Functional analysis of extrafloral nectaries and nectar of Australian native wild cottons

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Declaration

I certify that the material of this thesis has not been previously submitted as part of the requirements for a higher degree to any other university or institution.

This thesis contains no material previously published or written by any other person. I certify that all information sources and literature used are indicated in the thesis.

I wish to acknowledge the following assistance with the research detailed in this report:

- The high performance liquid chromatography analysis of sugars was conducted by the Australian Proteome Analysis Facility supported under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS);
- Aaron Phillips assisted with extrafloral nectar collection and the ^{14}C labelling experiment;
- Elisse Sutton assisted with the processing of ^{14}C labelled samples; and
- Dr Brian Atwell and Dr Lesley Hughes, my supervisors, assisted with method development and editing my drafts.

All other research described in this report is my own original work.

Belinda Fabian

Note to examiners

This thesis is written in the form of two journal articles for Plant, Cell & Environment. The majority of the author guidelines (Appendix III) have been followed, except for minor deviations detailed here and where the guidelines clash with Macquarie University thesis formatting requirements. All figures and tables have been presented at the appropriate places in the text to enhance readability. References would normally be presented at the end of each journal article, but are presented here in one combined list at the conclusion of both journal articles to reduce double entries. Some data have been used in both articles as they are needed for different comparisons.

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Abbreviations

aCO_2	Ambient carbon dioxide
CO_2	Carbon dioxide
DPM	Disintegrations per minute
eCO_2	Elevated carbon dioxide
EDTA	Ethylenediaminetetra-acetic acid disodium salt
EF	Extrafloral
FW	Leaf fresh weight
HPLC	High performance liquid chromatography
IKI	Iodine-Potassium Iodide
PAR	Photosynthetically active radiation
PBS	Phosphate buffered saline
RO	Reverse osmosis
RT	Room temperature
SE	Standard error

General Abstract

Extrafloral (EF) nectar is the foundation of many ant-plant interactions worldwide. EF nectar is a resource that encourages ant presence, in turn resulting in protection for the plant against herbivores. There is a paucity of research on this relationship and the plant structures that underpin it in Australian species. An examination of the morphology and anatomy of EF nectaries of four Australian cottons (*Gossypium* spp.) revealed a suberised layer in the nectaries which indicates solutes must travel through the symplasm. $^{14}CO_2$ labelling showed the studied leaves were carbon sources and the EF nectar carbon cost was 1% of net photosynthates.

Any environmental changes that affect EF nectar could have flow-on effects for ecological communities, for example rising atmospheric carbon dioxide (CO_2) levels. This study tested the response of EF nectar to elevated CO_2; the first investigation of elevated CO_2 impacts on any EF nectar system. The total volume and composition of EF nectar did not change, but there was evidence of accelerated plant development and a change in EF nectar allocation within plants. Developmental changes due to elevated CO_2 could affect the timing of EF nectar production which could have flow-on effects to ant mutualists and the defence of plants.

Chapter 1

Extrafloral nectary anatomy and carbon metabolism of Australian native wild cottons (*Gossypium* spp.; Malvaceae)

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Running title: Extrafloral nectary anatomy and carbon metabolism

1.1 Abstract

Extrafloral (EF) nectar is the foundation of many ant-plant interactions worldwide. EF nectar is a resource that encourages ant presence, in turn resulting in protection for the plant against herbivores. There is a paucity of research on this relationship and the plant structures that underpin it in Australian species. Firstly, this project characterised the gross morphology and cellular anatomy of EF nectaries of four Australian native wild cottons (*Gossypium* spp.) with prominent nectaries that produce abundant nectar. Histochemical staining identified starch granules in the nectary and the presence of a suberised layer around the glandular trichomes, indicating that nectar solutes must travel through the symplasm to reach the secretory cells. The phloem sap and EF nectar sugar compositions were quite distinct, so phloem sap must be processed before secretion as nectar. Secondly, this project used $^{14}CO_2$ labelling to examine the fixation and secretion of carbon in *G. sturtianum* EF nectar. The studied leaves were carbon sources which produced EF nectar and exported carbon. The carbon cost of EF nectar production by *G. sturtianum* was 1% of net photosynthates. These results increase our knowledge about Australian EF nectary-bearing species and their metabolism of carbon into EF nectar.

Keywords: anatomy, carbon metabolism, cotton, extrafloral nectaries, glandular trichome, histochemistry, nectar secretion, phloem sap, sugar, symplastic

1.2 Introduction

Extrafloral (EF) nectaries have been of interest to plant scientists for over 140 years (Delpino 1874 as cited in Mancuso 2010) and many studies have been conducted on them and the nectar they produce. The term nectary does not refer to a well-defined anatomical structure, but rather is a functional definition (Pacini et al. 2003). Extrafloral nectaries are structures which produce nectar, a sugar-rich solution, but are not involved with pollination (Nicolson et al. 2007). Their location and function varies between species and they can occur on almost any above-ground tissue, including leaves, petioles, stems, sepals and bracts (Koptur 1992). The morphology and anatomy of EF nectaries are highly diverse (Koptur 1992) and they can be classified into seven major groups based on their external morphology: formless, flattened, pit, hollow, scalelike, elevated and embedded nectaries (Zimmermann 1932 as cited in Elias 1983).

Extrafloral nectaries are very common; they are found in over 100 plant families worldwide (Weber & Keeler 2013). The production of EF nectar plays an important indirect defensive role for many plants. Ants visit the plant to locate and consume the nectar and in many cases they disturb or prey upon herbivores, resulting in a reduction in herbivory (Beattie 1985). To protect the most vulnerable plant tissues (Coley & Barone 1996), EF nectaries are generally located close to developing tissues, such as shoots and fruits (Wäckers & Bonifay 2004; Holland et al. 2009), to encourage the presence of ants.

Extrafloral nectar secretion involves three main classes of cells: the secretory cells on the plant surface, the nectary parenchyma and the vascular tissue (Pacini et al. 2003). The nectar is secreted at the plant surface through specialised secretory cells (glandular trichomes) or through stomates that remain permanently open (Fahn 1988). Extrafloral nectaries can be vascularised with phloem and/or xylem, but in most cases they have no specific vascular supply (Heil 2011). If an EF nectary is vascularised then the vascular tissue extends to within a few cells of the nectary epidermis, such as the phloem supply of the inflorescence stalk nectaries of *Vigna unguiculata* (Kuo & Pate 1985).

It was once believed that EF nectar was purely secreted phloem sap, but later studies have shown that EF nectar contains substances that in phloem sap have low concentration and/or are absent (Heil 2011). The current commonly held view is that phloem supplies some of the raw materials for EF nectar, but the phloem sap is filtered, concentrated and added to by the EF nectary tissues before excretion (Pacini & Nepi 2007). As a result, the composition of EF nectar is quite different to the composition of phloem (Orona-Tamayo et al. 2013). Extrafloral nectar has three major components; sugars (glucose, fructose and sucrose) are the largest component, followed by amino acids and then proteins in much lower concentrations (González-Teuber et al. 2009). The ratio of hexoses to sucrose in EF nectar varies considerably among species; some species have nectar dominated by hexoses, some are dominated by sucrose and others have more equal proportions (Fahn 1979).

There are only a few studies in which the composition of EF nectar has been compared directly to the phloem sap of the same species (Baker et al. 1978a; Pate et al. 1985; Lohaus & Schwerdtfeger 2014; Chanam et al. 2015). This is possibly due to the difficulty associated with extracting phloem sap from the majority of plants (Patrick et al. 1999). The majority of the sugar in phloem sap is sucrose, with other sugars present in minor amounts (Pate et al. 1985). In contrast, EF nectar can contain high levels of hexoses which are only present in phloem sap at low levels (Lohaus & Schwerdtfeger 2014).

The details of how sugars move from phloem unloading through the underlying tissues to the site of nectar secretion are being actively investigated (Heil 2011). In general, the two major ways the sugars can move through the tissues are via the apoplastic or symplastic pathways (Fahn 2000). The apoplastic pathway consists of the zone external to the cell membranes of the tissues (Läuchli 1976) and the symplastic pathway involves moving through cell membranes, the cytoplasm and connecting plasmodesmata (Spanswick 1976). The secretion of nectar is not restricted to only one pathway; the process could involve both pathways. The transport of sugars can be a direct process or can involve the intermediate step of starch storage (Heil 2011). The storage of carbohydrates as starch is common for floral nectaries, but has only been shown to occur in EF nectaries for a small number of species (Gaffal 2012).

There is little research identifying the tissue where the EF nectar sugars are generated by photosynthesis. There are three possibilities for the source of the sugars: (1) a single leaf generates the photosynthates and these are converted into the sugars that the leaf secretes in its EF nectar; (2) the photosynthates are generated elsewhere in the plant, are converted into sugars, travel through the phloem, are converted into EF nectar and secreted from other leaves; or (3) the sugars in EF nectar are partially generated in the same leaf and partially transported from other locations in the plant. A few studies have attempted to trace the source of the sugars in EF nectar (Radhika et al. 2008; Millán-Cañongo et al. 2014), but no clear picture has yet emerged about the movement of the carbon fixed by photosynthesis that is eventually secreted in EF nectar.

Nectar is metabolically costly for plants to produce (Lüttge 1977), so the investment level in EF nectar has to be balanced with other requirements (e.g. growth, reproduction and direct defences). The investment of plants in EF nectar production has only been measured in a few species; these studies show investments of approximately 1% of the total leaf energy (O'Dowd 1979), 0.1 - 2% of net photosynthates (Pate et al. 1985) and 0.9 ± 0.1% of total carbon fixed by the plant (Xu & Chen 2015).

Despite the breadth of research on EF nectaries generally, there is a paucity of research on Australian species with EF nectaries and their nectar. To my knowledge, there are 20 studies of Australian species with EF nectaries, and 50% of these studies focus on *Acacia* species (Table S-1). Australia is a mostly arid continent and nectar can impose a substantial water and nutrient penalty on plants, so Lamont (1979) suggests this may be the reason there are not a lot of Australian species with EF nectaries. However, aridity does not affect the prevalence of EF nectaries in other parts of the world (Pemberton 1988; Marazzi et al. 2013; Aranda-Rickert et al. 2014). For example, EF nectaries are abundant in the Cactaceae (Ruffner & Clark 1986; Oliveira et al. 1999; Holland et al. 2010). In addition, recent studies identified 34 EF nectary-bearing species in the Australian tropical rainforest (Blüthgen & Reifenrath 2003; Blüthgen et al. 2004) of which 12 genera with EF nectaries had not previously been reported. This discovery highlights just how little is known about the prevalence and function of EF nectaries in Australian vegetation.

The aim of this study is to broaden the range of studies on Australian EF nectary bearing species. This study focuses on Australian native wild cotton species which produce abundant nectar. There are 17 native Australian wild cotton species found in tropical, temperate and arid regions (Wendel et al. 2009). These native wild cotton species are from the same genus as cultivated cotton species (Fryxell 1978). Many species of *Gossypium* have EF nectaries present on a variety of their tissues (Butler et al. 1972; Elias 1983) and some species produce a large amount of nectar. Four species of Australian native wild cotton with foliar and bracteal EF nectaries are used in this study, *Gossypium sturtianum*, *G, australe*, *G. bickii* and *G. robinsonii*.

Due to the commercial importance of cotton, studies of EF nectaries have been completed on *G. hirsutum*, a species of cultivated cotton (Mound 1962; Wergin et al. 1975; Eleftheriou & Hall 1983), but there are no published studies on the EF nectaries of Australian native wild cotton species. Wild cotton species are a potential source of useful genes for cultivated cotton breeding programs to introduce new characteristics that improve yield without the use of more pesticides. Physiological knowledge of wild cottons is the first step towards identifying characteristics which may be of interest for genetic engineering or introgression with cultivated cotton.

The first objective of this study was to characterise the EF nectary morphology, anatomy and histochemistry of four species of Australian native wild cotton. As these plants have different natural ranges (Figure 1-1) it was expected that plants from similar environments would have similar external morphologies, but the cellular anatomy of the nectaries would be similar in all four species. The second objective was to determine the sugar profile and proportions of sugars in *G. sturtianum* foliar nectar and compare this with the sugars in phloem sap. The third objective was to measure the rate of carbon assimilation, trace the movement of carbon through leaf tissues and calculate the carbon cost of *G. sturtianum* EF nectar production.

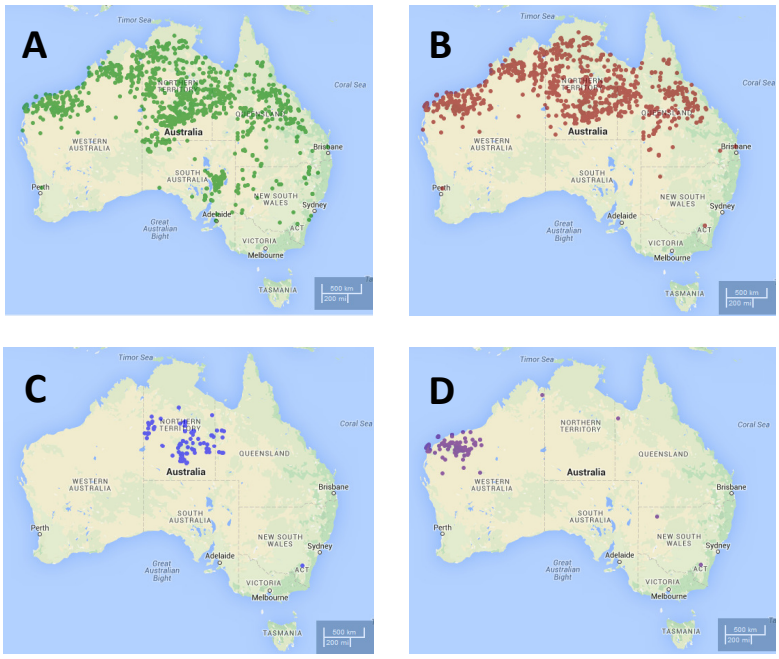

Figure 1-1. Distribution of (A) *G. sturtianum*, (B) *G. australe*, (C) *G. bickii*; and (D) *G. robinsonii* (Atlas of Living Australia 2015).

