height and biomass growth of blue gum (*E. globulus*) seedlings. A, height growth variation and B, biomass growth variation due to different rate of fertiliser application. Small letter shows significant variation among three groups of seedlings (control, 50% and 0%), small letters indicate significant differences (*p* ≤0.05) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., *n* = 25.

4.3.2. Relationships between leaf and phloem δ^13^C

Leaf and phloem δ^13^C were correlated with each other (*r^2^ = 0.36, *p* = 0.0008) with no impact of nutrient treatment observed (Figure 4.1).
Figure 4.1. Leaf and phloem $\delta^{13}C$ abundance with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, ○ denotes 50%, and ▼ denotes 0% fertilised seedlings.

4.3.3. Fertiliser effect on leaf and phloem $\delta^{13}C$

Whilst fertilizer treatments elicit a trend in metabolite and $d^{13}C$, statistically they are not significantly variable (Figure 4.2). For the leaf $\delta^{13}C$, it was observed that mean value of $\delta^{13}C$ of control, 50% and 0% fertilised seedlings were -29.10‰ (s.e. 0.25), -29.59‰ (s.e. 0.24), and -29.55‰ (s.e. 0.11), respectively.
Figure 4.2. Nutrient effect on leaf and phloem $\delta^{13}$C, (a) leaf $\delta^{13}$C and (b) phloem $\delta^{13}$C of *E. globulus* seedlings in response to different fertiliser treatments (control, 50% and 0%), data shown are means ± s.e., n= 12.

In case of phloem carbon isotope abundance ($\delta^{13}$C), it was observed that mean carbon isotope abundance of control group, 50% and 0% seedlings were -28.84‰ (s.e. 0.22), -28.65‰ (s.e. 0.17) and -28.93‰ (s.e. 0.18), respectively.

4.3.4. Relationships between phloem $\delta^{13}$C and carbohydrates

Phloem $\delta^{13}$C and phloem carbohydrate concentration showed a positive linear regression ($r^2 = 0.36, p = 0.0001$), with no impact of nutrient treatment observed (Figure 4.3).
Figure 4.3. Phloem $\delta^{13}C$ and carbohydrate relation in response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, ○ denotes 50%, and ▼ denotes 0% fertilised seedlings.

4.3.5. Relationships between leaf and phloem carbohydrates

Leaf and phloem carbohydrates concentration showed a positive linear regression ($r^2 = 0.28, p = 0.006$) with no impact of nutrient treatment observed (Figure 4.4).
4.3.6. Fertiliser effect on leaf and phloem carbohydrate concentrations

Leaf total carbohydrate concentrations were found to be not significantly variable among the treatments (Figure 4.5A). However, the sum of all carbohydrate concentrations in phloem was found to significantly differ between the control and 50% fertilised seedlings ($p = 0.001$, Figure 4.5B).
Figure 4. 5. Nutrient effect on leaf and phloem carbohydrate conc. A, leaf total carbohydrates, and B, phloem total carbohydrates conc. (µg mL⁻¹) of *E. globulus* seedlings in response to different fertiliser treatments, such as control, 50% 0%, small letters indicate significant differences (p≤0.05) among the treatments (Bonferroni post hoc test), data shown are means ± s.e., n= 12.
Table 4.2. Fertiliser effect on leaf and phloem carbohydrates concentration (µg mL⁻¹) and their variation between the groups (control vs 50% and control vs 0% seedlings)

Carbohydrates (CH) | Control (X± Se) | 50% fertilised (X± Se) | p value (Control vs 50%) | 0% fertilised (X± Se) | p value ++ (Control vs 0%)
--- | --- | --- | --- | --- | ---

**Leaf carbohydrates**

Pentaerythitol  | 11.77 ± 0.421 | 13.08 ± 0.417 | ns | 12.45 ± 0.470 | ns
Aminobutyric acid  | 0.204 ± 0.028 | 0.111 ± 0.019 | 0.012 **a** | 0.037 ± 0.014 | <0.001 **b**
Citric acid  | 2.990 ± 0.453 | 3.926 ± 0.396 | ns | 1.554 ± 0.485 | 0.085
Fructose  | 5.735 ± 0.616 | 7.436 ± 0.584 | 0.054 **a** | 7.323 ± 0.358 | 0.054 **a**
Allo-Inositol  | 4.344 ± 0.542 | 5.468 ± 0.431 | ns | 5.693 ± 0.396 | ns
Glucose 1  | 4.552 ± 0.572 | 5.760 ± 0.459 | ns | 5.979 ± 0.424 | ns
Glucose 2  | 4.277 ± 0.558 | 5.321 ± 0.443 | ns | 5.309 ± 0.372 | ns
Myo-Inositol  | 3.996 ± 0.291 | 3.639 ± 0.158 | ns | 3.207 ± 0.256 | ns
Maltose  | 0.640 ± 0.023 | 0.656 ± 0.012 | ns | 0.872 ± 0.070 | 0.002 **a**
Isomaltose  | 0.847 ± 0.042 | 0.845 ± 0.025 | ns | 0.908 ± 0.039 | ns
Sucrose  | 5.869 ± 3.566 | 1.914 ± 0.779 | ns | 2.845 ± 0.895 | ns
Total leaf CH  | 45.404 ± 3.228 | 48.429 ± 1.593 | ns | 46.320 ± 2.145 | ns

**Phloem carbohydrates**

Malic Acid  | 11.866 ± 1.781 | 7.581 ± 0.914 | 0.004 **a** | 5.986 ± 0.508 | 0.048 **b**
Aminobutyric acid  | 2.005 ± 0.324 | 1.206 ± 0.078 | 0.039 **a** | 1.558 ± 0.168 | ns
Citric acid  | 7.841 ± 2.200 | 4.377 ± 1.554 | ns | 1.869 ± 0.951 | 0.046 **a**
Fructose  | 10.212 ± 1.358 | 6.062 ± 2.232 | ns | 6.607 ± 0.910 | ns
Allo-Inositol  | 4.822 ± 1.224 | 2.158 ± 1.397 | ns | 1.501 ± 0.864 | ns
Glucose 1  | 6.345 ± 1.100 | 2.860 ± 1.397 | 0.05 **a** | 2.976 ± 0.738 | 0.05 **a**
Glucose 2  | 6.745 ± 1.209 | 2.956 ± 1.375 | 0.043 **a** | 3.153 ± 0.768 | 0.043 **a**
Myo-Inositol  | 6.641 ± 0.859 | 3.258 ± 0.394 | <0.001 **a** | 3.886 ± 0.336 | 0.006 **a**
Chiro-Inositol  | 0.664 ± 0.033 | 0.322 ± 0.061 | 0.003 **a** | 0.367 ± 0.086 | 0.013 **a**
Mannose 2  | 6.378 ± 0.897 | 2.149 ± 0.214 | <0.001 **a** | 2.707 ± 0.231 | <0.001 **a**
Sucrose  | 584.289 ± 63.171 | 342.518 ± 33.850 | 0.001 **a** | 490.398 ± 26.273 | ns
Total phloem CH  | 647.787 ± 69.686 | 375.446 ± 38.210 | 0.001 **a** | 520.468 ± 27.336 | ns

Glucose and mannose isomers are presented separately (1 and 2). Data shown as mean (X) ± standard error (se), where n = 12, **a** denotes p value between control and 50%, **++** denotes p value between control and 0% fertilised seedlings, small letters denote significant (p≤0.05), and ns denotes non-significant (p value > 0.05) variation, post hoc comparison was done by Bonferroni test.

Eleven carbohydrates were reliably quantified in the phloem. Concentrations varied among treatment groups. Many of the carbohydrates, such as malic acid, aminobutyric acid, glucose 1, glucose 2, myo-inositol, chiro-inositol, mannose 2 and sucrose concentrations significantly differed among the treatments. Sucrose was the most concentrated carbohydrate in the phloem and varied significantly between the treatments (Table 4.2).
4.3.7. Relationships between phloem carbohydrates $\delta^{13}C$ and growth

Total dry biomass of the blue gum ($E. globulus$) seedlings was positively correlated phloem carbohydrate concentration ($r^2 = 0.45$, $p<0.001$). With the increase rate of fertiliser, phloem carbohydrate concentrations and dry biomass were consistently increased (Figure 4.6).

![Figure 4.6. Total biomass and phloem carbohydrate concentration with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control group seedlings (100% fertilised), o denotes group A seedlings (50% fertilised), ▼ denotes group B seedlings (0% fertilised).](image)

**Table 4.3. Relation between phloem carbohydrates conc. ($\mu g \ mL^{-1}$) and dry biomass (g)**

<table>
<thead>
<tr>
<th>Phloem Carbohydrates</th>
<th>Linear Regression Model</th>
<th>$r^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malic Acid</td>
<td>BM = 48.40 + 1.14xMalic Acid</td>
<td>0.18</td>
<td>0.016*</td>
</tr>
<tr>
<td>Aminobutyric acid</td>
<td>BM = 50.69 + 4.13xAminobutyric Acid</td>
<td>0.03</td>
<td>0.292</td>
</tr>
<tr>
<td>Citric acid</td>
<td>BM = 32.28 + 0.68xCitric acid</td>
<td>0.12</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fructose</td>
<td>BM = 44.88 + 2.08xFructose</td>
<td>0.32</td>
<td>0.050*</td>
</tr>
<tr>
<td>Allo-Inositol</td>
<td>BM = 51.25 + 2.99xAllo-Inositol</td>
<td>0.44</td>
<td>0.001*</td>
</tr>
<tr>
<td>Glucose 1</td>
<td>BM = 48.52 + 3.08xGlucose 1</td>
<td>0.39</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Glucose 2</td>
<td>BM = 48.87 + 2.81xGlucose 2</td>
<td>0.37</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mannose 2</td>
<td>BM = 44.39 + 4.24xMannose 2</td>
<td>0.52</td>
<td>0.001*</td>
</tr>
<tr>
<td>Chiro-Inositol</td>
<td>BM = 45.19 + 32.21xChiro-Inositol</td>
<td>0.27</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>BM = 40.04 + 4.74xMyo-Inositol</td>
<td>0.35</td>
<td>0.001*</td>
</tr>
<tr>
<td>Sucrose</td>
<td>BM = 31.42 + 0.06xSucrose</td>
<td>0.48</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Total Carbohydrates</td>
<td>BM = 31.27 + 0.05xTotal Carbohydrates</td>
<td>0.45</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

BM biomass (g), small letter shows the significant correlation ($p \leq 0.05$) between growth and phloem carbohydrates concentration.
The highest concentrated metabolite, sucrose, showed a positive linear regression with biomass \((r^2 = 0.48, p < 0.001\) see Table 4.3).

Phloem \(\delta^{13}C\) showed a positive correlation with biomass growth of \(E.\ globulus\) seedlings \((r^2 = 0.35, p = 0.001,\) Figure 4.7).

![Figure 4.7. Total dry biomass and phloem \(\delta^{13}C\) with response to different rates of fertiliser treatments. The solid line shows regression line and the symbol ● denotes control, o denotes 50%, and ▼ denotes 0% fertilised seedlings.](image)

### 4.4. Discussion

#### 4.4.1. Fertilizer effect on plant growth

The imposition of the nutrient deficiency caused a growth restriction under the conditions of this study. Reductions in both plant height and total biomass infer that a significant reduction in the available nutrient was imparted on plants, however, even in the treatment whereby fertiliser was completely removed, growth still occurred. This indicates that the treatments were within the bounds of severity that still allowed for carbon fixation during the treatment period. In this study nutritional effect showed a significant variation of height between the
seedlings group (control vs 50%, control vs 0% fertilised seedlings and 50% vs 0% fertilised seedlings), however the pattern of biomass allocation was only significant in control group seedlings comparing to other groups. Though there might have height variation in between 50% and 0% fertilised group, their root/shoot ratio might not significantly varied, which may cause no significant variation of dry mass between these group. Further investigation can reveal information about the allocation of biomass with response to the fertilizer effect. Photosynthesis is the major physiological process to plant growth.

4.4.2. Nutrient effect on leaf and phloem $\delta^{13}C$

Heterotrophic enrichment of carbon as it is transferred from the phloem to leaves was detected in this study but was not influenced by nutrient supply. Heterotrophic enrichment was previously investigated by several authors (Cernusak et al. 2009; Smith et al. 2016). Merchant et al. (2010) described the important implications of heterotrophic enrichment for the use of both leaf and phloem $\delta^{13}C$ for the prediction of gas exchange. In this study, phloem sap $\delta^{13}C$ was found to be less negative than leaf $\delta^{13}C$, in agreement with both Bowling et al. (2008) and Cernusak et al. (2009). After fixation of carbon in leaves, several processes include packaging for transport of carbon occurred though bio-chemical conversion and solutes are transported across membrane, which eventually causes additional isotopic discrimination (Merchant et al. 2010). Importantly, it appears that the enrichment is represented by a consistent offset. These results are similar to that of Smith et al. (2016) which revealed a strong relationship between bulk leaf and phloem $\delta^{13}C$. Combined, these results present further evidence to support the notion that phloem sap $\delta^{13}C$ may be used as a surrogate for leaf $\delta^{13}C$ offering considerable advantages by removing the need for foliage collection, extraction and metabolite purification.
4.4.3. Nutrient effect on leaf and phloem carbohydrates

This study provides a detailed analysis of the effect of nutrients on leaf and phloem metabolites of *E. globulus* species and how they respond to growth. Nutrient availability influences metabolite concentrations in both leaf and phloem. Effect of nutrient addition on physiology was presented in chapter III. In this chapter nutrient effect on leaf and phloem carbohydrate contents reflecting plant growth are discussed. This study shows leaf carbohydrates are not significantly reduced with reducing the availability of fertilizer whereas phloem carbohydrates are significantly reduced with reducing nutrient availability, probable cause may be enriched carbohydrates are transporting from source to sink at the early stage of plant development, reflected in phloem transport. In the previous chapter, low fertilizer availability reduces CO₂ assimilation rate of plant. Similarly, Araya et al. (2009) observed low nitrogen treatment causes the repression of photosynthesis due to possibly relation to the carbohydrate content, rather than to sensitivity to carbohydrates.

Understandably, these patterns differed between leaf and phloem tissues due to the heterotrophic enrichment. Whereas an array of metabolites were found to be in similar concentrations among leaf tissues, sucrose was the dominant metabolite observed in the phloem. Previous experiments investigating the effect of reduced N supply on leaf metabolites showed that field grown cotton had significantly lower sucrose, higher starch and higher total non-structural carbohydrate concentration in low N plants compared to high N-fertilised plants (Zhao and Oosterhuis 2000). For conifers, nitrogen fertilisation had no effect in scots pine needles (Ericsson 1979). Equally foliar total sugar contents were not influenced by N availability in beech (Adams et al. 1986) presumably, as the fertiliser effect is short-lived and the balance between carbohydrate production and nutrient supply was immediately restored in unfertilised trees. However, Bassi et al. (2018) shows that plants supplied with high N contain increased levels of glucose, fructose and raffinose. Overall, interactions between metabolite
abundance and nutrient availability are poorly understood, restricted by the background of cellular metabolism and the complexity of metabolite networks. Further work on the relationships between individual metabolite abundance will undoubtedly prove useful, perhaps simplified by the use of phloem sap pools that are somewhat independent from the background of cellular metabolism.

In agreement with previous studies, sucrose was found to be the dominant carbohydrate in phloem sap, but this concentration did not appear to respond in a systematic way to the effects of treatment. Sucrose transport and hydrolysis play key regulatory roles in carbon allocation and sugar signal generation (Rolland et al. 2006). Carbon metabolism and sugar accumulation appear to play important roles in vegetative plant growth and development, presumably in part through sugar signal generation (Rolland et al. 2006). Sugars showed a key regulatory function to boosting plant growth (Dahiya et al. 2017). Previous studies have shown that non-reducing sugars are transported in the phloem stream to avoid cellular damage (Raven 1991) and sucrose is the major constituent of phloem sap (Dinant et al. 2010). Metabolic and developmental pathways draw on common resource pools and respond to environmental change and resource supply (Tonsor et al. 2004). Growth and associated passing through of metabolites to the cell needs to be adjusted for the metabolic capacity e.g. the ability to supply enough organic compounds. This type of regulation was noticed under growth depression with reducing primary metabolism (Chen et al. 2005; Fernie et al. 2002). Meyer et al. (2007) stated that the interaction between metabolism and the growth regulatory mechanism may operate in two ways: a high supply of metabolites triggers growth, or growth grains metabolites to a minimum tolerable level upon which growth is restricted. Our study did not show such patterns, perhaps due to the severity of our treatments, reallocation of metabolites throughout the plant or a combination of the two.
4.4.4. Phloem $\delta^{13}C$ and plant growth

Phloem $\delta^{13}C$ was positively correlated to the biomass growth. Our study suggests the possibility of using $\delta^{13}C$ to measure tree growth where nutrient availability is a growth limiting factor. The relationships that are often used to estimate water use efficiency are not affected by nutrient availability (within the range examined). The $\delta^{13}C$ can be useful in estimating plant water use efficiency (Farquhar et al. 1989a; Diefendorf et al. 2010; Yang et al. 2015). Similar results to those studies were also found in this study which describes a positive correlation leaf $\delta^{13}C$ with the biomass growth. Fertilisation with N and P can increase WUE (Sheriff et al. 1986), and it follows that nutrient deficiency will decrease WUE. Squire et al. (1987) showed that WUE in radiata pine ($Pinus radiate$) seedlings (in this case calculated as biomass per unit water transpired) was increased by both decrease in soil moisture and increase in soil nitrogen. Equally, in assessing the drought related growth limitation of 5 year old $E. globulus$ seedlings, Tausz et al. (2008) found a positive correlation between phloem $\delta^{13}C$ and basal area increment. In relation to these studies the current results also reveal that, phloem $\delta^{13}C$ can be a good tool to assess plant growth while nutrient availability is a limiting factor of growth.

4.5. Conclusion

Nutrient availability influences growth through a range of physiological and chemical mechanisms. Carbon isotope abundance ($\delta^{13}C$) obtained from phloem sap is often used to infer leaves processes. Here we show that $\delta^{13}C$ obtained from both leaf and phloem material is correlated. This relationship appears resilient to changes in nutrient supply. Sucrose is the dominant carbohydrate in phloem tissue and is not related to plant growth under the conditions experienced in this study. Combined, these results suggest that phloem sap carbon constituents analysis can be used to infer estimates of tree water use efficiency and growth. Further investigations are needed to determine the growth response of $E. globulus$ with different site
and environmental conditions in relation to changes of plant material carbohydrate and $\delta^{13}$C under field conditions.

**References**


Chapter V: Interactive effects of water and nutrient availability on wood density and carbon isotope abundance of phloem metabolites and cellulose in field grown *Eucalyptus globulus*

Abstract

Naturally occurring carbon isotope abundance (δ13C) is a useful tool in predicting leaf level gas exchange. Despite this, little is known regarding post-photosynthetic fractionation events. Tree ring wood cellulose δ13C was determined to identify potential relationships with phloem sap δ13C where the trees are treated with different rates of nutrient availability. Two and a half year old *Eucalyptus globulus* plantation grown trees treated with different rate of nutrient application under rainfed conditions. Phloem sap δ13C was correlated with the corresponding year grown wood cellulose δ13C. It was observed that from the bark side to the interior, the first, second and third wood sections (5 mm) showed a series of positive correlations with r² values of 0.21 (p = 0.02), 0.27 (p = 0.003), and 0.44 (p<0.001) respectively, however the fourth and fifth section of wood showed negative correlation with r² value of 0.14 (p = 0.02) and 0.24 (p = 0.005). The cellulose δ13C value increased from the bark side wood section to interior section of wood, having a range of -27.5‰ to -26.5‰. The basic densities of those wood sections did not significantly vary. The relationship between wood cellulose δ13C and basic density of the respective wood sections were significant but did not consistently show the same relation (e.g. 1st and 5th section of wood showed positive relations but 2nd to 4th section of wood showed negative relations). However, positive correlations between dry density and δ13C were observed for all the five sections with r² value of 0.30 (p = 0.001), 0.22 (p = 0.005), 0.29 (p = 0.002), 0.25 (p = 0.005), and 0.32 (p = 0.0007), respectively. The dry volume of those wood
sections decreased with decreasing nitrogen (N) availability and showed increasing δ\(^{13}\)C values, but the nitrogen-phosphorous (NP) treated plot had no consistent effect on wood growth and δ\(^{13}\)C. E. globulus did not show any significant variation of carbon sequestration rate (CSR) due to different rate of nutrient application. Implications for monitoring plant growth and gas exchange are discussed.

5.1. Introduction

Industrial activities and deforestation are the most significant causes of increased greenhouse gases emissions (Althoff and Chandler 1999). Forests play an important role in mitigating climate change by sequestering carbon in tree tissues. Trees assimilate CO\(_2\) via photosynthesis and store carbon in biomass and soils (Brown et al. 1996; Trexler and Haugen 1995; Watson et al. 2000). Therefore, afforestation or reforestation can be one of the possible methods to mitigate CO\(_2\) accumulation (Marland and Schlamadinger 1997). Trees accumulate carbon in above ground biomass, below ground biomass and added carbon through litter fall. The absorption of CO\(_2\) from the atmosphere by planting new forests is a viable contribution towards combating the effects of climate change. The idea of carbon offset plantings, originally proposed by Dyson (1977), is now being implemented worldwide under the Kyoto Protocol and other national strategies. In addition to the productive or protective role of plantation forests under a changing climate, species selection for enhancing carbon uptake and storage in forest ecosystems and optimising fertiliser or irrigation management options is critical (Schoene and Netto 2005). Madeira et al. (2002) described that the increase of carbon (C) in irrigated and fertilised mineral soils was six times higher (1.21 kg C m\(^{-2}\)) than in the control (0.21 kg C m\(^{-2}\)) for a 6 year old Eucalyptus globulus plantation. The increase of C was proportional to tree biomass production and allocation of C to the mineral soil was enhanced by increasing nutrient supply both in rain-fed and irrigated treatments.
For trees, carbon (C) sequestration rate is not linear through time with significant seasonal variation reflected in the width and density of growth rings. The width of the annual rings often varies sufficiently in different trees to allow determination of the exact year in which each wood material was laid down. Estimation of above ground biomass as well as net carbon sequestration potential through analysis of tree rings measurement is possible. Above ground biomass of individual trees and at the stand scale are describe by several authors (Brown et al. 1989; MacDicken 1997; Houghton et al. 2001; Achard et al. 2002; Chave et al. 200; Chave et al. 2014; Ramankutty et al. 2007) based on predictive relationships from a core set of measurements. To estimate the dry biomass of an individual tree, most allometric equations are based on developing regressions between diameters at breast height (DBH), or tree height, with above ground biomass. In more detailed but rare cases, studies such as Chave et al. (2014) used wood specific gravity to estimate dry biomass. However, the variation in density across the radial dimension of the stem was not considered. Whilst wood density is clearly important to the calculation of C sequestration and can be integrated across whole stems easily, studies characterising the distribution of density across the radial section remains rare, particularly when correlating such patterns with environmental drivers of C sequestration.

Stem core analysis has great capacity to characterise tree ring width and wood density along the ring which can be correlated with seasonal DBH increments and tree height to estimate above ground carbon sequestration rate without inducing mortality. This stem core also contains variations in $\delta^{13}C$, which may be used as a surrogate measure of water use efficiency (Seibt et al. 2008). The naturally occurring $\delta^{13}C$ is the ratio of $^{13}C$ to $^{12}C$ and is expressed in ppt (‰), as $\delta^{13}C = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$, where $R_{\text{sample}}$ is the $^{13}C/^{12}C$ ratios of a sample and $R_{\text{standard}}$ is the $^{13}C/^{12}C$ ratios of an international standard Vienna-PDB or VPDB (Coplen 1995). 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 of $^{13}$C as CO$_2$ diffuses into leaves and is fixed into plant components – termed fractionation (McCarroll and Loader 2004). Fractionation due to diffusional or biochemical processes can be influenced by environmental factors, consequently the abundance of carbon isotopes can allow the inference of environmental factors at the time of C deposition into plant tissues (McCarroll and Loader 2004).