1.3 Materials and Methods

1.3.1 Plant propagation

At the beginning of the project there were a few mature Australian native cotton plants at Macquarie University, but additional plants were required to complete this project. No reliable information on the germination of Australian native cotton seeds was available, so both seed germination and propagation from cuttings were attempted to increase the number of plants. Not all successfully propagated plants were used for experimental purposes.

Seeds were collected from mature *G. sturtianum*, *G. bickii* and *G. australe* plants propagated at Macquarie University. *G. robinsonii* seeds were obtained from Nindethana Seed Company (Albany, Western Australia). *G. sturtianum* (n = 245), *G. australe* (n = 210), *G. bickii* (n = 70) and *G. robinsonii* (n = 59) seeds were surface sterilised to reduce the occurrence of mould. Several pre-germination methods were compared on a subset of seeds of each species. Ten to eighty seeds were used for each treatment (Table S-2) and

not all treatments were applied to all species. After treatment, the seeds were placed in humid container and incubated at 30°C. Germinated seeds were planted into a 1:1 sand:loam mixture with a surface vermiculite layer and watered with automated misters three times a day for five minutes. When the seedlings were 2 - 3cm tall, they were transferred to a greenhouse with the following conditions: temperature 30/22 ± 2°C (day/night 14/10 hours), supplementary lighting (Phillips Contempa High Pressure Sodium lamps) of 400 µmoles m^{-2} s^{-1} added when ambient light level dropped below 400 µmoles m^{-2} s^{-1} and watered at least every second day. Seedlings were fertilised once a fortnight with 0.5 g/L Aquasol water soluble fertiliser (N:P:K = 23:3.95:14; Yates Australia, Padstow, NSW, Australia).

Twenty-four *G. sturtianum*, fifteen *G. bickii* and ten *G. robinsonii* cuttings were taken from mature plants and trimmed to 15 cm length. All but the two terminal fully expanded leaves were removed. The stem was dipped in a 1:1 mix of Clonex® rooting hormone (Yates Australia, Padstow, NSW, Australia) and 10^{-8} M 2,4-D auxin analogue (2,4-dichlorophenoxy-acetic acid; Sigma-Aldrich, Castle Hill, NSW, Australia). The cuttings were planted into a 1:1 sand:loam mixture with a surface vermiculite layer and watered with automated misters for five minutes three times a day.

1.3.2 Extrafloral nectary morphology, anatomy and histochemistry

Plant material

Four species of Australian native wild cotton (*G. sturtianum, G. australe, G. bickii and G. robinsonii*) grown in the Macquarie University glasshouses (see section 1.3.1) were used in all microscopy procedures. A minimum of two plants per species were used for all foliar nectary examinations. Cotton flowers are short-lived and not all species flowered during the project, so bracteal nectaries were examined only where bracteal tissue was available. Tissues used for microscopy were the youngest fully expanded leaves with functional nectaries or bracteal nectaries harvested from flowers on the day of anthesis.

Light microscopy

The gross morphology of foliar (four species) and bracteal (*G. sturtianum* and *G. bickii*) EF nectaries (fresh tissue) was observed using an Olympus SZX16 research stereo-microscope and images were captured using Colorview IIIu Digital Camera and analysis® Five software (Olympus Soft Imaging Solutions, Münster, Germany).

Foliar nectaries were excised and fixed in 4% v/v paraformaldehyde (Sigma-Aldrich, Castle Hill, NSW, Australia) in 10 mM phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl) for 24 hours. The nectaries were washed four times in PBS at half hour intervals and embedded in 6% w/v agarose. The nectaries were attached to a vibratome stage (VT1000S, Leica Biosystems, North Ryde, NSW, Australia) using superglue. Transverse and longitudinal sections of the foliar nectaries were cut at 30 µm thickness. Sections of each species were stained independently with four stains (Methylene Blue, Sudan Black B, iodine-potassium iodide and iron (III) chloride) to ascertain the histochemistry of the nectary tissue.

Methylene Blue staining was used for visualisation of the cell anatomy in the nectary tissue. Sections were placed in 0.006% v/v aqueous Methylene Blue (1% Methylene Blue, 0.6% sodium bicarbonate, 40% glycerol) for one minute then washed (concentration and staining time determined empirically).

Lipids (including suberin) were detected in sections by staining with Sudan Black B (Ruzin 1999). Sections were placed in 50% ethanol and stained with 0.07% w/v Sudan Black B (Gurr, London, UK) in 70% ethanol. Sections remained in the Sudan Black B solution for five minutes then were differentiated in 50% ethanol for one minute.

To detect starch molecules, an IKI solution (Johansen 1940) was used to stain the sections. The IKI solution consisted of 1% w/v potassium iodide (Thermo Fisher Scientific, Scoresby, VIC, Australia) and 1% w/v iodine flakes (VWR International, Tingalpa, QLD, Australia) dissolved in RO water. Sections were placed on a slide and two drops of IKI solution added directly to the sections. The sections were visualised without removal of the IKI solution.

Phenolic compounds in vibratome sections were detected using an iron (III) chloride solution (Mace 1963). A solution of 2% w/v iron (III) chloride (VWR International, Tingalpa, QLD, Australia) in 95% v/v ethanol was used to stain the sections for five minutes. Sections were differentiated in 70% v/v ethanol for 30 seconds and mounted on glass slides.

Unstained and stained sections were visualised using an Olympus BX53 brightfield compound microscope and images captured using DP26 digital camera and cellSens Entry imaging software (Olympus Corporation, Notting Hill, VIC, Australia). Compound images were created using differently focused images (EFI projections) using an Olympus BX63 epifluorescence/DIC/brightfield imaging system. Images were captured using a DP80 digital camera and compiled in cellSens Dimension imaging software (Olympus Corporation, Notting Hill, VIC, Australia).

Scanning electron microscopy

Nectaries were excised from the surrounding tissue and fixed for 24 hours with 3% v/v glutaraldehyde (ProSciTech, Kirwan, QLD, Australia) in 0.1 M phosphate buffer pH 7.2. The nectaries were washed in 0.1 M phosphate buffer pH 7.2 three times at 30 minute intervals. A serial dehydration with ethanol (30 - 100%) was conducted and the nectaries were dried using an Emitech K850 Critical Point Drier (Quorum Technologies, Kent UK). The dried nectaries were mounted on stubs with carbon paint, left to dry for 24 hours then gold coated using an Emitech K550 Gold Coater Unit (Quorum Technologies, Kent, UK). The nectaries were examined using a JSM-6480 Scanning Electron Microscope (JEOL, Tokyo, Japan) at 5 kV and images captured using JEOL SEM software.

Fluorescence microscopy

Vibratome cut sections of foliar nectaries were mounted on a glass slide with Fluoro-Gel mounting medium (ProSciTech, Kirwan, QLD, Australia). These sections were obtained from leaves fixed in paraformaldehyde (as above) as the auto-fluorescence of compounds commonly associated with nectaries (lignin and suberin) is not diminished by this type of

fixation (O'Brien & McCully 1981). Unstained sections were visualised using DAPI (UV light; 330 - 385 nm) and FITC (blue light; 450 - 480 nm) filters in an Olympus BX63 epifluorescence/DIC/brightfield imaging system. Images were captured using a DP80 digital camera and cellSens Dimension imaging software.

1.3.3 Carbon metabolism

Carbon budget

To calculate the proportion of the leaf carbon pool allocated to EF nectar, a comparison was made between estimated leaf sugar production and the amount of sugar exuded in EF nectar. The nectaries of three leaves per *G. sturtianum* plant (total of eight plants) were washed with MilliQ water and the nectar allowed to accumulate for 48 hours (Figure S-1). The nectar from each leaf was collected using 5 µL disposable microcapillaries (Drummond Scientific Company, Broomall, PA, USA). The nectar volume was determined by measuring the nectar length in the microcapillary, calculating the proportion containing nectar and then multiplying this by the volume of the microcapillary. The photosynthetic rate of the leaves was measured using a LiCor Li-6400XT portable photosynthesis system (LiCor Biosciences, NE, USA) with settings of 30^{o}C, 400 ppm CO_2 and PAR of 1800 µmoles m^{-2} s^{-1} (Figure S-1). Leaf area was calculated from photographs using Image J software (Schneider et al. 2012).

The photosynthetic rate and leaf area were used to estimate the sugar (sucrose-equivalents) able to be generated by a leaf in 28 hours (14 daylight hours per day over a two day collection period). The amount of sugar exuded in the nectar was calculated from the nectar volume and nectar sugar concentration, as determined by high-performance anion-exchange chromatography (see section 2.3.5). As the sugar profile contained sugars other than sucrose, the total sugar pool was re-calculated in sucrose-equivalents for the purpose of the leaf carbon budget calculations.

$^{14}CO_2$ labelling of leaves

To determine the origin of the sugars excreted in EF nectar, an atmospheric $^{14}CO_2$ pulse was used to label leaves, followed by a chase period (unlabelled CO_2 in ambient atmosphere). Single leaves from each of 20 *G. sturtianum* plants were labelled with $^{14}CO_2$, allowing a time course of sampling (four replicates per time point) to be conducted to trace the movement of ^{14}C through the leaf tissue. The ^{14}C labelling was conducted in a glasshouse with supplementary lighting (Philips GreenPower LED toplighting) of 600 µmoles m^{-2} s^{-1} switched on at the beginning of the $^{14}CO_2$ pulse (day/night 14/10 hours). The photosynthetic rate of an analogous leaf was measured during the $^{14}CO_2$ labelling with a LiCor Li-6400XT portable photosynthesis system (settings: 30°C, 400 ppm CO_2 and PAR 1800 µmoles m^{-2} s^{-1}).

Individual leaves were sealed inside 23 x 30 cm ziplock bags and tape was used to seal the plastic bag around the petiole (Figure 1-2). The leaves were exposed to $^{14}CO_2$ generated by the chemical reaction between 21.3 mM $NaH^{14}CO_3$ solution pH 9.5 (1.739 GBq/mmol; Perkin Elmer, Waltham, MA, USA) and 1 M HCl for 1.5 hours (see Appendix I for CO_2 depletion calculations), followed by unlabelled free air for zero, three, five, nine or 29 hours. After each chase period the labelled leaves and an adjacent leaf from each plant (fully expanded leaf closer to the stem terminus) were harvested, separated into sections (leaf blade, mid vein, nectary and petiole; Figure 1-3) and stored in 80% ethanol at -20°C until required for analysis. After the 29 hour chase, the EF nectar produced by the labelled leaf was collected using filter paper wicks (McKenna & Thomson 1988).

Figure 1-2. Experimental set up for 14C labelling. (A) Whole set up; (B) A single leaf was enclosed in a ziplock bag, sealed with tape around the petiole and labelled with $^{14}CO_2$ (n = 20); and (C) $^{14}CO_2$ was generated by injecting HCl through the septum into the Warburg flask containing $NaH^{14}CO_3$.

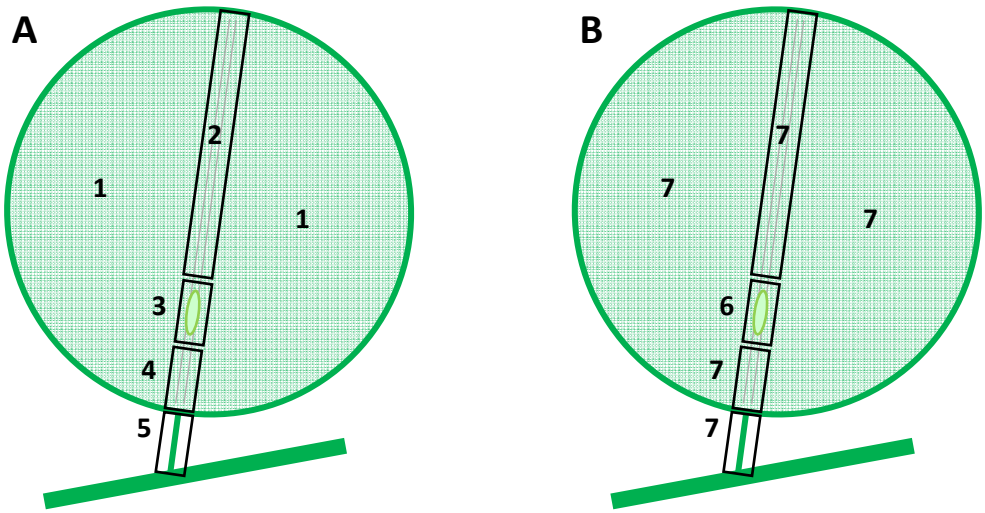

Figure 1-3. Diagrammatical representation of sampled sections of (A) labelled leaf; and (B) adjacent leaf. Leaf sections are classified as: (1) leaf blade; (2) mid vein, terminal section; (3) mid vein, with nectary; (4) mid vein, proximal to petiole, (5) petiole; (6) mid vein, with nectary; and (7) rest of leaf.

Extraction of the soluble sugars from each leaf section was carried out by boiling in 80% ethanol in individual scintillation vials for three minutes. The hot supernatant was removed and the samples were heated again in MilliQ water for three minutes. The supernatants were combined to form the final extract solution. A 1 mL subsample of solutions with a large amount of suspended particles was used for scintillation counting. Solutions with low amounts of suspended particles were evaporated at 100^{o}C and re-suspended in 1 mL MilliQ water. The filter paper wicks with nectar were soaked in 1 mL MilliQ water, vortexed and the wicks discarded. Chlorite bleach (100 µL; 33% NaClO) and scintillant (four parts to one part sample; Pico-Fluor Plus; Perkin Elmer, Waltham, MA, USA) were added to each vial. The radioactivity of the solution was measured using a scintillation counter (Tri-Carb 2910 TR; Perkin Elmer). To determine the background radiation four negative control leaves were sampled using the same methodology after exposure to unlabelled $NaHCO_3$. To check the levels of quenching using internal standards, a known amount of $NaH^{14}CO_3$ was used to spike a subset of vials and their radioactivity was measured again. The drop in the efficiency of counting the spike revealed the quenching effect.