Dominating fractionation events such as diffusion of CO$_2$ through the stomatal aperture and carboxylation reactions of photosynthesis are well characterised (Farquhar et al. 1989b) 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 remains promising (Cernusak et al. 2009). The $\delta^{13}$C from bulk wood tissue has been used previously (Craig 1954; Farmer and Baxter 1974; Libby et al. 1976) but purified cellulose is now used (Wilson and Grinsted 1975) to avoid overwhelming fractionation events associated with lignin formation (McCarroll and Loader 2004). Large isotopic variability has been demonstrated to occur within individual tree ring (Loader et al. 1995; Schleser et al. 1999; Switsur et al. 1995) consequently the homogeneity of sample material remains an important consideration, particularly when pooling material from many trees or blocks of annual rings.

Previous experiments conducted under field grown (see Chapter II,) and in control environments conditions (see Chapters III and IV) with varying nutrient availability, showed that nutrient availability influences the composition of phloem sap metabolites and patterns of $\delta^{13}$C in growth of *Eucalyptus globulus* and this is reflected in growth performance. Based upon this prior knowledge, this study seeks to determine the influence of environmental factors on tree ring cellulose $\delta^{13}$C and phloem metabolite $\delta^{13}$C and investigate the relationship of these parameters with wood density as a means of developing surrogate measures for tree carbon
sequestration. Specifically we hypothesise that: 1) phloem and cellulose δ\(^{13}\)C are tightly correlated, 2) this correlation varies among cellulose obtained from different growth rings 3) the δ\(^{13}\)C of wood cellulose varies in accordance with wood density, 4) volumetric growth and δ\(^{13}\)C of wood varies in accordance with nutrient and water availability; and 5) nutrient and water availability influences carbon sequestration when correcting for changes in wood density.

5.2. Material and methods

5.2.1. The study site

Nutrient treatments (see Table 5.1) were applied to a commercial plantation stand of *E. globulus* located in the Mount Gambier district of South Australia located at 37.75\(^{0}\) S latitude, and 140.77\(^{0}\) E longitude and an elevation of 63 m above sea level. This region is characterised as having a Mediterranean type climate with the majority of rainfall occurring in the winter and spring months (June to November). In the period 2014 to 2018, mean maximum and minimum air temperatures of Mount Gambier, South Australia were 27.72 \(^{0}\)C and 13.34 \(^{0}\)C, respectively. The Mean annual rainfall was recorded as 739.12 mm and mean annual daily evaporation was 3.5 mm (ABOM 2018).

5.2.2. Experimental design

A factorial treatment design was established for fertiliser doses consisting of 54 trees per plot, each with 9 trees per row. Row spacing is maintained at 4 m × 2.5 m. The total size of each plot was 0.06 ha. (24 m × 25 m). There are 42 plots consisting of seven treatments in six replicated plots. The total trial area is 2.52 ha (168 m wide and 150 m length). Treatments were randomly applied to each individual plot. The fertiliser treatments consisted of three rates of N fertiliser applied as ammonium and three rates of ammonium + phosphorus applied as superphosphate (Table 5.1), and the nil control plot had no fertiliser application.
Table 5.1. Composition of fertiliser and associated element(s) added to each treatment with the plantation of *Eucalyptus globulus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fertiliser application (ha(^{-1}))</th>
<th>Elemental amounts ha(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>250N</td>
<td>250 kg anhydrous ammonia</td>
<td>206 kg N</td>
</tr>
<tr>
<td>350N</td>
<td>350 kg anhydrous ammonia</td>
<td>289 kg N</td>
</tr>
<tr>
<td>450N</td>
<td>450 kg anhydrous ammonia</td>
<td>371 kg N</td>
</tr>
<tr>
<td>250NP</td>
<td>250 kg anhydrous ammonia + 250 kg superphosphate</td>
<td>206 kg N + 22 kg P + 28 kg S</td>
</tr>
<tr>
<td>350NP</td>
<td>350 kg anhydrous ammonia + 350 kg superphosphate</td>
<td>289 kg N + 31 kg P + 39 kg S</td>
</tr>
<tr>
<td>450NP</td>
<td>450 kg anhydrous ammonia + 450 kg superphosphate</td>
<td>371 kg N + 40 kg P + 50 kg S</td>
</tr>
</tbody>
</table>

5.2.3. *Collection of phloem sap*

Phloem sap was collected from *Eucalyptus globulus* tree trunks using the “razor blade technique” following the methods described by Merchant et al. (2011). Phloem sap droplets were progressively collected using a glass disposable pipette from 1000 h to 1400 h and kept in a single micro tube for each tree, with an addition of 200 µL of methanol into the micro tube for sample preservation. Samples were immediately transferred to a -20 °C freezer and within 48 h were stored in a -80 °C freezer. Samples collected from individual trees were bulked into one sample per plot. The sampled trees were randomly selected within the plot.

5.2.4. *Collection of wood core*

Wood core samples were collected from within each trial plot from a randomly selected single tree at breast height (1.3 m) in October 2018. Wood core samples were collected using a 16 inch, two-threaded increment borer of 5.15 mm core diameter (Haglof, Sweden). Extracted cores were immediately placed into a plastic drinking straw and wrapped with masking tape to reduce evaporation from the wood to determine green volume and calculation of basic density. Wood core sample preparation is described in paragraph 5.2.7.
5.2.5. **Phloem sap preparation and $\delta^{13}C$ analysis**

A 5 µL of phloem sap solution was placed into an aluminium capsule (dimensions: 2.88/16mm, IVA Analysentechnik e. K. Meerbusch, Germany) and dried in an oven at 60 °C for 48 h (see details in Chapter II). $\delta^{13}C$ of samples 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) are expressed as delta notation, $\delta^{13}C = \frac{R_{sample}}{R_{standard}} - 1$, $R$ is the ratio of $^{13}C$ and $^{12}C$ in a sample and standard VPDB. The standard material precision are 0.06 ‰ and 0.11‰.

5.2.6. **Growth and wood density**

The estimated stand volume under bark (ESVUB) over the two successive growing period (2016 and 2017) was collected from the Australian Blue Gum Plantation Pty. Ltd. Growth during the treatment period was calculated by subtraction (ESVUB of the year 2017 – ESVUB of 2016). The authority used the equation to calculate the ESVUB as

\[
\text{ESVUB} = (G*MDH*FF)BT2 = (G*MDH*0.344)0.09446 \dots \dots \dots \text{Eq (5.1)}
\]

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

Fresh wood sections were weighed and green volumes determined for all the prepared samples. Core samples were put in the oven at 60 °C for 48 h then oven dry weights were determined. 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 constant dry weight was achieved. Green volumes of all wood sections and their basic densities were calculated following the formulas: $v_g = \pi r^2h$ and $\rho = \frac{dw}{v_g}$ respectively (gm cm$^{-3}$), where $v =$ volume (cm$^3$), $r =$ radius of core (cm), $h =$ length of core (cm) and $\rho =$ basic density of wood, $dw =$ dry weight (gm), $v_g =$ green volume.
Dry volume of wood was calculated as $v_d = \pi r_d^2 h$, where $v_d$ = dry volume, $r_d$ = the dry radius of core (cm) and $h$ = length of the core and dry density as $\rho_d = dw/v_d$.

5.2.7. Sample preparation for cellulose $\delta^{13}C$ analysis

Annual DBH growth was around 3.5 cm with average bark width of 0.5 cm. Annual wood growth at DBH was therefore, calculated as $2.5 \left[3.5 - (0.5 + 0.5)\right]$ cm. As phloem sap was collected in November 2016 and February 2017, a 3 cm section of the wood core was removed before five sections (each section having 5 mm) of wood core were separated from the stem core to encompass wood formed during this time. For our analysis we avoided current year (2018) wood growth and to analyse wood of the year 2017. These five sections encompassing 2.5 cm of wood grown in 2017 were separated and extracted. For the cellulose extraction, the total sample size ($n$) was therefore 210 (1 stem $\times$ 5 section of wood core $\times$ 42 sample plot).

Each wood section (5 mm) was ground with mortar and pestle manually. Each time of individual grinding, ethanol was used to clean the mortar and pestle and allowed to evaporate between samples.

5.2.8. Extraction of cellulose

Cellulose extraction from wood was carried out following Brendel et al. (2000). Wood core samples (5 mm) were finely ground and 2-3 mg weighed into 1.5 ml polypropylene tubes. 120 $\mu$L acetic acid (80% acetic acid, reagent grade) and then 12 $\mu$L nitric acid (69% nitric acid, reagent grade) was added. Tubes were capped and inserted into heating blocks at 120 °C for 30 min. Tubes were agitated every 5 min. Capped tubes were then allowed to cool after which 400 $\mu$L of ethanol added, recapped, and agitated then centrifuged for 5 min at 10,000 rpm (Eppendorf, Centrifuge 5424, Hamburg, Germany). The resulting supernatant was then removed carefully and discarded. 300 $\mu$L 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 removed carefully and discarded. To get cleaner cellulose, steps using deionised water and ethanol were repeated for another 2 times and then again 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.

5.2.9. Analysis of isotope ratios in tree rings

A weight of 0.35 mg of cellulose was placed into the tin capsule for analysis via Isotope Ratio Mass Spectrometry (IRMS). The δ¹³C were determined by a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The oxidation reactor was set to 1000 °C.

5.2.10. Carbon sequestration rate

Carbon sequestration rate was calculated using the model for South Australia suggested by Hobbs et al. (2016) was followed as:

\[ \log(B_{ag} + 1) = 0.9161 \times \log(SV + 1) + 0.5444 \] .............................. (Eqn 5.2)

Where, \( B_{ag} \) above ground biomass in kg, \( SV = \) stem volume (m³ plant⁻¹ X 1000). SV was calculated with \( \pi r^2 h \), considering the cylindrical volume, where \( r = \text{DBH}_{1.3m}/2 \) and \( h = \) total height of tree.

The root biomass was calculated as:

\[ \log(B_{GB,plant} + 1) = 0.7426 \times \log(A_{GB,plant} + 1) + 0.6073 \] .............................. (Eqn 5.3)
Where AGB\text{plant} = \text{Above-ground biomass (kg plant}^{-1}\text{)}, BGB\text{plant} = \text{Below-ground biomass (kg plant}^{-1}\text{)}. Hence, carbon sequestration rate (CSR) was calculated as (Total Biomass X 0.5)/age of tree (yrs)/area (ha) and expressed as kg ha\textsuperscript{-1} yr\textsuperscript{-1}.

5.2.11. Statistical analysis

Analysis of variance and Post-hoc comparisons was made using Bonferroni test ($p \leq 0.05$). Linear regression analysis was performed to identify relationships between parameters. These statistical analyses were carried out in Sigmaplot (version 12.5, Systat Software, Inc. San Jose, CA, USA).

5.3. Results

5.3.1. Relationship between phloem and wood cellulose $\delta^{13}C$

The $\delta^{13}C$ abundance of phloem sap was found to be correlated $\delta^{13}C$ of wood cellulose but the relationship changed substantially across the radial direction of the growth ring. A gradual progression from a positive correlation to that of a negative correlation was observed with increasing depth of the sample (Figure 5.1).
Figure 5.1. Relation between phloem $\delta^{13}C$ with wood cellulose $\delta^{13}C$ across the radial direction, A, B, and C showed positive correlation between phloem $\delta^{13}C$ and first, second and third section of 5 mm wood cellulose respectively, D and E showed negative correlation between phloem $\delta^{13}C$ and fourth and fifth section of 5 mm wood cellulose respectively. The solid line shows regression line.