An additional leaf from each plant was harvested for determination of the size and diurnal fluctuation in the leaf sugar pool. Soluble sugars were extracted from the leaf using ethanol extraction (as above). The supernatant from the ethanol extraction was made up to 14 mL with MilliQ water. A 100 µL subsample of the extract mixture was added to 2.5 mL of anthrone solution (200 mg anthrone in 100 mL 71% v/v H_2SO_4), vortexed and boiled for exactly 10 minutes in a water bath. The samples were placed on ice for 10 minutes, allowed to come to RT and the absorbance at 630 nm was measured with a spectro-photometer (UV-1201; Shimadzu Corporation, Kyoto, Japan). The sugar concentration in glucose-equivalents was determined using a glucose absorbance standard curve.

1.3.4 Collection of phloem sap and extrafloral nectar

Phloem sap exudation

Methods to obtain phloem sap include bleeding plants (Patrick et al. 1999; Turgeon & Wolf 2009), aphid stylectomy (Munns & Fisher 1986) and EDTA-facilitated phloem exudation (King & Zeevaart 1974). EDTA-facilitated phloem exudation uses chelation to prevent wound healing so the phloem sap can be collected. In this study, phloem sap was collected using EDTA-facilitated exudation as described in Tetyuk et al. (2013). The tissue is washed and only the subsequent exudates into water are used for analysis, so the damaging effects and interference of EDTA during analysis are minimised (Tetyuk et al. 2013).

Ten leaves were harvested from each of six *G. sturtianum* plants and immediately placed in 20 mM Na_2-EDTA solution (ethylenediaminetetra-acetic acid disodium salt; Thermo Fisher Scientific, Scoresby, VIC, Australia) The petioles were re-cut under the solution's surface and kept in the solution for one hour in a humid chamber in a glasshouse with 600 µmoles m^{-2} s^{-1} of supplementary lighting (Philips GreenPower LED toplighting). The leaves were washed thoroughly with MilliQ water to remove the EDTA, placed in tubes containing MilliQ water and the exudates collected for a further five hours. The leaves were discarded, the resulting solution was concentrated under vacuum (Refrigerated CentriVap Benchtop Vacuum Concentrator; Labconco, Kansas City, MO, USA) and stored at -20^{o}C until required for analysis.

Extrafloral nectar collection

Methods for EF nectar collection include using filter paper wicks (McKenna & Thomson 1988), capillaries (Corbet 2003), micropipettes (Dafni 1992) and rinsing leaves/flowers (Grunfeld et al. 1989). *G. sturtianum* EF nectaries are located on the surfaces of leaves and bracts; they are easily accessible and there was a relatively large volume of nectar present. The capillary method was selected for EF nectar collection as under these conditions this method is simple and effective.

Twenty *G. sturtianum* plants aged between six and nine months were randomly allocated to two growth cabinets (model PG.15.18.9 TD.5x100R; Thermoline Scientific, Wetherill Park, NSW, Australia) and watered every second day. The cabinet conditions were: atmospheric CO_2 400 ppm (ambient), temperature 30/18 ± 1°C (day/night 14/10 hours), light at 1800 ± 50 µmol s^{-1} (metal halide multi vapour lamps; GE Appliances & Lighting, Richmond, VIC, Australia; measured using LiCor Biosciences light meter, model LI-250A). The relative humidity was maintained at 90 ± 10% using evaporation from trays of water. The plants in each cabinet were re-randomised once a week to minimise any intra-cabinet variation. The EF nectaries were washed with RO water to remove any accumulated nectar and allowed to air dry while isolated from ants with trays of water. After four weeks, EF nectar was collected after allowing the nectar to accumulate for five days. The nectar from all foliar nectaries (one per leaf) was collected with 75 µL microcapillaries and pooled for each plant. The nectar was stored at -20°C until required for analysis.

1.3.5 Sugar composition

The sugar composition of *G. sturtianum* foliar nectar (n = 8) and phloem sap (n = 6) was analysed using high performance liquid chromatography (HPLC) to determine the sugar profile and concentrations. The volume of nectar collected was low for some plants, so not all replicates were used for analysis. A 1 mg/mL solution was prepared in MilliQ water and an internal standard was added. The analysis was carried out on a high-performance anion-exchange chromatograph system with pulsed amperometric detection (HPAEC-PAD) fitted with a BioLC amino trap guard column (3 x 50mm) connected to a CarboPac

PA10 column (4 x 250 mm) held at 25°C. A 20 µL sample was injected into the HPAEC-PAD and analysed using basic solvents, at a flow rate of 0.5 mL min^{-1}. The analytes detected were quantified with internal standards for arabinose, rhamnose, galactose, glucose, sucrose, xylose, mannose and fructose. Each sample was divided into three technical replicates for analysis. For foliar nectar, the standard deviations were within 2.2%, 8.4% and 3.2% of the mean for concentrations of glucose, sucrose and fructose, respectively. For phloem sap, the standard deviations were within 14.5% and 12.9% of the mean for concentrations of glucose and sucrose, respectively. The mean of the technical replicates is used in all calculations and statistical analyses.

1.3.6 Statistical analysis

Statistical analysis was conducted using Minitab® Statistical Software (Minitab Inc 2015). The significance level for all analyses was 0.05 and error bars represent the standard error of the mean. One way ANOVA with Tukey's HSD post hoc test was used for monosaccharide equivalents over the chase period, the radioactivity of the adjacent leaf over the chase period and the hexose to sucrose ratio in foliar nectar and phloem sap. Two-way ANOVA with Tukey's HSD post hoc test was used for the radioactivity of labelled leaf sections over the chase period. Natural log data transformation was used for the radioactivity of the labelled leaf sections and the adjacent leaf over the chase period.

1.4 Results

1.4.1 Extrafloral nectary morphology, anatomy and histochemistry

Location of extrafloral nectaries

The EF nectaries of the four species occur only on leaves and bracts. In *G. sturtianum*, *G. bickii* and *G. australe* there is only one nectary per leaf, located on the mid vein on the abaxial leaf surface, proximal to the petiole (Figure 1-4). Each *G. robinsonii* leaf can present up to five nectaries on the abaxial surface; the leaves are palmate with a nectary

on the major vein of each lobe. The nectaries are located approximately halfway between the petiole and the leaf tip (Figure 1-4).

Bracteal nectaries were observed in *G. sturtianum*, *G. australe* and *G. bickii*, all located at the base of the bract, close to the peduncle attachment point (Figure 1-4). *G. robinsonii* did not flower during this study, so the presence of bracteal nectaries in this species cannot be confirmed. *G. sturtianum* and *G. australe* have three bracteal nectaries, whereas *G. bickii* has three bracteal nectaries on the majority of flowers, but instances of five bracteal nectaries were observed.

External morphology

Stereo microscopy shows the foliar nectaries of the four species have very different morphologies. Foliar nectaries of *G. australe*, *G. bickii* and *G. robinsonii* have a pink-red colouration making them stand out against the leaf tissue. *G. sturtianum* foliar nectaries are a very similar colour to the mid vein and are difficult to discern (Figure 1-5). *G. sturtianum* and *G. australe* foliar nectaries are elongated along the length of the mid vein, *G. bickii* foliar nectaries have a round shape and *G. robinsonii* nectaries have are an arrow shape and point towards the terminus of the mid vein (Figure 1-5).

Scanning electron microscopy shows the cells of the nectaries appear round, smooth and tightly packed and are similar in all four species and at all nectary locations (Figure 1-6). The presence, number and type of non-glandular trichomes in and around EF nectaries varies with species. *G. australe* and *G. bickii* have multi-radiate stellate non-glandular trichomes interspersed among the round nectary cells, on the nectary margins and covering the surface of the surrounding leaf (Figure 1-6). *G. robinsonii* also has non-glandular trichomes within the nectary, but these are bi-, tri- and tetra-radiate stellate trichomes (Figure 1-6). Apart from one or two bi-radiate stellate non-glandular trichomes at the petiole-end of the nectary, *G. robinsonii* does not have non-glandular trichomes on the nectary margins or the surrounding leaf. *G. sturtianum* foliar nectaries do not have non-glandular trichomes within the nectary, on the margins of the nectary or on the surrounding leaf tissue (Figure 1-6).

Cellular anatomy & histochemistry

The foliar nectaries of all four species are comprised of epidermal cells with a glandular trichome layer on the outermost surface (Figure 1-7). Each glandular trichome comprises a unicellular head and a stalk cell that connects to a basal epidermal cell. Below this is parenchyma and then deeper in the tissue is the vasculature. There is no evidence of additional vascular tissue that specifically supplies to the nectary tissue, nor of any chloroplasts or stomates in the EF nectaries. Calcium oxalate crystals were observed in all four species (Figure 1-7).

Unstained sections visualised with an epifluorescence microscope show auto-fluorescence with DAPI (UV light; 330-385 nm) and FITC (blue light; 450-480 nm) filters (Figure 1-8). The DAPI filter shows strong fluorescence of the xylem and nectary glandular trichomes, but fluorescence of these cells under the FITC filter is much weaker. Histochemical staining shows that the anatomy of the nectaries in the four species is the same, but there are some minor variations in histochemistry between the species (Figure 1-9). Starch molecules are visible (stained black with IKI solution) in the nectary parenchyma of *G. sturtianum* and *G. australe*, but not in *G. bickii* or *G. robinsonii*. ith Sudan Black B stains lipids blue which are visible in the cuticle of all four species. The stalk cells at the base of the glandular trichomes also have blue margins, indicating that they are suberised. Staining with iron (III) chloride indicates there are phenolic compounds present (stained brown-black) in the glandular trichomes and their subtending epidermal cells, but the concentration of phenols varies with species.

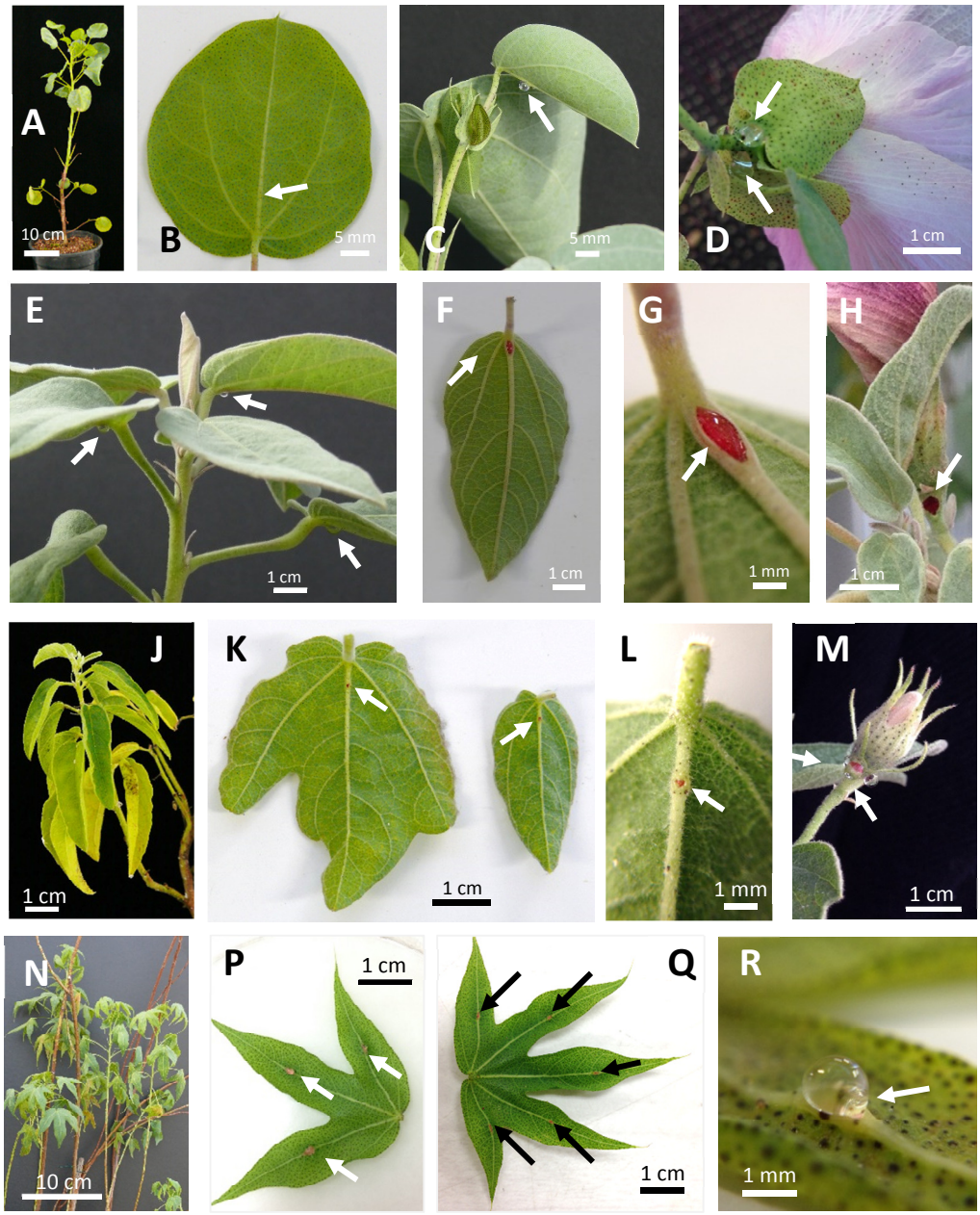

Figure 1-4. Australian native wild cotton plant morphology (A, E, J, N), leaf abaxial surfaces (B, F, K, P), foliar nectaries (B-C, E-G, K-L, P-R) and bracteal nectaries (D, H, M) of *G. sturtianum* (A-D), *G. australe* (E-H), *G. bickii* (J-M) and *G. robinsonii* (N-R). Arrows indicate nectary locations and nectar droplets are visible in (C-D, G, L-M, P, R). Leaf morphology variations are shown in *G. bickii* (K) and *G. robinsonii* (P-Q).

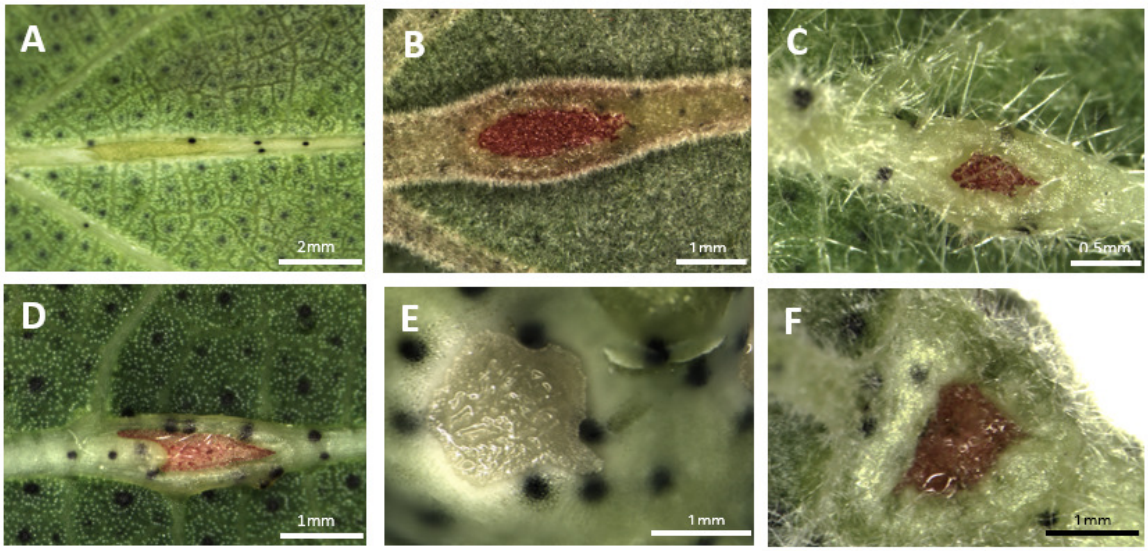

Figure 1-5. Gross morphology of foliar (A-D) and bracteal (E-F) nectaries of *G. sturtianum* (A, E), *G. australe* (B), *G. bickii* (C, F); and *G. robinsonii* (D).

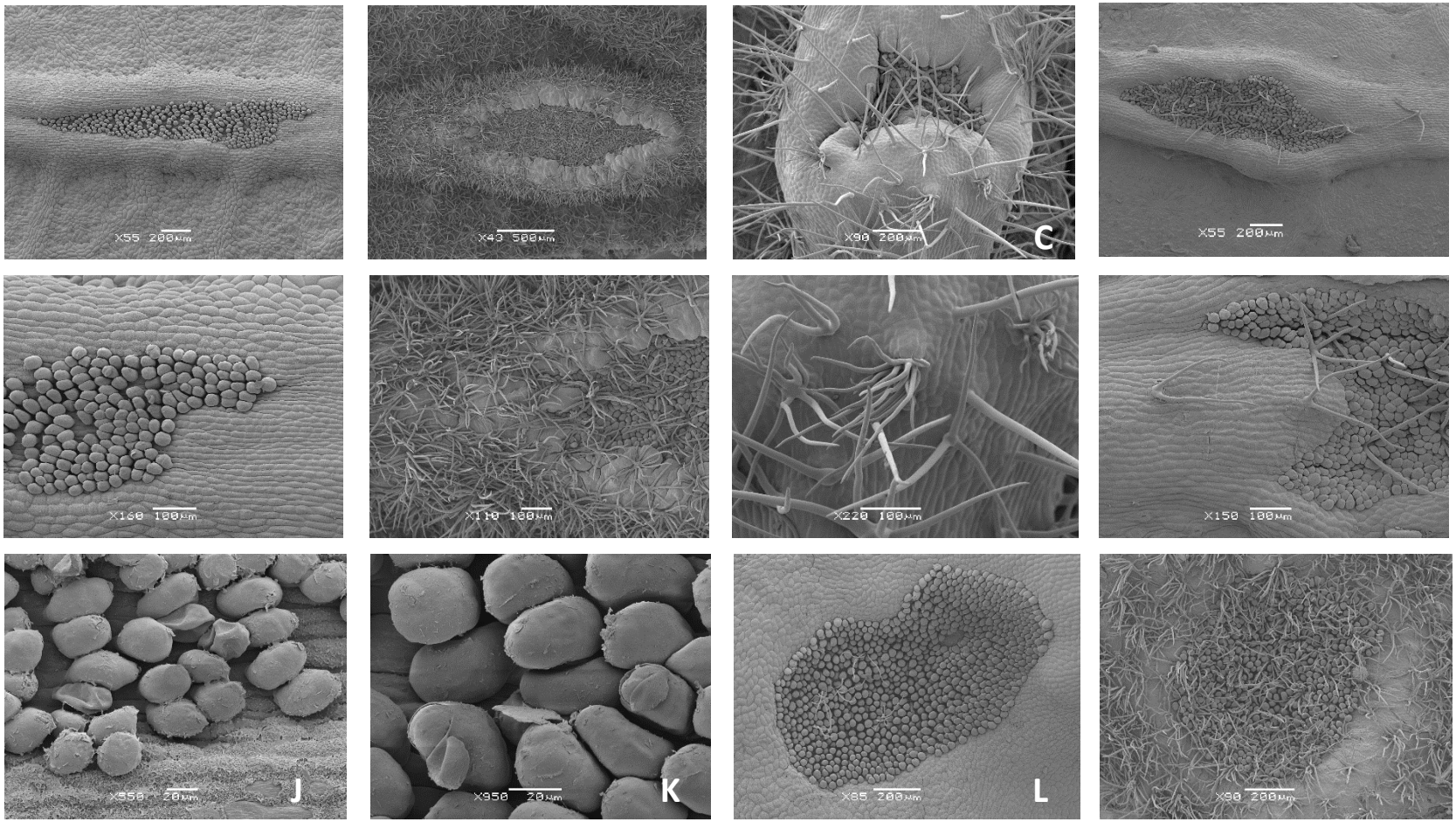

Figure 1-6. Scanning electron micrographs of foliar (A-K) and bracteal (L-M) nectaries from *G. sturtianum* (A, E, J, K, L), *G. australe* (B, F, M), *G. bickii* (C, G); and *G. robinsonii* (D, H). Whole foliar nectaries (A-D), magnified sections of foliar nectaries (E-K) and whole bracteal nectaries (L-M).

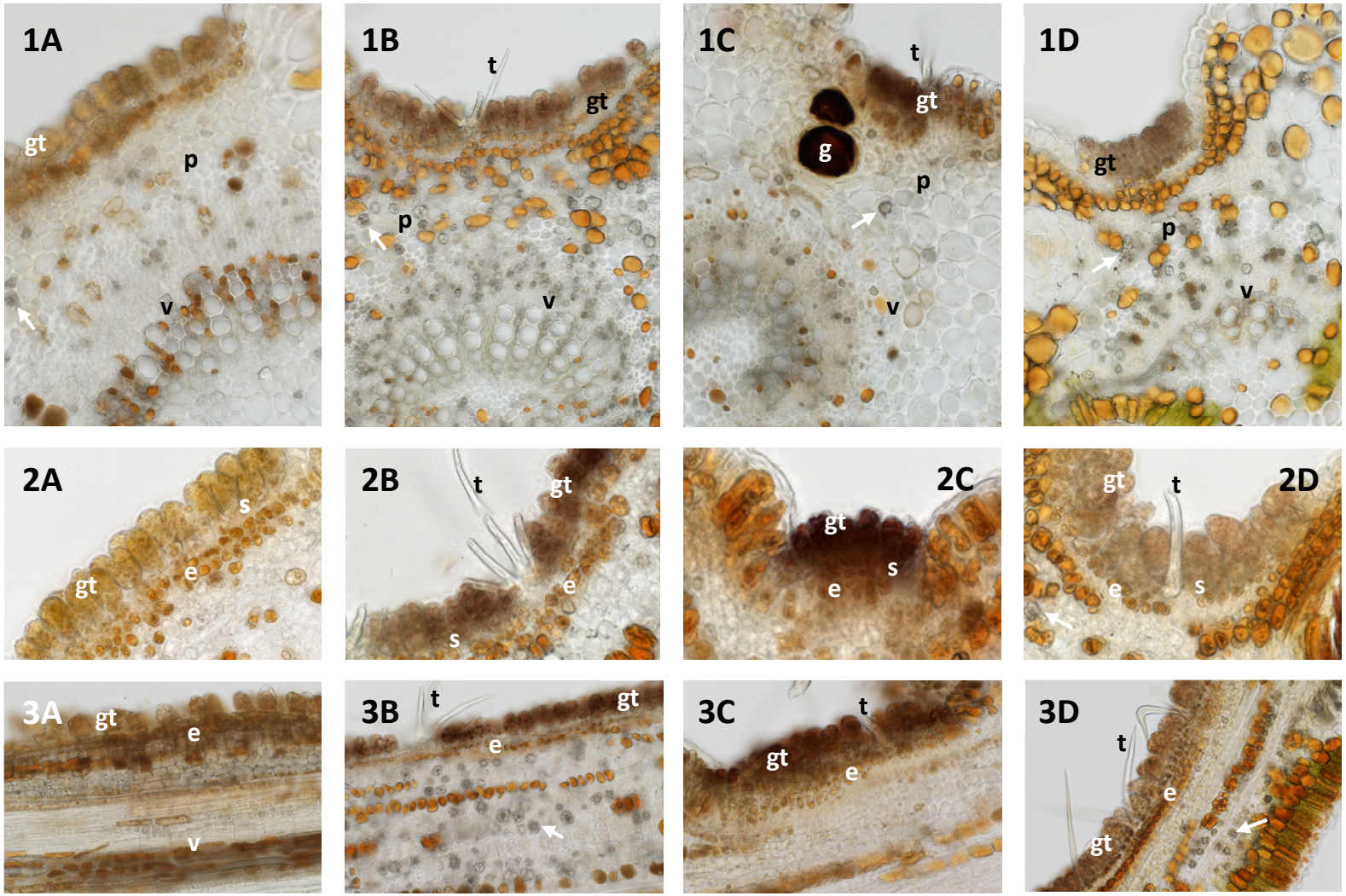

Figure 1-7. Unstained foliar transverse (1-2) and longitudinal (3) cross-sections from foliar nectaries of *G. sturtianum* (A), *G. australe* (B), *G. bickii* (C) and *G. robinsonii* (D). Sections shown at 20x (1, 3) and 40x magnification (2). e = epidermal cell; g = gossypol gland; gt = glandular trichome; p = nectary parenchyma; s = stalk cell; t = non-glandular trichome; v = vascular tissue. Arrows indicate calcium oxalate crystals.

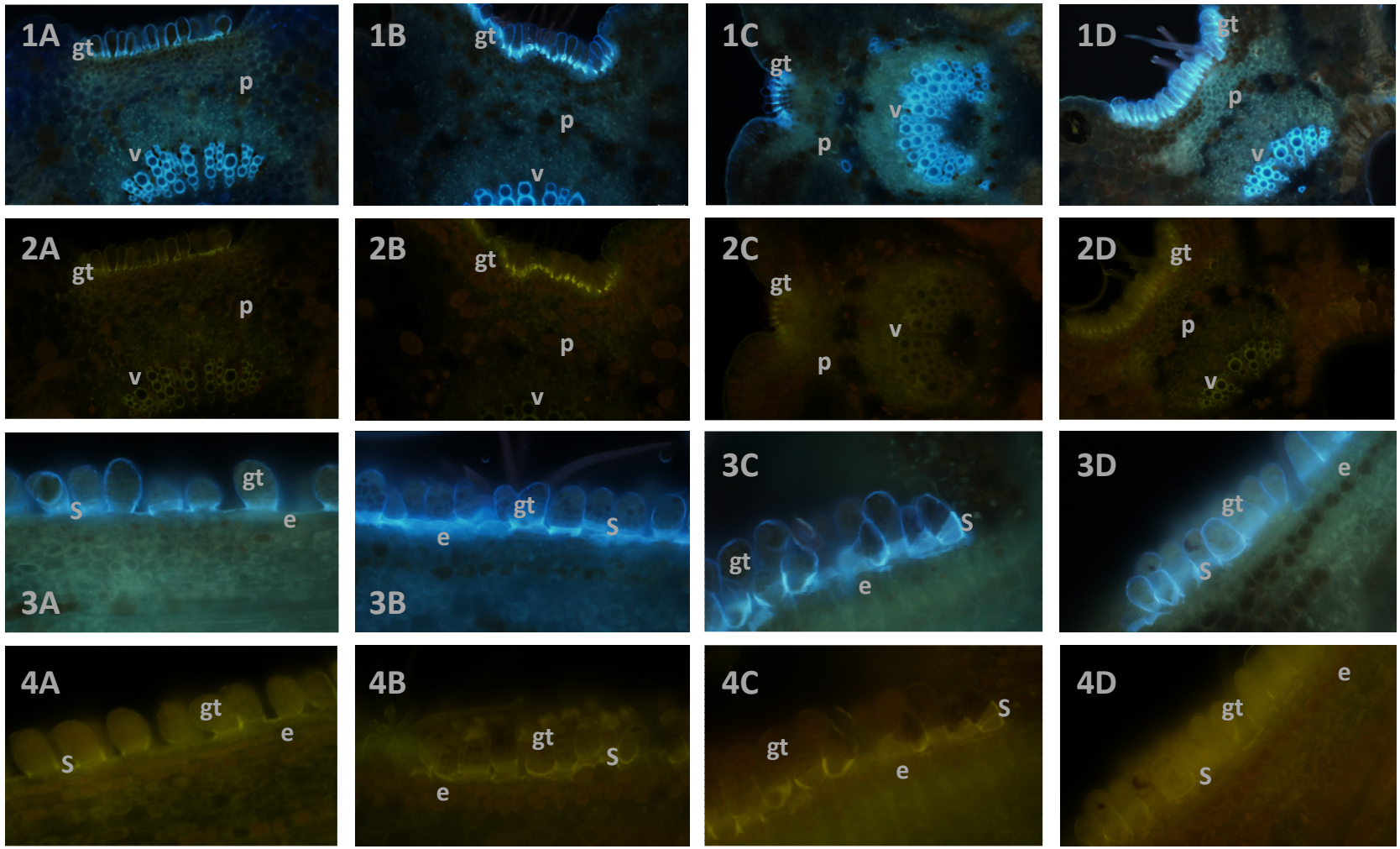

Figure 1-8. Unstained transverse (1,2) and longitudinal (3,4) cross-sections of foliar nectaries from *G. sturtianum* (A), *G. australe* (B), *G. bickii* (C) and *G. robinsonii* (D) visualised with DAPI (330-385 nm) (1,3) and FITC (450-480 nm) (2,4) filters. Sections shown at 10x (1-2) and 40x magnification (3-4). e = epidermis; gt = glandular trichome; s = stalk cell; p = nectary parenchyma; v = vascular tissue.