The $\delta^{13}C$ of first, second and third sections of the wood core cellulose showed positive linear correlations with phloem $\delta^{13}C$ with $r^2$ value of 0.21 ($p = 0.02$, Figure 5.1A), 0.27 ($p = 0.003$, Figure 5.1B) and 0.44 ($p < 0.001$, Figure 5.1C), respectively. In contrast, $\delta^{13}C$ of fourth and fifth sections of wood core cellulose showed negative linear correlations with phloem carbon isotopes abundance ($\delta^{13}C$) with $r^2$ value of 0.14 ($p = 0.02$, Figure 5.1D) and 0.24 ($p = 0.005$, Figure 5.1E), respectively.

5.3.2. Basic wood density and cellulose $\delta^{13}C$

No significant variation in basic density of wood was observed across the radial direction of the growth ring (Figure 5.2) however, a gradual increase of mean value of $\delta^{13}C$ across the first
to fifth section of wood cellulose was identified (Figure 5.2). Cellulose δ^{13}C of the 1st section of wood significantly differed with that of the 4th and 5th section of wood (p values were 0.05 and 0.01 respectively).

![Figure 5.2](image)

**Figure 5.2.** Basic density of wood and cellulose δ^{13}C of corresponding outer (1st section of wood) to inner part of the tree trunk (5th section of wood) across the radial direction. Small letter shows significance difference (Bonferroni test, \(p \leq 0.05\)), the data shown as means ± s.e., \(n = 6\).

### 5.3.3. Intra-specific growth ring density and cellulose δ^{13}C

Positive correlations were observed between cellulose δ^{13}C and basic density of wood in the first and fourth sections across the radial direction, whereas negative correlations were observed in second, third and fourth sections of wood (Figure 5.3). The δ^{13}C of first and fifth sections of wood core cellulose were positively correlated to the basic density of wood with \(r^2\) value of 0.26 (\(p = 0.002\), Figure 5.3A) and 0.21 (\(p = 0.006\), Figure 5.3E), respectively.
Figure 5.3. Relationships between cellulose $\delta^{13}$C with basic density of wood across the radial direction, A and E showed positive correlation between cellulose $\delta^{13}$C and basic density of first and fourth sections of wood, B, C and D showed negative correlation between cellulose $\delta^{13}$C and basic density of second, third and fourth sections of wood. The solid line shows regression line.

The $\delta^{13}$C of second, third and fourth sections of wood core cellulose showed different relationships with wood density. The $\delta^{13}$C of second, third and fourth sections of wood core cellulose were negatively correlated to the basic density wood with $r^2$ value of 0.24 ($p = 0.001$, Figure 5.3B), 0.30 ($p = 0.001$, Figure 5.3C) and 0.19 ($p = 0.01$, Figure 5.3D) respectively.

In case of dry density, there were positive correlations observed between wood cellulose $\delta^{13}$C and dry density of concurrent wood sections. The $\delta^{13}$C of first, second, third, fourth and fifth section of wood core cellulose showed positive linear correlation with dry density of wood with $r^2$ value of 0.30 ($p = 0.001$, Figure 5.4A), 0.22 ($p = 0.007$, Figure 5.4B), 0.29 ($p = 0.002$, Figure 5.4C), 0.25 ($p = 0.005$, Figure 5.4D) and 0.32 ($p = 0.0007$, Figure 5.4E) respectively.
Figure 5.4. Relation between cellulose $\delta^{13}C$ with dry density of wood across the radial direction of the growth ring, A, B, C, D, and E showed positive correlation between cellulose $\delta^{13}C$ and dry density of first, second, third, fourth and fifth sections of wood respectively. The solid line shows regression line.

5.3.4. Nutrient effect on growth and cellulose $\delta^{13}C$

Nutrient availability showed a positive variation with dry volume of wood sections of 0.1 cm$^3$ green volume of wood across the radial direction of the growth ring. An increase in dry volume of wood growth was observed with the increasing rate of nitrogen fertiliser availability while nitrogen-phosphorous fertiliser did not show any distinct pattern in growth of dry wood volume. However, for both types of fertiliser applications, it was observed that there was an increase of dry wood volume while comparing with the nil control plot though the variation was not significant (Figure 5.5).
Figure 5.5. Nutrient effect on average dry volume growth (black bar) and corresponding cellulose $\delta^{13}C$ (dark grey line graph) of 5 sections of 1 cm wood across the radial direction of the growth ring. The data shown as means ± s.e., n = 6.

The $\delta^{13}C$ of wood cellulose from respective treatment plot were plotted against the growth of estimated stand volume under bark (ESVUB) of the respective plot. The $\delta^{13}C$ was found to be positively correlated with an average estimated stand volume under bark ($r^2 = 0.30$, $p = 0.001$, Figure 5.6).
Figure 5.6. Nutrient effect on average estimated volume under bark (ESVUB) and corresponding wood cellulose $\delta^{13}$C of trees grown in treatment plots. The solid line shows regression. Plots with different nutrient treatments are denoted by the symbols $\bullet$ = Nil control, $\nabla$ = 250 kg ha$^{-1}$ N, $\circ$ = 350 kg ha$^{-1}$ N, $\bullet$ = 450 kg ha$^{-1}$ N, $\square$ = 250 kg ha$^{-1}$ NP, $\blacksquare$ = 350 kg ha$^{-1}$ NP, $\blacktriangle$ = 450 kg ha$^{-1}$ NP.

5.3.5. Nutrient effect on carbon sequestration rate

Carbon sequestration rate (CSR) did not show any significant variation in response to the nutrient treatment. However, an albeit non-significant decreasing trend of CSR was observed when nitrogen fertiliser rate was increased. Combined nitrogen-phosphorous application did not show any significant changes in CSR (Figure 5.7).
5.4. Discussion

5.4.1. Phloem and wood cellulose $\delta^{13}C$

The relationship between phloem $\delta^{13}C$ and wood cellulose $\delta^{13}C$ varied in accordance with the inter-specific location of the cellulose within or along the radial direction of the growth ring. For *E. globulus*, Pate and Arthur (1998) predicted seasonal fluctuation in bulk phloem $\delta^{13}C$ would be reproduced in xylem laid down approximately a 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 the phloem into the cellulose, as would heterotrophic fractionation (see, for example, Smith and Merchant 2018). For the present study, wood cellulose $\delta^{13}C$ rather than bulk wood $\delta^{13}C$ was successfully used to determine such relations, similar to that of Loader et al. (2003). In the current study, it was observed that phloem sap $\delta^{13}C$ was positively correlated with wood cellulose $\delta^{13}C$ in the first three sections of wood core (see Figure 5.1). These contrasting directions of the correlation may indicate contrasting limitations on carboxylation, notably that
of diffusional (stomatal and mesophyll conductance to CO₂) and biochemical (nutrient and/or light limitations) origins.

The development of vascular tissues are very much sensitive to the availability of water (Kozlowski 1982) and it influences the mass of water transport. For example, in many tree species early wood (spring wood) has large vessels comprising more water content than the late wood (summer wood). Variation of δ¹³C has been found as large as 2-3% within the growth rings obtained from the same species growing at different microenvironments (Leavitt and Long 1989). Similarly, variations across the radial direction of the growth rings of δ¹³C in three types of *Eucalyptus* species has been observed (*E. diversicolor*, *E concinna*, and *E. phaenophylla*) grown in South Australia (Schulze et al. 2006). This study observed that at the onset of earlywood formation, a scope of δ¹³C values between -27‰ to -25.67‰. In the present study, cellulose δ¹³C across the five sections of wood shows variation that suggests that these segments progressed from late to early wood (Figure 5.2) driven largely by rainfall patterns increasing from summer to winter. Similarly, summer rainfall may contribute to late wood growth with previously preserved carbohydrates while winter rainfall could produce early wood (Schulze et al. 2006).

Measures of δ¹³C can be used as a tool to estimate water use efficiency in a rapid, cost effective and non-destructive manner compared to leaf gas exchange. δ¹³C is correlated with water use efficiency (Farquhar et al. 1989a; Farquhar et al. 1982), and under water limited environments can often be correlated with plant growth (Merchant et al. 2010). Trees may demonstrate high WUE through either low transpiration or high net photosynthesis (or both). Stomatal limitations determine isotope composition of plant tissues which have important implication for growth and survival in water limiting condition (Ehleringer 1993). The model of sensitivity of photosynthetic discrimination was described as a function of stomatal openness, expressed as the ratio of CO₂ inside and outside the leaf (Farquhar et al. 1982), while Pate and Arthur (1998)
explained the $\delta^{13}C$ variation in phloem sugars, corresponding with seasonal patterns of plant water stress. Here we step further to find the relation of $\delta^{13}C$ at phloem and tree ring wood cellulose level under different nutritional status of the soil. The correlation between phloem $\delta^{13}C$ and tree ring $\delta^{13}C$, as observed in this study, indicates that predictions of WUE based upon phloem $\delta^{13}C$ may be extrapolated to cellulose $\delta^{13}C$ to estimate the WUE over the life of the tree if inter- and intra-ring variation is considered.

5.4.2. Wood density and cellulose $\delta^{13}C$

Wood density has important implications for calculations of biomass and the carbon content of a tree (Yeboah et al. 2014). The observed relation between wood basic density and $\delta^{13}C$ of different sections of wood sample (from the direction of bark to pith) was inconsistence because a positive correlation detected in the first and fourth sections of wood whilst negative correlations were observed in the inner segments (second to fourth sections of wood, see Figure 5.3). However, for dry density, all relationships were positive (Figure 5.4). These results differ from results obtained by Macfarlane and Adams (1998) which showed that for drought stressed *E. globulus* basic density was not correlated to the $\delta^{13}C$ and that this poor relationship may be influenced by water availability. Water availability influences $\delta^{13}C$ of wood through changing stomatal conductance and CO$_2$ concentration in leaves (Farquhar et al. 1982) and influences wood density with photosynthates and auxin supply to cambium (Kozlowski 1982). Macfarlane and Adams (1998) 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. Whilst not conclusive to determine the functional relationship, our results support the notion that under conditions by which water is significantly limiting growth, $\delta^{13}C$ may offer a suitable surrogate measure of forest carbon sequestration.
Previous studies have characterised variation in $\delta^{13}$C within growth rings (see for example, Skomarkova et al. 2006), indicating significant variation not commensurate with water availability. In the present study, *E. globulus* tree growth was likely limited by a combination of water and nutrient deficiency not by other factors such as temperature. Also, under the conditions of our study, the relationship between wood density and its cellulose $\delta^{13}$C differed between the seasons. In the present study the basic density was positively correlated with $\delta^{13}$C in the first and fourth section of wood, suggesting this wood is grown in late summer or beginning of summer at the end and the inner part of the wood (second, third and fourth section of wood core) are grown in the wet season, for which the relationship was negative (see Figure 5.3). Previous research by Skomarkova et al. (2006) found that $\delta^{13}$C was negatively correlated to wood density in the wet season while in the dry season it was a positive correlation. The influence of inter-annual changes of water availability in wood $\delta^{13}$C was examined previously by several authors (Leavitt and Long 1989; Kagawa et al. 2003; Warren et al. 2001). The positive correlation between those parameters suggested accumulation of more carbon content reflected through enriched signals of $\delta^{13}$C. Combined, these results may assist industry applications to spatially and temporally integrate stand $\delta^{13}$C with carbon capture and wood growth independent of destructive whole plant biomass measurements. Predictive models may also be developed to assess future growth and C sequestration at the stand level as suggested by numerous authors (Graumlich et al. 1989; Yanai et al. 2000; Acker et al. 2002; Bond-Lamberty et al. 2002; Mund et al. 2002).