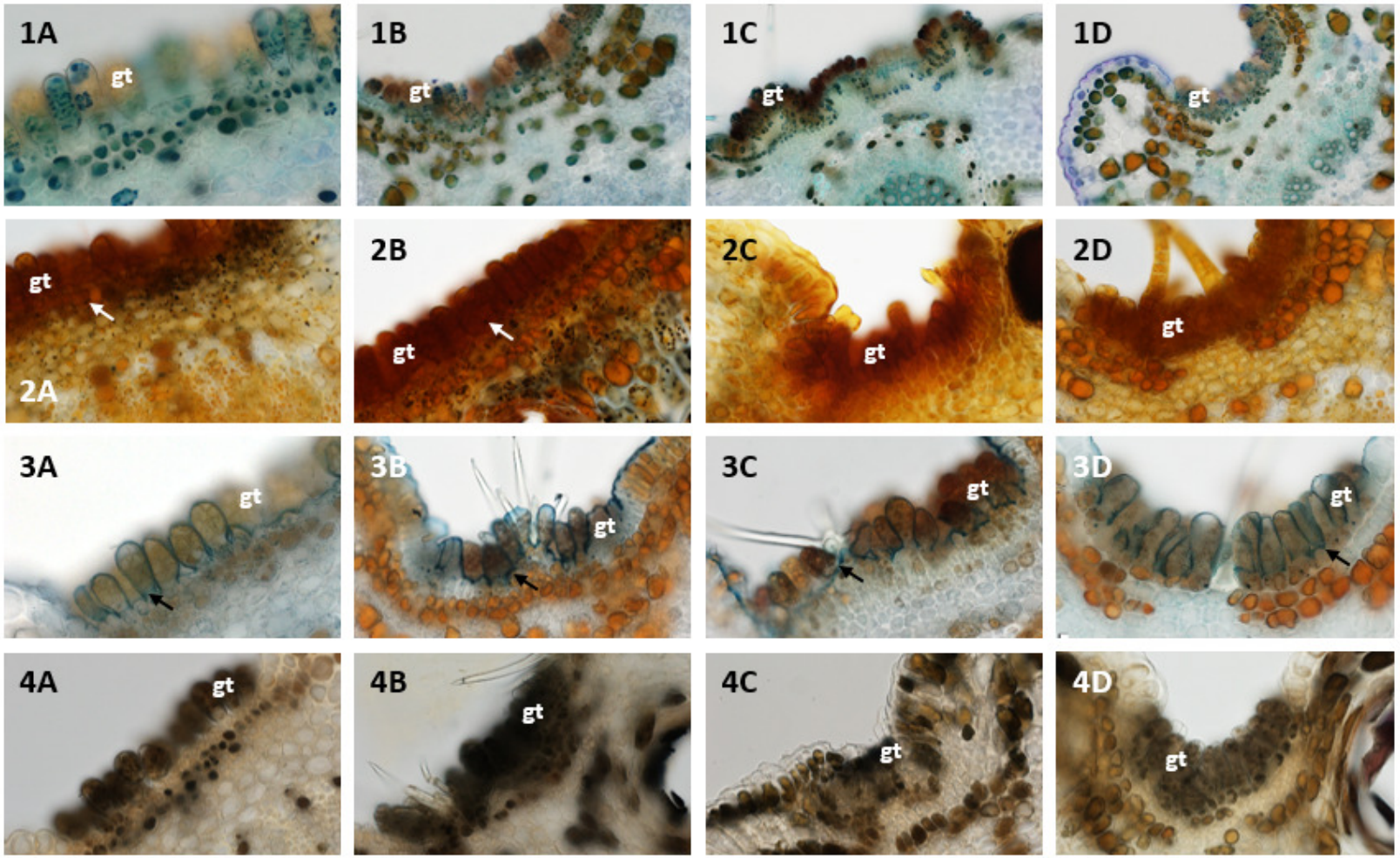

Figure 1-9. Transverse cross-sections of foliar nectaries from *G. sturtianum* (A), *G. australe* (B), *G. bickii* (C) and *G. robinsonii* (D) stained with Methylene Blue (1), IKI solution (2), Sudan Black B (3); and iron (III) chloride (4). Sections shown at 20x (1) and 40x magnification (2-4). gt = glandular trichome; white arrows = representative starch granules; black arrows = stalk cells with a suberised layer.

1.4.2 Carbon metabolism

Carbon budget

The mean (± SE) percentage of the estimated sugar production per leaf that was exuded in EF nectar over a two day period was 0.98% ± 0.21%.

Establishing a near steady state carbon metabolism

There were significant changes over time in the leaf soluble sugar pool ($F_{4,15}$ = 7.87, p = 0.001). The leaf soluble sugar increased at the beginning of the chase period, declined steadily over the remainder of the day and then overnight returned to the levels of the previous day, indicating a steady state in carbon metabolism (Figure 1-10). There was no significant difference between the photosynthetic rate at the beginning and during the chase period ($F_{1,18}$ = 0.17, p = 0.69), consistent with steady-state conditions. The mean ± SE photosynthetic rate at the beginning of the chase period was 25.5 ± 1.5 µmol CO_2 m^{-2} s^{-1} and after four hours was 26.6 ± 1.8 µmol CO_2 m^{-2} s^{-1}.

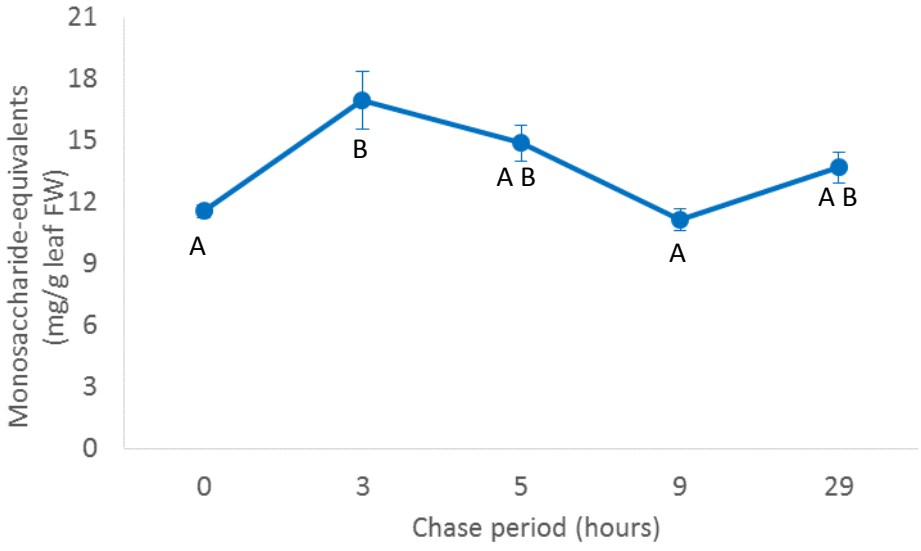

Figure 1-10. Monosaccharide-equivalents per gram of leaf fresh weight (FW; n = 4) at each time point (n = 4) in the chase period (measured in unlabelled leaves after $^{14}CO_2$ labelling of separate leaves for 1.5 hours). Points that do not share a letter are significantly different (Tukey's HSD). Vertical bars represent ± SE of the mean.

$^{14}CO_2$ labelling of leaves for tracing photo-assimilates

The radioactivity of the vascular tissue in the labelled leaf was statistically different between chase periods, i.e. over time ($F_{4,57} = 139.5$, $p < 0.001$) and between leaf sections ($F_{3,57} = 41.3$, $p < 0.001$; Figure 1-11). There was no significant interaction between leaf section and time ($F_{12,57} = 1.71$, $p = 0.09$). At zero hours post-labelling, the radioactivity of the nectary section was higher than the terminal vein (but not statistically different), but it was significantly higher than the proximal vein and the petiole (Tukey's HSD). This pattern was repeated at three and twenty nine hours post-labelling. At five hours post-labelling, radioactivity of the four vascular sections were not statistically different (Tukey's HSD). At nine hours post-labelling, the radioactivity of the terminal vein was significantly higher than the proximal vein, but not statistically different from the vein with nectary or the petiole (Tukey's HSD).

The rate of decline in radioactivity in the labelled leaf sections over the chase period was approximately exponential (Figure 1-12), so an exponential decay curve was fitted for each labelled leaf section. Decay constants for the terminal vein, vein with nectary, proximal vein and petiole were very similar (range: -0.62 to -0.80; Table S-3).

At 29 hours post labelling the mean (± SE) percentage of the total radioactivity in each section of the labelled leaf was: leaf blade 83.3 ± 14.3%; terminal vein 2.9 ± 0.6%; mid vein with nectary 0.5 ± 0.1%; proximal vein 0.5 ± 0.2%; petiole 3.9 ± 0.7%. In the unlabelled adjacent leaf the mean (± SE) percentage of the total radioactivity was 0.11 ± 0.05% in the nectary and 7.6 ± 4.0% in the remainder of the leaf.

The radioactivity of an unlabelled leaf adjacent to the labelled leaf was the same as background levels until the 29 hour chase period, when the radioactivity increased significantly ($F_{5,17} = 4.16$, $p = 0.01$). The radioactivity of the nectary from the adjacent unlabelled leaf did not rise above background levels any time during the chase period. Two labelled leaves generated nectar, which accounted for 1.4 ± 0.9% (mean ± SE) of the ^{14}C in the labelled leaf at 29 hours, while the other two did not release nectar in the labelling period.

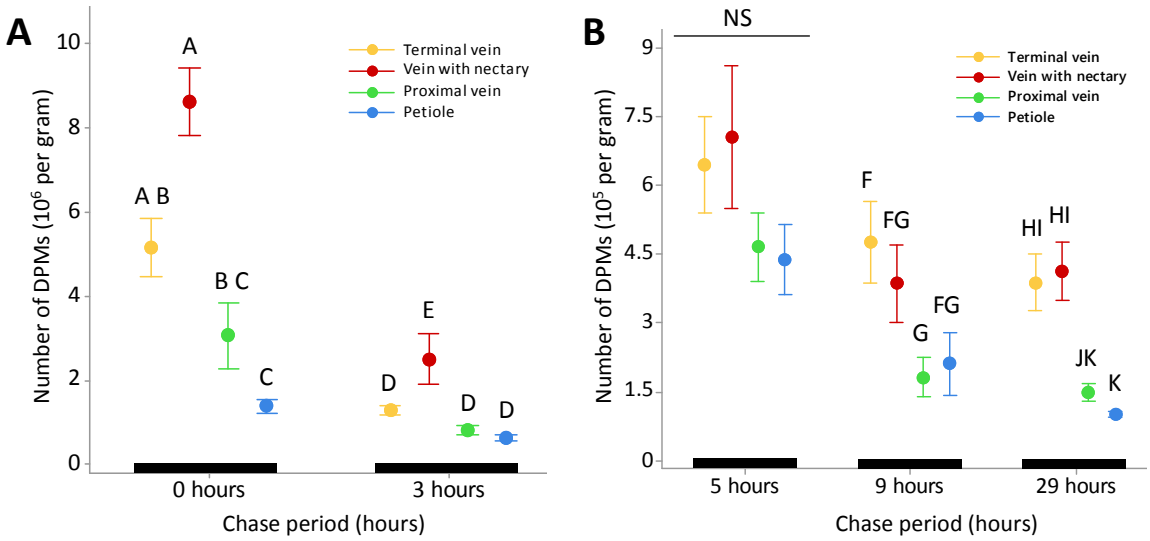

Figure 1-11. Radioactivity of the four vascular tissues at each time point in the chase period. Vertical bars represent ± SE of the mean; NS = non-significant at $p < 0.05$; n = 4; means that do not share a letter are significantly different (Tukey's HSD), comparisons only valid within each period. Note: the scale of (A) is an order of magnitude greater than the scale of (B).