5.4.3. Nutrient effect on wood growth and its cellulose $\delta^{13}$C

In the present study, the average dry volume of wood sections across the radial direction of the growth ring was found to be not significant with respect to fertiliser treatments, suggesting that *E. globulus* dry volume accumulation is not only limited by nutrients, but there may be other micro environmental factor such was water availability, temperature and light. The decrease of
wood growth with decreasing nitrogen availability corresponded with an increasing trend of wood cellulose $\delta^{13}C$ (see Figure 5.5). However, a good correlation was observed in between estimated stand volume under bark and cellulose $\delta^{13}C$. Since nutrient input improves the capacity of a crop to absorb and transfer water and nutrients from soil, water intake by plants is significantly increased (Du et al. 1995; Shengxiu 1994). Water use efficiency (WUE) was found to be higher with sufficient N supply in agreement with previous studies (Ghashghaie and Saugier 1989) indicating that larger plants are using water resources thus increasing competition for a limited supply. Livingston et al. (1999) found a significant positive correlation between WUE and needle $\delta^{13}C$ and biomass growth in both fertilised and nitrogen stressed conditions. Combined, our results indicate that relationships between nutrient effect and water use efficiency is not straightforward and plants interactively control WUE by physiological and morphological process in complex manner (Brueck 2008).

5.4.4. Nutrient effect on carbon sequestration rate

Carbon sequestration rate of fast growing species such as *Eucalyptus* have significant potential in mitigating climate change effects through absorbing CO$_2$ from atmosphere, offsetting emissions from industrial sources and supplying wood for the paper industry. The yield of *Eucalyptus* can be 20 t ha$^{-1}$ yr$^{-1}$ if the soil and moisture conditions are optimal and appropriate fertilisers are used (Mosiej et al. 2012). The yield is also varies depending on species type, site condition, plant density, age and silvicultural management (de Moraes Goncalves et al. 2004; Lodhiyal 2014). Neumann et al. (2011) estimated that 11 year old *E. globulus* produce approximately 90 kg of dry biomass which is approximately 8.18 kg ha$^{-1}$ yr$^{-1}$. In the present study, the dry biomass accumulation was measured as 7.72 kg ha$^{-1}$ yr$^{-1}$ for the trees of 2.5 years. To calculate the biomass, the simple allometric equation suggested by Hobbs et al. (2016) for South Australia (SA) region was considered, without the complicated model that includes other
environmental and soil parameters due to lack of information available. However, results give some indication on the role of nutrients in plant carbon sequestration. Enhancement of growth by nutrient supply may not increase C sequestration due to changes in morphology (such as wood density). Therefore, it is on this basis that the rate of fertiliser may not influence CSR in E. globulus over such a short term period (2.5 years old). However, in long-term, nutrient application may induce substantial variation that warrants investigation.

5.5. Conclusions

Wood core analysis is a tool through which tree ring information (ring width, density, carbon isotopes abundance etc.) can be collected from a standing tree. Tree rings can be used to assess the trend of C-sequestration, historical growth and infer physiological processes involved in the formation of wood through patterns in $\delta^{13}$C. Under different seasons and nutrient treatments, this study sought to determine the relationship of wood density and $\delta^{13}$C of carbon obtained from intra-growth ring segments and metabolites obtained from the phloem stream. $\delta^{13}$C obtained from metabolites within the phloem sap correlated with $\delta^{13}$C obtained from cellulose with distinct intra-specific patterns observed within the growth rings. These parameters produced both positive and negative relationships indicating contrasting limitations (water or nutrient) to the growth of trees under field conditions. Additionally, we illustrate that $\delta^{13}$C of wood cellulose is influenced by both water (seasonal) and nutrient regimes and that relationships with wood density vary considerably, reflecting the interactive effects of water availability, nutrient availability on a background of seasonal variation in climatic conditions. Combined, these results illustrate that intra-ring variation in wood density and $\delta^{13}$C needs to be considered in the application of these tools in predicting historical patterns in forest productivity.
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Chapter VI: Effects of drought on carbon sequestration potential of tropical trees in Bangladesh

Abstract

Tropical forests play a crucial role in the mitigation of global climate change by absorbing CO$_2$ from the atmosphere, subsequent storage of carbon in their biomass and transfer to the soil. Assessing the effects of drought on carbon sequestration and uncertainties in measurement are therefore important for implementing the Reducing Emissions from Deforestation and Forest Degradation (REDD+) programs and carbon trading. The objective of the study was to understand how drought affects carbon sequestration potential in plantation species teak (*Tectona grandis*), hickory wattle (*Acacia mangium*), and red gum (*Eucalyptus camaldulensis*) growing in Bangladesh. Sampling was done at two different sites with contrasting annual precipitation. Site and species inter-specific variation in $\delta^{13}$C was observed, and higher (less negative) values were found at the low precipitation (dry) site compared to the high precipitation (wet) site indicating differences in water use efficiency (WUE). Trees at the dry site showed lower growth rate and higher wood (basic) density compared to the trees at wet site. The $\delta^{13}$C was positively correlated with wood density for all species in both sites suggesting that under water stress conditions, higher wood density is reflected with increasing $\delta^{13}$C. Trees at the dry site showed significantly lower carbon sequestration rate (CSR) than those of the wet site. Inter-specific species differences in CSR were observed. Considering the CSR and stress tolerance under water limited conditions, *E. camaldulensis* and *A. mangium* have higher potential for carbon sequestration especially in response to drought. These results provide insights in intra- and inter-specific CSR variations and confirm the importance of
considering site-specific radial variation of wood density to render more accurate accounting of carbon sequestration and storage.

6.1. Introduction

Increases in atmospheric CO₂ concentration is a central driver of climate change, leading to a substantial increase in temperature and to a shifted seasonal precipitation across the globe (Stocker et al. 2013). Tropical (including subtropical) forests are considered key components of the terrestrial carbon cycle and play a crucial role in regulating global climate by reducing CO₂ from the atmosphere and sequestrating it in different organs of plants (Beer et al. 2010; Malhi et al. 2009). In such kinds of ecosystems, carbon is mainly stored in living biomass (above- and below-ground) of standing trees (42%) and soils (44%), while a smaller amount is stored in litter (5%) and dead wood (8%) (Malhi et al. 2009; Ngo et al. 2013; Pan et al. 2011; Sierra et al. 2007). However, the stocks of sequestrated carbon in tropical forests may differ considerably both within and between the regions (Slik et al. 2010) due to varying biomass dynamics through growth, recruitment, mortality and climate (Chave et al. 2008; Chave et al. 2014; Djomo et al. 2011; Malhi et al. 2009; Malhi et al. 2004). Moreover, the process of quantification contains large uncertainties inhibiting our understanding of the potential for tropical forests in mitigating climate change (Feldpausch et al. 2014; Houghton 2005). Therefore, accurate estimation of carbon stocks and sequestration rate of forests is essential in Reducing Emissions from Deforestation and Forest Degradation (REDD+) mechanisms in order to establish reliable Forest Reference Emission Levels (FREL) and to estimate carbon stock changes for carbon trading under Kyoto Protocol.

Forest carbon stocks are not measured directly but derived mostly from living tree biomass which is typically estimated using allometric models (Chave et al. 2005; Chave et al. 2014). However, one of the important sources of uncertainty in biomass or carbon estimates lies in the
choice of a particular allometric model (Chave et al. 2008; Lewis et al. 2013; Nam et al. 2018; Wayson et al. 2015). Most of the allometric equations require input data on tree diameter at breast height, wood (basic) density, and tree height (Chave et al. 2005; Chave et al. 2014). However, field measurement of tree height is rather tedious compared to tree diameter because the observation of tree height is often affected by the complexity of canopy closure, topography and landforms (Feldpausch et al. 2012; Larjavaara and Muller-Landau 2013). Despite the importance of wood density, much less is known about its variation within individual trees, especially in tropical forests where within tree variation may be extreme apart from inter and/or intra-specific variations (Hietz et al. 2013; Williamson and Wiemann 2010). From earlier studies, it can be interpreted that wood density variations between and within trees are related to the radial growth of trees which depends on the activity of the cambium, age and seasonality in climatic conditions, such as drought stress. Even small differences in wood density can have important consequences on the total carbon estimation (Pretzsch et al. 2018). Therefore, understanding of wood density variation is crucial for estimating the potential magnitude of carbon sequestration.

Due to the effects of global climate change, the occurrence of droughts in tropical ecosystems is expected to drastically affect gas exchange and water use efficiency (WUE, the ratio of assimilation to transpiration) of trees that resulting growth variation in tree species (Ehleringer and Cerling 1995; Huang et al. 2017). Increased drought stress causes a reduction of the CO₂ assimilation rate, as well as a decrease of the stomatal conductance resulting in a lower ratio of the intercellular to atmospheric CO₂ concentration (Cᵢ/Cₐ) and consequently in a higher δ¹³C (Farquhar et al. 1982; Lin and da SL Sternberg 1992; Medina and Francisco 1997). Discrimination is linearly related to Cᵢ/Cₐ, which reflects the balance between the rate of inward CO₂ diffusion, mediated by stomatal conductance, and the rate of CO₂ assimilation in photosynthesis (Warren et al. 2001). Thus the δ¹³C signature of trees is widely used as a reliable
proxy for WUE or tree water stress (Kruse et al. 2012). So far, whether variations in $\delta^{13}C$ linked to drought are linked with declines in carbon sequestration and/or allocation to stem growth remains unclear (Jucker et al. 2017). Therefore, it is crucial to study how trees respond to droughts and to understand the impacts of climate change on carbon sequestration.

Even though making only a small contribution (0.36%) to global emissions (WRI 2017), Bangladesh is one of the most vulnerable countries (ranked sixth in the Global Climate Risk Index) in the world to climate change (Kreft et al. 2017). Having a substantial area of plantations and natural forests, Bangladesh is contributing in mitigating global climate change under the Clean Development Mechanism (CDM) which was emphasized in the Kyoto Protocol (UNFCCC 2004). Strong commitments to international communities and the recognized significance of forests in mitigating climate change have motivated government and scientific communities to study carbon sequestration by forests and the maintenance of forest resources under REDD+ programs in Bangladesh. However, studies are mainly focussed on carbon stock assessment using pan-tropical or few locally developed allometric models (Rahman et al. 2015; Khan et al. 2018; Mahmood et al. 2019; Majumder et al. 2019), rather incorporating the issues of uncertainties in estimation, annual sequestration rate and/or climate change. Therefore, the objective of the study was to understand how drought affects carbon sequestration potential in plantation species growing in Bangladesh. We thus hypothesized that under drought conditions, trees exhibit higher $\delta^{13}C$ which is largely driven by stomatal regulation as per Farquhar et al. (1989). This increase in $\delta^{13}C$ is coupled with a decline in tree growth and increase in wood density. On this background of increases wood density and reductions in volume, carbon sequestration rate (CSR) of trees under water limitation (dry site) and that such relationships differ among species (Chave et al. 2008; Nam et al. 2018). In this study, we examined the variation in carbon sequestration rate (CSR) at sites of contrasting
water availability by comparing trees in three plantation species at two sites with a significant precipitation difference (dry versus wet site).

6.2. Material and methods

6.2.1. Study area

Study sites were selected based on contrasting annual precipitation and accessibility to the managed plantation forests. The study sites were located in the north western (Tanore Upazilla, Rajshahi 24.58°N and 88.53°E) region and in the north eastern (Sylhet Sadar Upazilla, 24.95°N and 91.93°E) region of Bangladesh (Figure 6.1).

![Figure 6.1](image-url)

**Figure 6.1.** Study areas are marked by rectangle: (i) the left side arrow head shows the dry site located at Tanore upazilla in Rajshahi, and ii) the right side arrow head shows the wet site located at Sylhet Sadar upazilla in the district of Sylhet).
6.2.2. Climate

A monsoonal climate prevails at both sites where precipitation follows a unimodal distribution (Figure 6.2). However, the mean annual precipitation is significantly lower \( (p<0.01, \text{t-test}) \) in the north western region (Tanore, 1422 mm) compared to north eastern (Sylhet) region (4012 mm). Therefore, the study site of the north western region (Tanore) is considered a dry site and the north eastern region (Sylhet) is wet site.