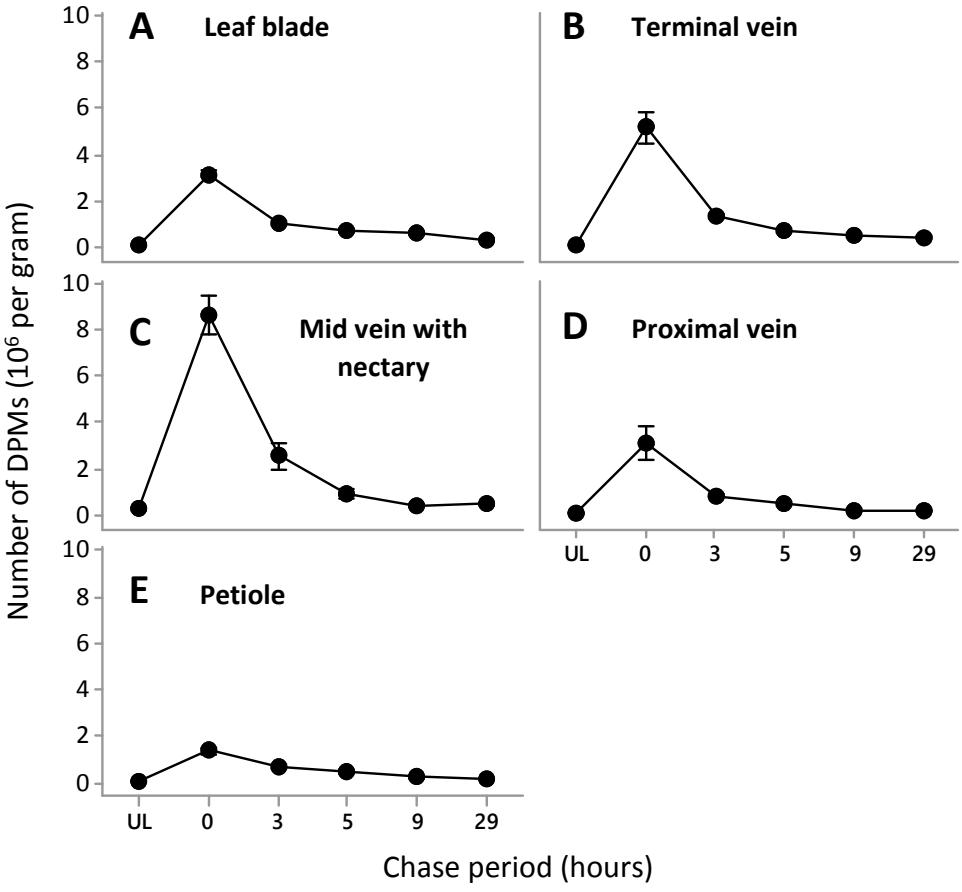

Figure 1-12. Patterns of decline in radioactivity over the chase period in the labelled leaf sections. Vertical bars represent ± SE of mean; n = 4, UL = unlabelled.

1.4.3 Sugar composition – phloem sap and extrafloral nectar

Both glucose and sucrose were present in foliar nectar and phloem sap, but fructose was only present in foliar nectar. Trace amounts of arabinose, galactose and rhamnose were detected in phloem sap, but these sugars were not present in foliar nectar. The mean (± SE) hexose to sucrose ratio in foliar nectar was 20-fold higher (10.0 ± 1.5) than in phloem sap (0.5 ± 0.1).

1.5 Discussion

1.5.1 Extrafloral nectary morphology and cellular anatomy

The EF nectaries of the four *Gossypium* species have different external morphologies, but their cellular anatomy and histochemistry are similar. Even though the colouration and shape of the EF nectaries are different, they are all 'pit nectaries' as classified by Zimmerman (1932, cited in Koptur 1992). The presence and abundance of non-glandular trichomes in and around the nectaries varies among species. The nectaries comprise glandular trichomes (unicellular head and a basal stalk cell) subtended by epidermal cells, nectary parenchyma and vascular tissue. The fluorescence of glandular trichomes under UV light indicated the presence of a suberised or lignified layer (Rost 1995). Histochemical staining showed the presence of starch only in the nectary parenchyma of *G. sturtianum* and *G. australe*. Sudan Black B staining revealed a suberised layer around the stalk cells and iron (III) chloride showed that all four species had phenols present in the glandular trichomes, but the concentration varied among the species. The presence and abundance of non-glandular trichomes was similar between *G. australe* and *G. bickii* and these were quite different to *G. sturtianum* and *G. robinsonii*. As expected, the cellular anatomy observed in all four species is the same and is consistent with the EF nectary anatomy of other members of the Malvaceae, such as *G. hirsutum* (Mound 1962; Wergin et al. 1975) and *Hibiscus pernambucensis* (Rocha & Machado 2009).

1.5.2 Nectar production pathway

Auto-fluorescence of the glandular trichomes and Sudan Black B staining showed there was a suberised layer in the glandular trichomes which acts in a similar fashion to a Casparian strip in the roots (Luttge 1971). This layer is likely located in the apoplastic zone and would force solutes to move through the symplastic pathway to enter the secretory cells (Duca 2015). A suberised layer is present in the secretory structures of many plants (Luttge 1971) and has been identified in other *Gossypium* species (Eleftheriou & Hall 1983). The presence of the suberised layer does not exclude the possibility of nectar moving through the apoplastic pathway before this point.

Staining with IKI solution showed starch granules in the nectaries of *G. sturtianum* and *G. australe*, suggesting that starch may be an intermediary form of carbohydrate storage in these structures. Until recently, EF nectar sugars were thought to be derived directly from photosynthesis (Pacini et al. 2003) and there are very few observations of EF nectaries using starch reserves for nectar secretion (Gaffal 2012). The presence of starch, however, is common in floral nectaries and is thought to be related to the high rates of nectar secretion required upon anthesis (Ren et al. 2007). Extrafloral nectar was abundant in *G. sturtianum* and *G. australe*, so starch granules may be present in EF nectaries of these species for a similar reason.

1.5.3 Sugar composition of extrafloral nectar and phloem sap

The ratio of hexoses to sucrose is 20-fold higher in EF nectar than in phloem sap which shows that EF nectar is not simply secreted phloem sap. These results are consistent with other studies that have compared the compositions of EF nectar and phloem sap within the same species e.g. Chanam et al. (2015) and Orona-Tamayo et al. (2013). It is generally accepted that sucrose is the dominant sugar in phloem sap with hexoses present only in very low concentrations, if at all (Dinant & Lemoine 2010). Recent studies by van Bel and Hess (2008) and Liu et al. (2012), however, have found high concentrations of hexoses in phloem sap, in some cases higher than the sucrose concentration. The hexose to sucrose ratio of 0.5 in phloem sap in the present study shows that hexoses and sucrose are both prevalent in *G. sturtianum* phloem sap.

1.5.4 Carbon metabolism

The ^{14}C labelling showed that carbon fixed by photosynthesis travels rapidly through the leaf and follows an exponential pattern of decline over time in each leaf section. The amount of ^{14}C present in the nectary section of the mid vein was the same as in the terminal vein and higher than the proximal vein and petiole in most of the chase periods. At 29 hours post-labelling, 7.6% of the ^{14}C was present in the adjacent unlabelled leaf and 1.4% was present in EF nectar from the labelled leaf. Throughout the chase period the ^{14}C levels in the nectary of the adjacent unlabelled leaf did not rise above background levels, showing that the labelled leaf was not a source of carbon for secretion of EF nectar by the adjacent leaf. The carbon cost of EF nectar for *G. sturtianum* was approximately 1% of net photosynthates for leaves actively secreting nectar.

The similar ^{14}C exponential decay curves for each vascular tissue section indicates that the nectary is not acting as a store for soluble sugars. The slightly higher radioactivity of the nectary section of the mid vein compared to other vascular tissues and the presence of ^{14}C in the EF nectar at the end of the chase period suggest that ^{14}C is moving through the nectary parenchyma to the glandular trichomes throughout the chase period. This species is known to store carbon in starch, so examining the radioactivity of EF nectar over a longer time period and the radioactivity of the insoluble leaf fraction would shed light on what proportion of the ^{14}C is captured as starch and how the starch is metabolised for secretion in EF nectar.

The absence of ^{14}C from the nectary of the adjacent unlabelled leaf does not rule out the possibility that carbon from other locations in the plant is being exuded in EF nectar. Radhika et al. (2008) traced the movement of ^{13}C in *Phaseolus lunatus* along a unidirectional gradient from older source leaves to younger sink leaves and into their EF nectar. Young leaves are carbon sinks (i.e. have a negative net photosynthetic rate), so they need to import carbon to synthesise defensive compounds (Arnold & Schultz 2002). Millán-Cañongo et al. (2014) showed in *Ricinus communis* that a reduction in photosynthesis of one leaf does not affect the EF nectar production of neighbouring leaves. The findings from the present study mean it is unlikely that dominant source of carbon in EF nectar is photosynthates from other places in the plant. Combining the

findings from the present study, Radhika et al. (2008), and Millán-Cañongo et al. (2014), suggests that the source of the sugars in EF nectar varies with the age of the leaf; older leaves fix enough carbon to produce the sugars for secretion in their EF nectar, whereas younger leaves are more likely to be importing carbon to use in EF nectar.

The carbon cost of EF nectar production for *G. sturtianum* in this study was just under 1% of the net photosynthates for leaves actively secreting nectar. This result is consistent with the small number of other studies that have calculated the investment in EF nectar. For example, Pate et al. (1985) showed that the investment by *Vigna unguiculata* in EF nectar was 0.1-2% of net photosynthates and Xu and Chen (2015) calculated that 0.9% of the total carbon fixed by *Clerodendrum philippinum* var. *simplex* plants is used in EF nectar. The method of calculating the investment of *G. sturtianum* in EF nectar in this study was an approximation because the photosynthetic rate used to calculate leaf sugar generation was based on a measurement at mid-morning. Photosynthetic rates vary diurnally and at mid-morning the rate is close to the maximum in many plants (Pallardy 2010). It is likely that these calculations overestimated the total leaf sugar generation and therefore the true carbon cost of *G. sturtianum* EF nectar may be higher than the 1% reported here.

1.5.5 Conclusions

This study is the first to examine the morphology and anatomy of EF nectaries in Australian native wild cottons. The results show that the EF nectaries of the four study species are comprised of glandular trichomes and have the same structure as other *Gossypium* EF nectaries. The phloem sap has a distinctly different composition from EF nectar, indicating that the phloem sap must be processed before secretion as EF nectar. The presence of a suberised layer around the stalk cells of the glandular trichomes indicates that the nectar solutes must travel, at least in part, through the symplasm. Further examination of the EF nectary ultrastructure and the formation and hydrolysis of starch in these four species is necessary to determine the full nectar pathway from the vascular tissue to the glandular trichomes for secretion.

The results of the $^{14}CO_2$ labelling show that the EF nectaries of *G. sturtianum* are not storing soluble carbohydrates and that carbon continuously moves through the EF nectary tissues. The labelled leaves in this study were carbon sources with enough photosynthates to produce EF nectar and export carbon to other locations in the plant. The carbon cost of EF nectar in this study is consistent with other species' investments in EF nectar. Further work with a broader focus could examine EF nectar and other indirect defence mechanisms to see how carbon allocation varies with development and assess the absolute carbon costs of nectar-based defence strategies.

Chapter 2

Response of Australian native wild cotton (*Gossypium* spp.; Malvaceae) extrafloral nectar production and composition to elevated carbon dioxide

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Running title: Responses of extrafloral nectar to elevated CO_2

2.1 Abstract

Extrafloral (EF) nectar underpins many plant-insect interactions worldwide and comprises an indirect defence mechanism. EF nectar attracts ants and their presence provides protection for the plant against herbivores. Any environmental changes that affect EF nectar production or composition could have flow-on effects for communities and ecosystems. In particular, it is important to understand the impact of rising atmospheric carbon dioxide (CO_2) levels on EF nectar. This study tested the response of EF nectar production and composition to increased CO_2, representing the first investigation of elevated CO_2 impacts on any EF nectar system. The total volume and composition of EF nectar from plants grown in elevated CO_2 conditions did not differ from plants grown in ambient CO_2 conditions, but there was evidence of accelerated plant development and a change in EF nectar allocation within plants. Developmental changes due to elevated CO_2 could affect the timing of EF nectar production which could have flow-on effects to ant mutualists and their defence of plants.

Keywords: carbon dioxide, cotton, development, extrafloral nectaries, indirect defence, nectar, sugar

2.2 Introduction

Interactions between plants and insects dominate ecosystems globally and are essential for ecosystem function (McCluney et al. 2012). One of the major resources sustaining plant-insect interactions is nectar (Heil 2011). Nectar is a sugary secretion produced by flowering plants and some ferns (Baker et al. 1978b; Koptur et al. 2013) to attract pollinators and mutualists for defence (Pacini et al. 2003; Mayer et al. 2014). Nectar can be produced both within flowers (floral nectar) and from specialised nectaries on vegetative tissues, such as leaves, stems and petioles (extrafloral (EF) nectar; Marazzi et al. 2013). Extrafloral nectar is produced by over 100 families of plants worldwide (Weber & Keeler 2013). Their presence, location and function varies during development, but EF nectar is generally produced around young leaves and developing flowers (Heil et al. 2000).

The production of EF nectar is a cost to the plant, requiring an investment of organic resources and thus energy; the trade-off for this cost is a benefit to the plant (O'Dowd 1980; Rutter & Rausher 2004). Ants are attracted to the nectaries and by virtue of their presence plants benefit from a reduction in herbivory (Heil & McKey 2003). Extrafloral nectaries and their associated nectar therefore form an indirect defence mechanism (Kessler & Heil 2011).

Changes in environmental conditions that alter the volume or composition of EF nectar could have flow-on effects on mutualistic ant populations (Rudgers & Gardener 2004; Wilder & Eubanks 2010). For example, Ness (2003) and Bixenmann et al. (2011) found that an increase in the sugar content of EF nectar was associated with an increased total number of ants attracted to leaves, indicating that higher EF nectar sugar concentration could increase the overall defence provided by ant mutualists. Similarly, an increased volume of EF nectar could maintain more ants on a plant and therefore provide a more comprehensive defence.

Extrafloral nectar is composed mainly of sugars and amino acids, but can also include small amounts of other substances (Bentley 1977a). The sugars in EF nectar are sucrose, fructose and glucose (Bentley & Elias 1983; Nicolson et al. 2007) and in the majority of cases EF nectar is dominated by hexoses. However, the proportions of the three sugars

can vary with species, type of nectar (e.g. foliar vs bracteal) and environmental conditions (Blüthgen et al. 2004; Heil 2015).

Extrafloral nectar can be produced constitutively to support obligate ant species (González-Teuber & Heil 2009; Escalante-Perez et al. 2012) or induced in response to herbivory (Ness 2003; Mondor et al. 2013). The volume of EF nectar produced varies widely between plants, between nectaries and is temporally variable, over timescales ranging from hours to days and even seasons (Escalante-Perez et al. 2012; González-Teuber et al. 2012).

Increasing atmospheric CO_2 is predicted to affect plant chemistry and phenology, and in turn potentially disrupt plant-insect interactions (Root & Hughes 2006). Atmospheric CO_2 levels have been rising since the industrial revolution (IPCC 2013) and in 2015 a global average CO_2 level of over 400 ppm was reached for the first time since record keeping began (Dlugokencky & Tans 2015). The sheer number of plant-insect interactions worldwide means that atmospheric changes could disrupt ecosystems across the world.