![Figure 6.2. Mean annual precipitation (mm) and temperature (°C) of the study sites: (a) mean annual precipitation and mean temperature at the dry site (b) mean annual precipitation and mean temperature at the wet site since 1997-2017). The bar graph represents the precipitation and the line graph represents temperature.](image)

At the dry site, mean annual temperature is 25 °C with monthly maximum and minimum temperatures ranging from 40.8 °C to 6.3 °C, respectively. Conversely, at the wet site, mean temperature is 24 °C in the wet site, monthly maximum and minimum temperature is 35 °C and 9 °C, respectively.

6.2.3. Soil properties

The recent floodplains made up of piedmont alluvial plains, meander floodplains, basin area, tidal flood plains, estuarine floodplains and sandy beaches constitute 79% of the total land of
Bangladesh. Approximately 13% of this area in the northern and eastern hills are of tertiary formations, and 8% area in Madhupur and Barind tract are of Pleistocene terraces. These lands are formed by sedimentary geological formations of Tertiary and Quaternary ages (Morgan and McIntire 1959; Wadia 1957). The study site 1(Tanore, Rajshahi) is associated with Amnura soil series, which are developed in deeply weathered Madhupur clay, part of Barind tracts on summits and slopes of dissected areas (SRDI 2000). The study site 2 (Khadimnagar, Sylhet) is associated with Khadimnagar soil series, which consists of steep, well drained soils developed in moderately coarse textured, unconsolidated, folded, potentially tertiary sediments. They occur on moderately to very steep topography with scattered hills. Most of the land are used for forest and bamboo plantation, tea cultivation in piedmont valleys (SRDI 2008). The table 1 shows soil chemical properties of the two study sites.

Table 1. Soil properties and nutrient contents of the study sites used in the present study

<table>
<thead>
<tr>
<th>Soil Properties</th>
<th>Tanore, Rajshahi</th>
<th>Sylhet Sadar, Sylhet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texture</td>
<td>Silt loam to silt clay</td>
<td>Sandy loam</td>
</tr>
<tr>
<td>Ph</td>
<td>4.7-6</td>
<td>4.1-5.2</td>
</tr>
<tr>
<td>Organic Matter (%)</td>
<td>1.18</td>
<td>1.7</td>
</tr>
<tr>
<td>Nitrogen (%)</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Phosphorus (µg/g)*</td>
<td>5.65</td>
<td>4.34</td>
</tr>
<tr>
<td>Potassium (mEq/100g)</td>
<td>0.11</td>
<td>0.18</td>
</tr>
<tr>
<td>Sulphur (µg/g)</td>
<td>11.6</td>
<td>16.01</td>
</tr>
<tr>
<td>Zinc (µg/g)</td>
<td>1.13</td>
<td>0.92</td>
</tr>
<tr>
<td>Boron (µg/g)</td>
<td>0.47</td>
<td>0.23</td>
</tr>
<tr>
<td>Calcium (mEq/100g)</td>
<td>3.65</td>
<td>0.79</td>
</tr>
<tr>
<td>Magnesium (mEq/100g)</td>
<td>1.24</td>
<td>0.31</td>
</tr>
<tr>
<td>Copper (µg/g)</td>
<td>2.63</td>
<td>0.61</td>
</tr>
<tr>
<td>Iron (µg/g)</td>
<td>124.28</td>
<td>56.45</td>
</tr>
<tr>
<td>Manganese (µg/g)</td>
<td>1.12</td>
<td>45.38</td>
</tr>
</tbody>
</table>

Source: (SRDI 2000; SRDI 2008)

6.2.4. Silviculture and management

Plantations were established at a 2 x 2 m spacing. Establishment fertilizer was set as 3.5 m³ organic fertilizer and 20 kg Double Super Phosphate per thousand seedlings. Rotation lengths vary throughout the region from 10-20 years (BFD 2018).
6.2.5. Sample collection and preparation

Three widely used important plantation species, *Tectona grandis*, *Acacia mangium* and *Eucalyptus camaldulensis* were selected to study. These species are common in the plantations of both study sites. These species were 17-30 years old during field measurements and sampling and raised in monoculture plantations with seedlings using 2 x 2 m initial spacing. Tree height (m) was measured by using a clinometer (Sunnto PM5-360), and diameter at breast height (dbh) over bark (cm) was measured by using a diameter tape at breast height (1.37 m above ground level). Ten individual trees were randomly selected from each species for coring in each site. Wood cores were collected at breast height (1.3 m) of standing trees using a two-threaded increment borer of 5.15 mm diameter (Haglof, Sweden). Collected wood cores were immediately put in plastic pipe and wrapped with masking tape to prevent loss of evaporation.

6.2.6. Radial growth and wood density measurements

Radial growth of sampled tree was calculated as dbh/age of the tree. Wood (basic) density often varies radially from pith to bark due the transition from juvenile to mature wood (Zobel and Sprague 1998). To minimize radial effects, mean wood density was calculated for whole radius. Wood cores were separated into 5 segments of 1 cm length in the direction of bark to pith. Green volume of each sample was calculated by measuring the fresh wood sample with a slide calliper according to the following formula, $v_g = \pi r^2 h$, where $v_g$ = green volume (cm$^3$), $r$ = radius of the increment core section and $h$ = sample length (Chave et al. 2009). All samples were placed in the oven at 60 °C for 48 h and then oven dry weights were measured. Samples were then kept in the oven for another 12 h and their dry weights were measured. After another 12 h interval of drying the wood samples the constant dry weight of them was achieved. Density was calculated following the formula, $\rho = dw/v_g$, where $\rho$ = density of wood (g cm$^{-3}$), $dw$ = oven dry weight (g).
6.2.7. Extraction of cellulose and measurement of $\delta^{13}$C

Wood samples were taken from the bark side to 5 cm interior section of the stem for cellulose extraction to minimize variation due to the juvenility of trees (Zobel and Sprague 1998). A total of 60 wood samples (20 per species) were used for cellulose extraction from each site to measure $\delta^{13}$C following the method of (Brendel et al. 2000). In this study, the value of $\delta^{13}$C was measured for cellulose which is more stable than that of bulk wood $\delta^{13}$C, as cellulose has a more consistent structure and a single biosynthetic pathway (Park and Epstein 1961). Moreover, the bulk $\delta^{13}$C has an offset value by 0.5 to 2% depleted compared to cellulose (Borella et al. 1998; Cullen and MacFarlane 2005; Helle and Schleser 2003; Helle and Schleser 2004; Hietz et al. 2005; Loader et al. 2003; Macfarlane et al. 1999; Tans and Mook 1980).

Wood samples (1 cm) were finely ground and 2-3 mg weighed into 1.50 ml polypropylene tubes. Then, 120 µL acetic acid (80%, reagent grade) was added to each tube followed by 12 µL nitric acid (69%, reagent grade). Tubes were capped securely and inserted into heating blocks at 120 °C for 30 min and agitated every 5 min. The capped tubes were then allowed to cool after which 400 µL of ethanol was added, agitated and then centrifuged for 5 min at 10000 rpm. The resulting supernatant was then removed carefully and discarded. In the tube, 300 µL distilled deionised water (DDW) was mixed with the remaining pellet and again capped, agitated and centrifuged for 5 min at 10,000 rpm (Eppendorf, Centrifuge 5424, Hamburg, Germany). 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 10000 rpm. Again, the supernatant was removed carefully and discarded. To get cleaner cellulose, steps using deionised water and ethanol were repeated for another 2 times and then again 150 µL acetone was used to separate the cellulose from the supernatant. The supernatant was then removed and samples placed at 45 °C for 24 h. The final product appeared as white, fine pellet of cellulose. Subsamples of cellulose (0.35 mg) were transferred into tin capsules and analysed.
for $\delta^{13}$C (‰) using Isotope Ratio Mass Spectrometry (IRMS: Thermo Fisher Scientific, Bremen, Germany) where the oxidation reactor was set at 1000 $^\circ$C.

6.2.8. Calculation of carbon sequestration rate

Aboveground biomass was estimated using a pan-tropical allometric equation that relates diameter (D), wood density ($\rho$) and an environmental stress factor ($E$) to the weight of above ground biomass, AGB (Chave et al. 2014). Due to a lack of species- or site-specific model in the study areas, we therefore used this pan-tropical model because of its robustness in estimation of AGB in the tropics:

$$(\text{AGB}_{est}) = \exp[-1.803 - 0.976E + 0.976 \ln(\rho) + 2.673 \ln(D) - 0.0299(\ln(D))^2] \quad \text{......... (Eqn 6.1)}$$

Where, $\text{AGB}_{est}$ = estimated above ground biomass, kg, $E$ = environmental stress value, calculated as,

$$E = (0.178 \times \text{TS} - 0.938 \times \text{CWD} - 6.61 \times \text{PS}) \times 10^{-3} \quad \text{................................. (Eqn 6.2)}$$

$E$ was calculated as 0.5754873 (dry site) and 0.2298245 (wet site) respectively (see for example: Chave et al. 2014), where TS = temperature seasonality, amount of time a plant is exposed to stressful temperature, CWD = climate water deficit, a negative value which increases with annual water stress, PS = precipitation seasonality, $\rho$ = wood density (oven dry weight/green volume, g cm$^{-3}$) and D = diameter at breast height of tree in centimeters. Root biomass was estimated as 15% of $\text{AGB}_{est}$ (MacDicken 1997) and biomass carbon content was 50% of the total biomass weight (WorldBank 1998). Therefore,

Total biomass of tree = $[\text{AGB}_{est} + (0.15 \times \text{AGB}_{est})] \quad \text{................................. (Eqn 6.3)}$

Total biomass carbon content = Total biomass x 0.50 \quad \text{................................. (Eqn 6.4)}

Hence, annual carbon sequestration rate (CSR) was calculated as,

$$\text{CSR} = \frac{\text{Total biomass carbon}}{\text{Basal area of tree (m}^2\text{)} / \text{Tree age (year)}} \quad \text{................................. (Eqn 6.5)}$$
Carbon sequestration rate (CSR) is expressed as kg m$^{-2}$ yr$^{-1}$, where biomass carbon content is annually measured with respect to the corresponding basal area of tree. The basal area of tree was measured as $\pi \times (D^2/4 \times 10000)$ in m$^2$, where D is the diameter at breast height of tree in centimeters.

6.2.9. Statistical analysis

Data was analysed using Sigmaplot (Version 12.5, Systat Software, Inc, San Jose, CA, USA). Variation between the sites was explored using t-tests. Variations among the species in both sites were analysed using one way ANOVA followed by a post-hoc (Bonferroni) test and simple linear regression analysis was carried out between the parameters.

6.3. Results

6.3.1. Variation of $\delta^{13}C$, growth rate and wood density

Cellulose $\delta^{13}C$ significantly varied between sites ($p<0.05$). T. grandis and trees at the dry site showed higher (less negative) values than the wet site (Fig. 6.3A). A. mangium and E. camaldulensis showed no significant variation of cellulose $\delta^{13}C$ between sites (Figure 6.3A). Cellulose $\delta^{13}C$ ranged from -25.58 to -28.12% at both sites (Table 6.1), which are within the range observed in C3 plants (-32 to -20%) (Ehleringer 1993). Inter-specific variation of cellulose $\delta^{13}C$ was also observed among the species at both sites. In the both study sites, cellulose $\delta^{13}C$ of T. grandis showed a significantly higher mean value of cellulose $\delta^{13}C$ than that of other species ($p<0.05$). A. mangium showed lower (more negative) mean values of cellulose $\delta^{13}C$ (-24.76%, dry and -26.28%, wet site) followed by E. camaldulensis (-24.62% at dry and -25.95% at wet site). Differences in cellulose $\delta^{13}C$ between species was significant ($p<0.05$).
Figure 6.3. A, wood cellulose $\delta^{13}C$ of *T. grandis*, *A. mangium* and *E. camaldulensis* showed variation between the study sites, B, radial growth variation between the study sites, C, density variation between the study sites. Small letters show the significant variation ($p \leq 0.05$) between the study sites and among the species, significance level was tested by t test for site difference and repeated ANOVA with post-hoc (Bonferroni) for inter-specific variation. Values are means ± s.e., n = 10.
At the dry site, T. grandis showed significantly lower growth rate than the wet site ($p<0.05$). Similarly, A. mangium E. camaldulensis had slightly lower growth rate at dry site, however the difference was not significant (Figure 6.3B). The mean radial growth rate varied from 0.45 (at the dry site) to 0.57 (at the wet site) cm year$^{-1}$ in T. grandis. The radial growth in A. mangium was 0.49 cm year$^{-1}$ at dry site whereas at the wet site it was 0.67 cm year$^{-1}$. The mean radial growth in E. camaldulensis was 0.59 cm year$^{-1}$ at dry site and 0.60 cm year$^{-1}$at the wet site (Table 6.1).

**Table 6.1.** Mean parameters of the species in both study sites (Rajshahi and Sylhet)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tectona grandis</th>
<th>Acacia mangium</th>
<th>Eucalyptus camaldulensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dry site (mean±se)</td>
<td>wet site (mean±se)</td>
<td>dry site (mean±se)</td>
</tr>
<tr>
<td>DBH (cm)</td>
<td>27.0 ± 2.8</td>
<td>18.3 ± 0.8</td>
<td>16.4 ± 1.2</td>
</tr>
<tr>
<td>Height (m)</td>
<td>17.1 ± 0.3</td>
<td>16.8 ± 0.2</td>
<td>9.4 ± 0.2</td>
</tr>
<tr>
<td>$\delta^{13}$C</td>
<td>(-25.9 ± 0.3)</td>
<td>(-26.5 ± 0.2)</td>
<td>(-28.9 ± 0.2)</td>
</tr>
<tr>
<td>Radial growth (cm year$^{-1}$)</td>
<td>0.45 ± 0.09 (0.29 - 0.73)*</td>
<td>0.57 ± 0.05 (0.44 - 0.70)*</td>
<td>0.49 ± 0.03 (0.35 - 0.76)*</td>
</tr>
<tr>
<td>Wood density (g cm$^{-3}$)</td>
<td>0.75 ± 0.02 (0.69 - 0.97)*</td>
<td>0.66 ± 0.02 (0.49 - 0.81)*</td>
<td>0.70 ± 0.02 (0.58 - 0.80)*</td>
</tr>
<tr>
<td>CSR (kg year$^{-1}$ m$^{-2}$)</td>
<td>122.16 ± 7.73 (96.63 - 181.93)*</td>
<td>153.37 ± 8.09 (121.18 - 210.91)*</td>
<td>180.90 ± 11.51 (141.21 - 233.74)*</td>
</tr>
</tbody>
</table>

$\delta^{13}$C, carbon isotope ratio; CSR, carbon sequestration rate; se., standard error, * indicates the range value.

Mean wood density of the studied species was significantly higher at the dry site than the wet site for each species ($p<0.01$). Inter-specific variation of wood density was also observed at both sites. Wood density in A. mangium was significantly lower than T. grandis and E. camaldulensis in both sites (Figure 6.3C). However, the variation between T. grandis and E. camaldulensis was not significant. Mean wood density was 0.75 g cm$^{-3}$ at the dry site and 0.66 g cm$^{-3}$ at the wet site in T. grandis (Table 6.1). The wood density in A. mangium was 0.70 g cm$^{-3}$ at dry site whereas at the wet site it was 0.60 g cm$^{-3}$. The mean wood density in E. camaldulensis was 0.83 g cm$^{-3}$ at dry site and 0.67 g cm$^{-3}$ was at the wet site. The variation in wood density reported in this study was within the range of global wood density (ca. 0.30 - 1.00 g cm$^{-3}$) variation (Chave et al. 2009).
6.3.2. *Relationship between cellulose δ¹³C and wood density*

Wood cellulose δ¹³C showed a positive relationship with wood density for all studied species (Figure 6.4). The mean wood densities of *T. grandis* showed a positive relationship with mean cellulose δ¹³C at both sites ($r^2 = 0.24$, $p<0.05$, dry site, and $r^2 = 0.17$, $p>0.05$, wet site respectively).

![Figure 6.4. Relationship between cellulose δ¹³C and wood density. A, B show dry and wet site relations between cellulose δ¹³C and wood density for *T. grandis*; C, D show dry and wet site relations between cellulose δ¹³C and wood density for *A. mangium* and E, F show dry and wet site relations between cellulose δ¹³C and wood density for *E. camaldulensis*. The solid line shows regression and the symbol ● denotes for dry site and ■ denotes for wet site relation between the parameter.](image-url)
Conversely, *A. mangium* showed a moderate positive relationship with cellulose $\delta^{13}C$ at the wet site ($r^2 = 0.24$, $p<0.05$), compared to the dry site ($r^2= 0.32$, $p<0.05$). Similarly, *E. camaldulensis* showed a stronger positive relationship with cellulose $\delta^{13}C$ at the dry site ($r^2= 0.59$, $p<0.05$) than the wet site ($r^2 = 0.18$, $p>0.05$).

### 6.3.3. Variation in carbon sequestration rate

All three species showed significant variation in carbon sequestration rate (CSR) between the sites ($p<0.01$), and trees at the dry site had lower CSR than the wet site (Figure 6.5A). The mean CSR of *T. grandis* ranged from 122.16 to 222.59 kg yr$^{-1}$ m$^{-2}$ at dry and wet site, respectively (Table 6.1). In case of *A. mangium*, the CSR was 153.37 and 180.90 kg yr$^{-1}$ m$^{-2}$ at dry and wet site, respectively. The mean CSR in *E. camaldulensis* was 165.16 and 248.64 kg yr$^{-1}$ m$^{-2}$ at dry and wet site, respectively.
Figure 6.5. Intra and inter-species specific variation carbon sequestration rate of *T. grandis*, *A. mangium* and *E. camaldulensis*. (A) Carbon sequestration rate calculated with the calculated wood density in this study and (B) Carbon sequestration rate calculated with the Global Wood Density (GWD) published wood density (https://datadryad.org/handle/10255/dryad.235), mean wood density of *T. grandis* 0.61, *A. mangium* 0.51 and *E. camaldulensis* 0.72, small letters shows the significant variation (*p*≤0.05) of CSR between the sites and among the species, significance level was tested by t test for the site difference and repeated ANOVA with post-hoc (Bonferroni) for inter-specific variation. Values are means ± s.e., n = 10.

Inter-specific variation of CSR was also observed however the pattern of variation was different in two sites (Figure 6.5A). The CSR in *T. grandis* was significantly lower than other species at dry site (Figure 6.5A). Alternatively, *A. mangium* showed significantly lower CSR
comparing to other species at the wet site. Using wood density figures obtained from the global database (Chave et al. 2009), the CSR variation was significant among the species as well as for site differences \((p<0.01, \text{ Figure 6.5B})\). However, wood density data from the global database underestimates the CSR (10 to 30\%) in this study, except for \textit{E. camaldulensis} at the wet site.

6.4. Discussion

6.4.1. Variation in \(\delta^{13}C\), tree growth and wood density

Tree radial growth is often strongly influenced by precipitation across a range of tree species. Species grown in the drier environment showed lower growth corresponding to higher \(\delta^{13}C\) values than those of the wet site. Whilst both biochemical and diffusional fractionation events (see Farquhar et al. 1989) are undoubtedly influencing the abundance of \(^{13}C\) as it moves from the atmosphere to incorporation into plant cellulose, it is diffusional fractionation events that dominate due to water limitation. Nevertheless, the higher \(\delta^{13}C\) of wood in trees located at the dry site suggests that, irrespective of the functional mechanisms governing these patterns, \(\delta^{13}C\) signature of wood may potentially be used as an indicator of growth for the studied species. The inferred influence of water availability on \(\delta^{13}C\) of wood indicates differences in water use efficiency (WUE) in the growing trees (Gebrekirstos et al. 2011; Rahman et al. 2019). As expected, trees at the dry site showed lower growth rate than those of the wet site in each species (Fig. 6.3B), possibly due to lower stomatal conductance and carbon gain which reduces cell turgor at the dry site (Tyree 2003; Zweifel et al. 2007). During water limitation, stomatal aperture will be reduced hence the \(C_i/C_a\) ratio is lowered as \(CO_2\) supply through the stomata is limited (Farquhar et al. 1989). The significantly higher value of cellulose \(\delta^{13}C\) in \textit{T. grandis} compared to the two other species at both locations may be explained by higher stomatal conductance common among deciduous species. In general, evergreen leaves tend to have
lower stomatal conductance than deciduous leaves (Sobrado 1986; Sobrado 1991) presumably reflecting a more profligate use of water by deciduous species during the growing season. Conversely, the lack of significant difference in δ¹³C values of the remaining two evergreen species (A. mangium and E. camaldulensis) between the sites may be considered as an adaptive strategy in response to drought. Furthermore, from a physiological perspective, low turgor pressure in the cambium may reduce radial growth of trees growing in arid areas (Steppe and Lemeur 2007).

The mean density of wood in the studied species was significantly (p<0.01) higher at the dry site in each species (Figure 6.3C). Positive relationships between δ¹³C and wood density in the studied tree species (Figure 6.4), suggest that the water stress condition (reflected by increasing δ¹³C) may be reflecting by higher wood density. However, relationships are mostly weak and indicate that trees might be influenced by different water use strategies during cell development. It is also suggested that δ¹³C of wood can indicate changes in the nature and duration of cambial activity which may result in less production of dense wood (Macfarlane and Adams 1998). Water deficit regulates δ¹³C of wood mostly by altering stomatal conductance and the concentration of CO₂ in leaves (Farquhar et al. 1982), and influences wood density by increasing photosynthates and auxins in the cambium (Kozlowski 1982). Stomatal conductance is however more sensitive to water deficits than in photosynthesis (Teskey et al. 1986). The low precipitation site elicited higher wood density in all species (Figure 6.3C), which is consistent with the previous studies on other species (Chave et al. 2006; Onoda et al. 2010; Pickup et al. 2005; Swenson and Enquist 2007). The general notion is that lower wood density at the wet site is due to higher volumetric growth rates for a given amount of carbon assimilation (Figure 6.3C). It is also noted that low density woody tissue has higher hydraulic conductivity and thus capacity to supply more water to a greater photosynthetic capacity in leaves than trees with high wood density (Chave et al. 2009; Keeling et al. 2008). Although
one explanation for this is that trees with higher growth at the wet site might have wider vessels than trees with lower growth at the dry site, the evidence for an inverse relationship between wood density and vessel size is reported in the earlier studies (Pfautsch et al. 2016; Preston et al. 2006). However, increasing vessel size is counteracted by decreasing vessel frequency so that the vessel fraction does not significantly change (Zanne et al. 2010) and wood density may be unaffected.

Apart from intra- and inter-specific disparity, wood density varied along the radius within the tree in each case. The radial variation in wood density was quite similar in the studied species in both sites where it was lower near the pith side compared to the rest of the radius. This type of radial variation might be due to changes in juvenile (inner) to mature (outer) wood, where transition is attained after certain age of tree (Chowdhury et al. 2013; Zobel and Buitjeenen 1989). The juvenile wood is more variable with density than the mature wood which could be due to wood anatomical variations (Wiemann and Williamson 2007). Due to the presence of considerable within tree (radial gradient), intra-specific (between sites) and inter-specific variations, using single-mean data of wood density might be erroneous in biomass or carbon estimations (Hietz et al. 2013; Nock et al. 2009). Thus, future research should be directed to determine wood density considering whole tree variations which are likely to be beneficial for both the accuracy and precision of biomass or carbon quantification.