In general, increased atmospheric CO_2 is expected to lead to an increase in the photosynthetic rate of C3 plants by increasing the rate of carbon fixation by Rubisco (Leakey et al. 2012). This increase in photosynthesis leads to increased availability of organic compounds, if no other resources are limiting, so more carbon-based resources are available for allocation to growth, reproduction and defence (Ainsworth & Rogers 2007). An increase in photosynthesis from elevated CO_2 may result in increased plant growth, but this growth may be constrained by other abiotic factors, such as low soil fertility or low water availability (Robinson et al. 2012). Stomatal conductance is reduced under elevated CO_2, leading to reduced evapotranspiration and increased water use efficiency (Eamus 1991). Thus, reduced stomatal conductance, through feedback control, constrains theoretical photosynthetic gains under climate change (Ainsworth & Rogers 2007).

While there have been several studies published on the response of floral nectar to elevated CO_2, none have been published on the impact on EF nectar volume and composition. Studies of floral nectar production and composition under elevated CO_2 conditions show a variable response to elevated CO_2 (Table S-4). Nectar volume can

increase (Lake & Hughes 1999), decrease (Rusterholz & Erhardt 1998) or remain the same (Osborne et al. 1997) under elevated CO_2. Multiple studies on nectar sugar concentration have shown that nectar sugar concentration does not change under elevated CO_2 (Lake & Hughes 1999; Dag & Eisikowitch 2000; Erhardt et al. 2005). Overall, the majority of studies show an increase in nectar volume but no change in nectar sugar concentration under elevated CO_2.

This study investigated the impact of elevated CO_2 on EF nectar production and composition in two native Australian wild cottons, *Gossypium sturtianum* and *G. australe*. These native wild cotton species are from the same genus as cultivated cotton species (Fryxell 1978). Australian native cottons have EF nectaries present on a variety of their tissues and some species produce a large amount of nectar. These characteristics make these species ideal for testing the impact of elevated CO_2 on EF nectar.

The first objective of this study was to determine whether the total volume of EF nectar produced, either per nectary or per plant, changes under elevated CO_2. The increase in carbon resources under elevated CO_2 conditions could have a number of impacts on the nectar volume per plant and/or per leaf (Figure S-2), such as:

- Increased resources could lead to an increase in the growth rate (Ainsworth & Rogers 2007), which could result in an increase in the total number of leaves. Assuming EF nectary formation is proportional to leaf number, this would result in a greater number of nectaries per plant;
- A change in the proportion of leaves which are actively producing nectar (hereafter referred to as 'active leaves');
- A change in the volume of nectar produced per nectary or per leaf (i.e. more nectaries active on each leaf).

Any or all of these changes could occur in response to elevated CO_2 conditions. When taken together, many of these changes could result in an increased total volume of EF nectar. The second objective of this study was to test whether there are qualitative changes in EF nectar composition under elevated CO_2. Even though an increase in carbon resources would be expected to translate into increased nectar production, results from the majority of floral nectar studies under elevated CO_2 show that nectar sugar composition does not change.

2.3 Materials and Methods

2.3.1 Plant material

G. sturtianum and *G. australe* plants were grown from seed at the Macquarie University Plant Growth Facility (Sydney, Australia; see section 1.3.1). Growth conditions were: 30/22 ± 2°C (day/night 14/10 hours), supplementary lighting (Philips Contempa High Pressure Sodium lamps) of 400 µmoles m^{-2} s^{-1} when ambient light level dropped below 400 µmoles m^{-2} s^{-1}. Plants were watered every second day and fertilised once a week with 0.5 g/L Aquasol water soluble fertiliser (N:P:K = 23:3.95:14; Yates Australia, Padstow, NSW, Australia).

2.3.2 Carbon dioxide treatment

Forty *G. sturtianum* and sixteen *G. australe* plants aged between six and nine months were randomly divided into equal groups and placed in four growth cabinets (model PG.15.18.9 TD.5x100R; Thermoline Scientific, Wetherill Park, NSW, Australia). Two cabinets had atmospheric carbon dioxide of 400 ppm (ambient) and two cabinets were maintained at 700 ± 20 ppm (custom built CO_2 Monitoring & Dosing System, The Canary Company, Lane Cove, NSW, Australia). All other conditions in the cabinets were constant: 30/18 ± 1°C (day/night 14/10 hours), light at 1800 ± 50 µmol m^{-2} s^{-1} (metal halide multi vapour lamps; GE Appliances & Lighting, Richmond, VIC, Australia; measured using LiCor Biosciences light meter, model LI-250A). Plants were watered every second day and the relative humidity was maintained at 90 ± 10%. The plants in each cabinet were re-randomised once a week to minimise any intra-cabinet variation.

2.3.3 Nectar collection

At four, six and eight weeks after carbon dioxide treatments commenced EF nectar was collected from the *G. sturtianum* and *G. australe* plants after allowing it to accumulate for five days each time (Table 2-1). The nectar from all the foliar nectaries and bracts was collected and separately pooled for each plant using the same methodology as outlined

in section 1.3.4. Some *G. australe* plants died during the experimental period so the number of plants from which nectar was collected varied at each time point. The number of surviving *G. australe* plants in the eCO_2 treatment was too small for statistical analysis at the third time point so no plants were sampled.

Table 2-1. Number of plants from which extrafloral nectar was collected after a five-day nectar accumulation.

Weeks in treatment	GS foliar nectaries		GS bracteal nectaries		GA foliar nectaries	
	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
4	20	20	3	7	8	6
6	20	20	9	12	8	3
8	20	20	4	12	NS	NS

GS = *G. sturtianum*; GA = *G. australe*; NS = not sampled.

2.3.4 Carbon budget

To calculate the proportion of the leaf carbon pool allocated to EF nectar under ambient and elevated CO_2 conditions, a comparison was made between the estimated amount of sugar produced by a leaf, based on net photosynthetic rates, and the amount of sugar exuded in the EF nectar. The nectar of three leaves per plant in each treatment (n =16) accumulated for 48 hours and was collected using the methodology outlined in section 1.3.3. The photosynthetic rates of the leaves were measured using a LiCor Li-6400XT portable photosynthesis system (LiCor Biosciences, NE, USA) and leaf areas were calculated from photographs using Image J software (Schneider et al. 2012).

2.3.5 Nectar sugar composition

The sugar composition of *G. sturtianum* foliar (n = 8) and bracteal (n = 4) nectar, and *G. australe* foliar nectar (n = 4) was analysed using osmometry, refractometry and HPLC. The volume of nectar collected was low for some plants, so not all replicates have been used for composition analysis. EF nectar sugar concentrations were measured using the HPLC method outlined in section 1.3.5.

The EF nectar osmolality was measured using freezing point depression with a Fiske One-Ten Osmometer (calibrated using MilliQ water and calibration standards; Fiske & Associates, Massachusetts, USA). Neat nectar was unable to be frozen by the osmometer. This was thought to be due to the high sugar concentration of the nectar, so the nectar samples were diluted 1:50 with MilliQ water. These samples were frozen by the osmometer and the osmolality of the nectar was determined. The osmolality of the samples was adjusted to account for the 1:50 dilution.

The EF nectar sugar concentration was determined using an Abbé refractometer (Officine Galileo, Florence, Italy). The sugar concentration was measured in sucrose-equivalents (% sucrose = g sucrose / 100 g water). The refractometer was calibrated using MilliQ water and readings were taken at 25^{o}C ± 1^{o}C. Refractometer readings are correct at 20^{o}C (Hirschmüller 1953) but as the maximum temperature correction for sucrose when working in a temperature range of 15-30^{o}C is less than 1% sucrose w/w (Corbet 2003) no correction has been applied. As EF nectar is not a pure sucrose solution (contains sucrose, glucose and fructose) measurements of the sugar content by a refractometer (calibrated for measuring sucrose) underestimate the actual sugar content (Marov & Dowling 1990). The underestimation of total sugars in a 100% hexose solution is not more than 2% when converted to sucrose-equivalents (Corbet 2003) so no correction has been applied.

Extrafloral nectar volume and concentration is affected by environmental factors, such as humidity and temperature (Corbet 2003), so a number of methods of reporting nectar composition have been used. Nectar volumes, concentrations, proportions and masses of sugars have been reported to provide information about investment by the plant.

2.3.6 Statistical analysis

Statistical analysis was conducted using Minitab® Statistical Software (Minitab Inc 2015). The significance level for all analyses was 0.05 and error bars represent the standard error of the mean. Photosynthetic rate and nectar volume per active leaf were correlated using multiple linear regression. The proportion of flowering plants for each time point

and treatment was compared using a Pearson's chi-squared. All other statistical analyses and data transformations for ambient vs elevated CO_2 treatment comparisons are outlined in Table 2-2. Due to the low rate of flowering by *G. australe* plants, no statistical analysis related to flowering was conducted.

A cabinet effect in the elevated CO_2 treatment was detected for nectar production data collected in week 8 (Table S-5). This effect is thought to be due to a temporary problem with the temperature control in one growth cabinet. As no cabinet effects were found in any other week it is thought to be an artifact and not a true biological difference. The cabinet effect resulted in a significant difference between CO_2 treatments for the number of active leaves per *G. sturtianum* plant when otherwise this may not have occurred.

Table 2-2. Statistical tests and data transformations applied for data comparisons between the ambient and elevated CO_2 treatments. Not all comparisons were conducted for both species and some did not require data transformation*. Tukey's HSD post-hoc test were conducted for two-way and one-way ANOVAs where necessary.

Comparison	Statistical test				Data transformation*	
	Repeated measures two-way ANOVA	Independent two-way ANOVA	One-way ANOVA	Student's t-test with unequal variances	Natural log	Square root
Photosynthetic rate			GS			
Proportion of total leaf sugar exuded in foliar nectar			GS			
Total number of leaves per plant	GS GA					
Number of active leaves per plant	GS GA					
Proportion of active leaves	GS GA					
Total foliar nectar volume	GS GA				GS	GA
Nectar volume per active leaf	GS GA					
Foliar nectar volume with and without flowers			GS[1]			GS
Total bracteal nectar volume	GS					
Concentration of each sugar species		GA[f]		GS[1]		
Hexose : sucrose ratio				GS[f] GA[f]	GS[f]	
Osmolality of foliar and bracteal nectar			GS[f,b] GA[f]		GA[f]	
% sucrose-equivalents in foliar and bracteal nectar			GS[f,b] GA[f]			
Total sugar production		GS[f] GA[f]			GS[f]	

GS = *G. sturtianum*; GA = *G. australe*; [1] = individual test performed for each week or sugar species; [f] = foliar nectar; [b] = bracteal nectar.

2.4 Results

2.4.1 Carbon budget

The photosynthetic rate under elevated CO_2 conditions was 21% higher than under ambient CO_2 conditions ($F_{1,30} = 4.73$, $p = 0.04$; Figure 2-1A). The proportion of estimated sugar production per leaf that was secreted in foliar nectar was 29% lower in elevated CO_2 conditions, but this difference was not statistically significant ($F_{1,14} = 1.36$, $p = 0.26$; Figure 2-1B).

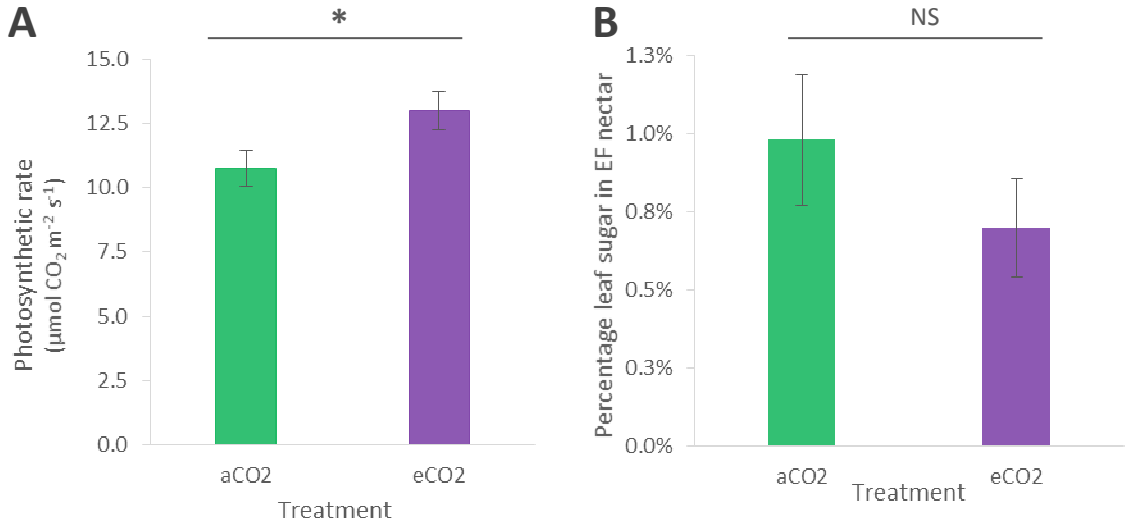

Figure 2-1. *G. sturtianum* (A) photosynthetic rate (n = 16); and (B) proportion of estimated sugar production per leaf exuded in foliar nectar after eight weeks in ambient (aCO_2) and elevated (eCO_2) conditions (n = 8). Vertical bars represent ± SE of the mean; * = significant at $p < 0.05$; NS = not significant at $p < 0.05$.

Multiple linear regression showed that nectar per active leaf depended on both the treatment and the photosynthetic rate ($F_{1,28} = 2.37$, $p = 0.025$) with photosynthetic rate accounting for 43% of the variability in nectar volume per active leaf (Figure 2-2). There was a positive correlation between photosynthetic rate and nectar volume per active leaf for the ambient CO_2 treatment, and a negative correlation for the elevated CO_2 treatment (Table S-6).