6.4.2. Species-specific variation of carbon sequestration potential

Trees at the dry site showed significantly lower carbon sequestration rate (CSR, kg m$^{-2}$ yr$^{-1}$) than the wet site in each species (Fig. 6.5A). At the dry site, the growth rate was lower and wood density was higher than those of the wet site (Figure 6.3B & C). The effects of wood density might be mirrored by slow growth rate of trees and resulting lower CSR at the dry site. Similarly, Hietz et al. (2017) suggested that biomass increment significantly correlated with
diameter increment of trees rather than wood density in the Panamanian rainforest. This indicates that there is a weak relationship between wood density and tree growth ($r^2 = 0.004$, $p > 0.05$), which is contrary to what has been found at the inter-specific level, where, in general, growth rates relate negatively to wood density (Chave et al. 2009; Fajardo 2018; Hietz et al. 2013; Van Gelder et al. 2006). In this study, even though the relationship was not significant with radial growth, wood density still has positive influences on biomass estimation. For example, using wood density data from global repositories (Chave et al. 2009), while the estimation model and other variables are constant, the CSR were significantly lower in each case (Fig. 6.5B), except *E. camaldulensis* at the wet site. Similarly, the importance of wood density variation on biomass studies was also highlighted in the earlier studies (Chave et al. 2008; Kairo et al. 2009; Komiyama et al. 2005; Njana et al. 2016).

Similar to forests across the globe, models for biomass or carbon estimates for Bangladesh forests typically include a measure of tree size (i.e., dbh and/or height) and mean wood density (Majumder et al. 2019). Here, emphasis is also given to model selection and field measurements rather than wood density, and values of wood density are mainly used from literatures or global databases, except few studies (Hossain et al. 2015; Mahmood et al. 2019), where site-specific wood density was used but not considering the whole radius. However, using wood density of single or few segments of wood is somewhat different than the mean radius value because wood density varies from pith to bark in the same tree (Nock et al. 2009; Zobel and Buitjheen 1989). Therefore, this study is suggesting that ignoring site-specific radial variation of wood density can propagate significant errors in carbon estimation. Accurate wood density data could become more crucial in the coming age because more reliable tree volume assessment methods using remote sensing techniques are being developed, such as airborne laser scanning or digital stereo imagery (Goodbody et al. 2019; Vastaranta et al. 2013).
Precipitation is one of the most important climatic parameters as it influences growth, wood properties such as wood density and carbon sequestration rate. The trend of global climate change increases the surface temperature and modifies the magnitude and frequency of seasonal precipitation (Stocker et al. 2013). The declining precipitation and/or increasing of temperature could lead to a decline forest biomass in tropical forests (Saatchi et al. 2007). Therefore, the variations in carbon sequestration depend critically on site-specific changes in tree biomass due to environmental changes via the relative contribution of tree growth and wood density. *T. grandis* showed significantly lower CSR at dry site compared to *A. mangium* and *E. camaldulensis*. On the other hand, *A. mangium* showed significantly lower CSR comparing to other species at wet site. According to the present study, species containing high wood density do not necessarily have a high CSR. However, species showing higher growth rate may be able to sequester more carbon than species with lower growth rate because of changes in wood density. These results indicate that under the precipitation scenarios experienced here, water limitation increases the $\delta^{13}C$ in wood that decreases the water use efficiency in trees and consequently reduce growth rate. It is suggested that under drought conditions, trees experience carbon starvation due to stomatal closure (McDowell et al. 2008), and therefore reduce the tree growth. Specifically, water deficit appears to constrain tree biomass by imposing a limitation the biomass of individual trees via its effect on embolism and hydraulic conductance (Stegen et al. 2011). Therefore, environmental (hydrological) variations may affect the CSR of tree species even within a small geographic area as stated in an earlier study (Montagnini and Porras 1998). Considering the CSR and stress tolerance under water limited conditions, (for example species with low $\delta^{13}C$ values indicating higher water use efficiency), is useful in species selection to enhance carbon sequestration in response to the effects of climate change.
6.5. Conclusion

Among the studied tree species, the cellulose $\delta^{13}C$ were strongly influenced by water limitations and trees at the dry site showed higher $\delta^{13}C$ values. The results suggest that $\delta^{13}C$ in wood can be useful in estimating changes in water use efficiency of trees and hence in screening drought tolerant tree species. At the dry site, the growth rate was lower and wood density was higher than those of the wet site. These results combined suggest that the $\delta^{13}C$ of wood cellulose may indicate variation in wood density in the studied species irrespective of the mechanistic link between the two. Substantial intra- and inter-specific difference in wood density was found and diverged substantially from values obtained from a common global database. This suggests not only the preferential use of species and site-specific values for enhancing CSR, but that estimations of CSR at the landscape scale must consider site-specific variation for precise biomass or carbon estimation. Drought significantly reduced the CSR at the dry site in each species, and inter-specific variations revealed that *E. camaldulensis* followed by *A. mangium* have higher potential for carbon sequestration under water stress conditions. Besides their potential to increase accuracy in carbon accounting, results of this study help to direct future management decisions to select site-specific species with potential for enhanced carbon sequestration. Future research should be directed at improving our understanding of the variability of carbon sequestration rate considering more diverse site conditions including nutrients, tree age, radial gradients of wood density and carbon quantification to provide improved quantitative data to support REDD+ and CDM programs.
References


Chapter VII: General discussion and future directions

7.1. General discussion

Determining the rate of forest growth and estimating carbon carrying capacity of a forest ecosystem is crucial as forest play major roles in climate change mitigation through assimilation of carbon from the atmosphere. Forest growth is commonly limited by nutrients and water, hence to understand their nutritional and water status is important to predict forest growth. Estimation of the carbon content of a tree is generally calculated from the total biomass (sum of above and below ground) multiplied by 0.50 as a proportion of carbon that constitutes the dry weight. However, the carbon content can be vary intra-specifically (e.g. carbon content of leave, stem, wood vary) or inter-specifically (e.g. Acacia and Eucalyptus differ in carbon accumulation) and also types of vegetation (e.g. herbaceous to woody species) or even the same species grown in different climatic condition (see details Ma et al. 2018). Accumulation of tree biomass is measured using allometric equations applied to specific forest types across a range of climatic zones.

To calculate the potential carbon carrying capacity of a forest, it is important to know the limiting factors forest growth such as nutrient or water. Such information will assist in developing more realistic predictions of future sequestration potential. The availability of water, nutrient and light is important for plant biomass production because CO₂ sequestration is driven by those factors (Körner 2015). Many tools exist to indicate the nutritional status of a forest based on both aboveground and belowground analysis. In many cases, these approaches are laborious, time consuming and costly. Throughout this thesis, a set of novel techniques are characterised based on phloem sap analysis to provide time and spatially integrated measures of plant physiological status and carbon sequestration.
This work builds upon previous characterisations of phloem sap analysis, but illustrates several novel aspects and confirms these patterns under field conditions. The phloem is the central conduit that determine carbon distribution among plant organs. It can be used as diagnostic tools to determine plant nutrition and water status (Chapters II – V and Merchant et al. 2010). Phloem analysis is a non-destructive process which has the great potential to assess the whole plant nutritional status (Peuke 2009). In addition, several authors (Merchant et al. 2010; Merchant et al. 2011; Pate and Arthur 1998; Tausz et al. 2008)) have shown that that phloem δ¹³C can be used as predicting tree water status.

This work is built upon by investigating the response of phloem sap properties to assess nutritional status under both field and glasshouse conditions, as well as its interaction with water status. This new information is used to address recognised gaps (see Chapter 1) in the calculation and inference of limitations to carbon stock calculations. In particular, the incorporation of percentage carbon and wood density into carbon stock calculations are made and placed into the context of existing models of Chave et al. (2014) and Hobbs et al. (2016).

This thesis began with a review of existing literature to outline existing methods of determining plant nutrition and water status. The limitations of those methods are outlined, then methods to improve existing tools are suggested. In addition, a critical analysis of existing tools is presented to estimate above and below ground biomass estimation to outline gaps and their limitations and suggest improvement to more accurately predict the carbon stock in a forest ecosystem.

In the first experimental chapter, a field experiment on a trial plantation of *Eucalyptus globulus* in Mount Gambier, South Australia was conducted to determine if nutrient availability influenced the components of phloem sap. The trial plantation was built with different rate of nitrogen, nitrogen-phosphorous and nil control treatment. Phloem sap was collected from the growing and non-growing season and it was observed that total amino acid concentrations of
phloem sap significantly varied in between seasons as well as nutrient availability imparted by the treatments. The amino acid, sucrose concentration and $\delta^{13}C$ was significantly correlated with the growth of the tree during the growing season, however sucrose concentration and $\delta^{13}C$ were found to be negatively correlated to the growth in the non-growing season. Most interestingly, patterns in amino acid abundance in the phloem were influenced by an interaction with P supply indicating N and P status is tightly linked. Overall, this illustrated that phloem sap may be used as an indicator of tree nutritional status, but must also be interpreted on a background of seasonal variation and interactive effects of different nutrient availabilities.

To investigate these relationships further, a glass house experiment was carried out where *E. globulus* seedlings were treated with three levels of nutrient (100% NPK fertiliser, 50% NPK fertiliser and 0% NPK). Here it was observed that leaf and phloem amino acids concentrations significantly varied with response to the rate of nutrient availability. Again, close correlations were found between treatments and both molecular and isotopic phloem sap constituents. Phloem amino acids were also found to be positively correlated with collar diameter growth of seedlings. Phloem and leaf carbohydrates were positively correlated with seedlings biomass growth and $\delta^{13}C$ of phloem exudates were also found to be positive correlated with biomass growth. These results indicate that phloem sap analysis is a valid diagnostic tool to assess plant growth performance where nutrient and water are limiting factors of growth.

In order to develop these tools specifically towards predictions of growth and thus carbon carrying capacity of a forest, this research expanded the work to encompass relationships with dendrochronological patterns. One field experiment was conducted in the same trail plot of *E. globulus* grown under different rates of nutrient availability. From the study site, stem core and other growth data were collected. Wood cellulose was isolated and then measured $\delta^{13}C$. An increase pattern of $\delta^{13}C$ was observed from the bark side wood section to the interior sections.
of wood. Cellulose $\delta^{13}C$ displayed patterns that were interpretable in the context of seasonal wood formation and relationships between $\delta^{13}C$, wood density and carbon content were used to calculate carbon sequestration using an existing model (Hobbs et al. 2016) for South Australia applicable to the geographic area under investigation. Here it was observed that nutrient availability does not show any significant variation of carbon sequestration in this environment.

To further investigate the effect of drought/water availability on carbon sequestration rate with broadening the scope of study in different geographical range, another experiment was conducted in the two types of forests in Bangladesh (tropical zone) through utilising the tools developed to determine the carbon sequestration rate of a diverse range of tree species. The studied species were teak ($Tectona grandis$), hickory wattle ($Acacia mangium$), and red gum ($Eucalyptus camaldulensis$). A significant intra- and inter-specific difference in wood density which diverge from values in global databases suggesting the preferential use of species and site-specific values for precise biomass or carbon estimation. Another observation of this study was that drought significantly reduced the carbon sequestration rate in each species, and there was also inter-specific variations. Besides their potential to increase accuracy in carbon accounting, results of this study will help to direct future management decisions to select site-specific species suitable for carbon sequestration.

### 7.2. Future directions

The development of new tools to monitor forest growth, especially to understand the nutritional and water status of forests presents many avenues for further research. In particular, further investigations are suggested to elucidate the interactive relationships between nutrients and their effect on phloem sap constituents to widen the scope of using phloem sap analysis for monitoring forest growth. It is clear from the work presented in this thesis, that interactive
effects are at play amongst different nutrients and compounds, patterns that are not characterised but likely influential over growth.

Similarly, many questions remain over our capacity to encompass spatial and temporal variation in carbon exchange from both individual trees and the forest estate. The relationship between rainfall, temperature and $\delta^{13}C$ in wood cellulose should be investigated at a mechanistic level to better understand predict tree growth and its interaction with site-specific environmental parameters. Such investigations will enhance our monitoring and predictive tools in forest growth and carbon sequestration. A further avenue for future investigations should be conducted to understand the carbon carrying capacity of tree species grown in a heterogeneous forest with the interactive effect of nutrient and water availability to more accurately predict the carbon stock in a specific forest ecosystem. Added to this is a clear necessity to incorporate seasonal carbon sequestration rates into the modelling of forest carbon sequestration rate (CSR) to enhance our estimations of global carbon exchange.

References