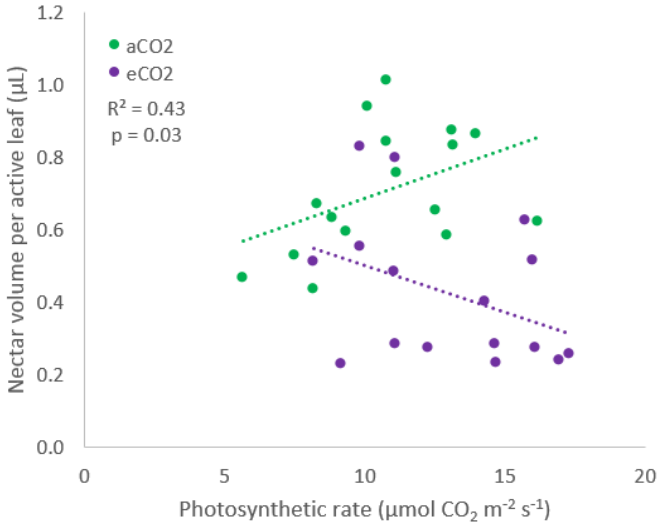

Figure 2-2. Linear regression of *G. sturtianum* photosynthetic rate and nectar volume per active leaf after eight weeks of ambient (aCO_2) and elevated (eCO_2) treatment (n = 16).

2.4.2 Nectar production

Foliar nectar – *G. sturtianum*

The total number of leaves per plant was not significantly different between the ambient and elevated CO_2 treatments ($F_{1,76} = 0.74$, $p = 0.396$). The total number of leaves increased with time ($F_{2,76} = 160.56$, $p < 0.001$). There was no significant interaction between time and CO_2 treatment ($F_{2,76} = 1.80$, $p = 0.17$). There was no change in total number of leaves between weeks four and six for either treatment, but by week eight the total number of leaves had increased in both treatments (Figure 2-3A).

There was a significant difference in the number of active leaves between the ambient and elevated CO_2 treatments ($F_{1,76} = 7.91$, $p = 0.008$) and at each time point ($F_{2,76} = 86.72$, $p < 0.001$). There was no significant interaction between time and treatment ($F_{2,76} = 2.37$, $p = 0.10$). There was a significant difference in the total number of active leaves between the two elevated CO_2 growth cabinets at eight weeks (Tukey's HSD). The number of active leaves was the same between treatments at four and six weeks, but diverged at eight weeks. The number of active leaves consistently rose in elevated CO_2 from four to eight weeks, and in ambient CO_2 the number of active leaves rose from four to six weeks then levelled off between six and eight weeks (Figure 2-3B).

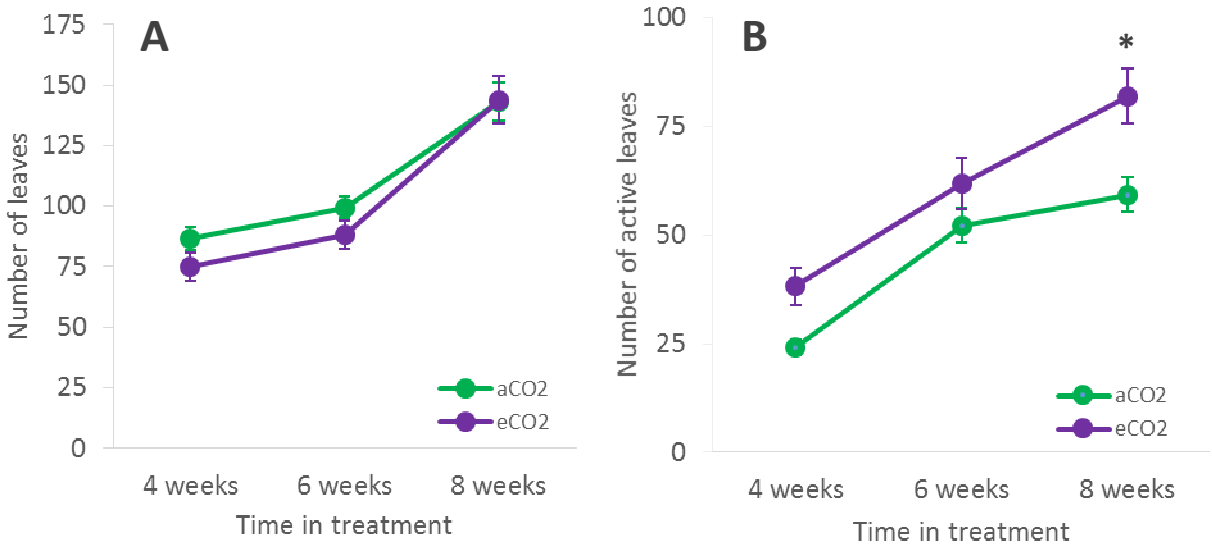

Figure 2-3. Number of (A) total leaves and (B) active leaves per *G. sturtianum* plant under ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the CO_2 treatment (n = 20). Vertical bars represent ± SE of the mean; * = significant at $p < 0.05$.

There was a significant difference in the proportion of active leaves between ambient and elevated CO_2 treatments ($F_{1,76} = 42.07$, $p < 0.001$) and over time ($F_{2,76} = 32.58$, $p < 0.001$). There was no significant interaction between time and treatment ($F_{2,76} = 0.73$, $p = 0.48$). The proportion of active leaves was variable over time in both treatments but was always significantly higher under elevated CO_2 compared with ambient CO_2 (Figure 2-4).

There was a significant interaction between treatment and time for total foliar nectar volume per plant ($F_{2,76} = 8.42$, $p < 0.001$). At each time point, the difference in total foliar nectar volume per plant between the two treatments was not statistically significant (Tukey's HSD). The total foliar nectar volume per plant in ambient CO_2 consistently increased over time, whereas it was constant over time in the elevated CO_2 treatment (Figure 2-5A).

A significant interaction between treatment and time was observed for nectar volume per active leaf ($F_{2,76} = 20.81$, $p < 0.001$). The nectar volume per active leaf in the ambient CO_2 treatment was constant, whereas nectar volume per active leaf in the elevated CO_2 treatment decreased over time, resulting in a significant difference between the treatments at eight weeks (Figure 2-5B).

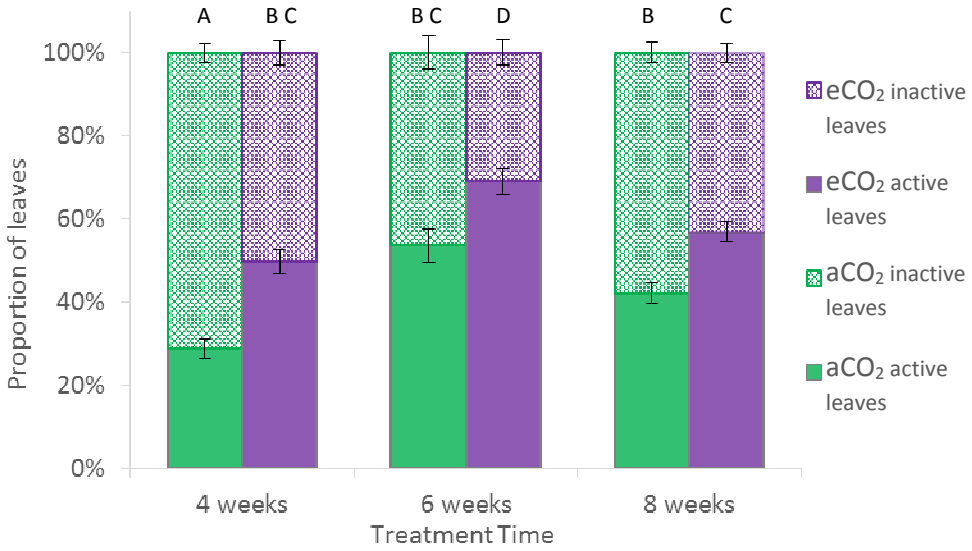

Figure 2-4. Proportion of *G. sturtianum* leaves actively producing nectar under ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the CO_2 treatment (n = 20). Vertical bars represent ± SE of the mean; columns that do not share a letter are significantly different (Tukey's HSD).

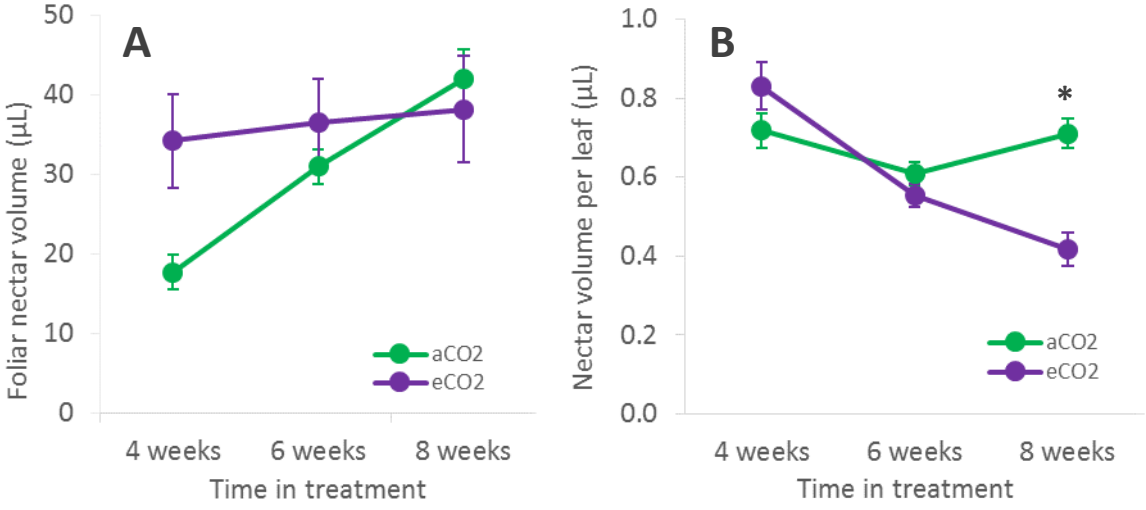

Figure 2-5. (A) Total foliar nectar volume per plant; and (B) Nectar volume per active leaf for *G. sturtianum* under ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the CO_2 treatment (n = 20). Vertical bars represent ± SE of the mean; * = significant at $p < 0.05$.

There was no significant difference in the number of flowering plants after four weeks (χ^2 = 1.29, df = 1, p = 0.26) and six weeks (χ^2 = 0.40, df = 1, p = 0.53) of treatment, but at eight weeks the number of flowering plants was significantly greater in elevated CO_2 (χ^2 = 6.67, df = 1, p = 0.01). At each time point, there were more plants flowering in elevated CO_2 than in ambient CO_2 (Figure 2-6A).

There was no significant difference between the total foliar nectar volume of flowering and non-flowering plants within each CO_2 treatment at four weeks ($F_{1,36}$ = 0.55, p = 0.46), six weeks ($F_{1,36}$ = 0.09, p = 0.76) or eight weeks ($F_{1,36}$ = 1.87, p = 0.18). Flowering did not affect the total volume of foliar nectar produced under ambient or elevated CO_2 conditions (Figure 2-6B).

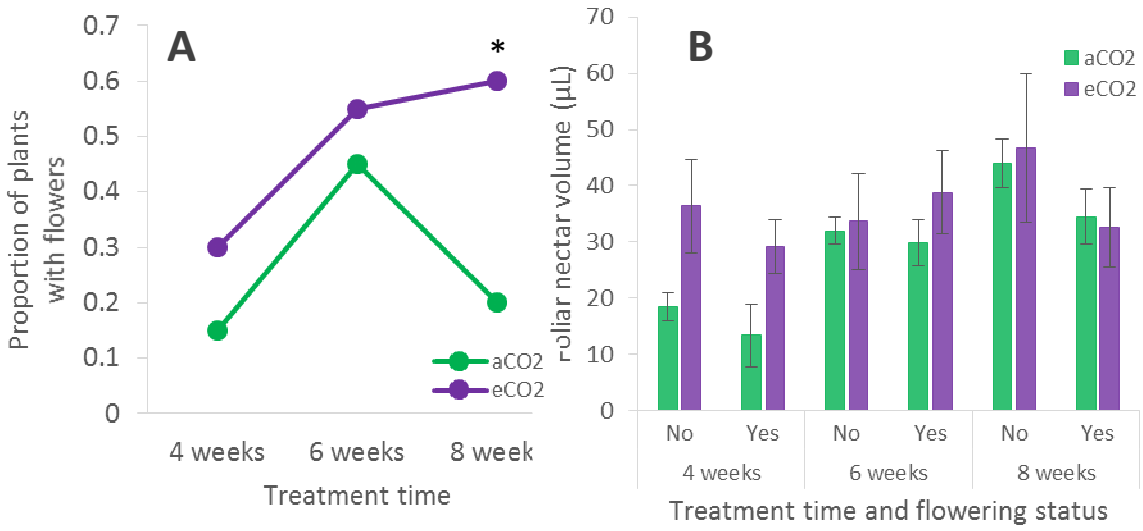

Figure 2-6. (A) Proportion of *G. sturtianum* plants flowering; and (B) Total foliar nectar produced by *G. sturtianum* plants with and without flowers at each stage of the ambient (aCO_2) and elevated (eCO_2) treatments. For all weeks and treatments total n = 20; number of flowering plants: four weeks aCO_2 n = 3, eCO_2 n = 6; six weeks aCO_2 n = 9, eCO_2 n = 11; eight weeks aCO_2 n = 4, eCO_2 n= 12. (A) shows proportions of plants, hence without variance. Vertical bars represent ± SE of the mean; * = significant at $p < 0.05$.

Foliar nectar – *G. australe*

The total number of leaves per plant was significantly different between the ambient and elevated CO_2 treatments ($F_{1,21} = 6.35$, $p = 0.02$), but not between time points ($F_{1,21} = 1.24$, $p = 0.28$). There was no significant interaction between time and treatment ($F_{1,21} = 0.56$, $p = 0.46$). At each time point, the difference in total number of leaves between the two treatments was not statistically significant (Tukey's HSD). The total number of leaves increased slightly between four and six weeks in both treatments (Figure 2-7A).

There was no significant difference in the number of active leaves between the ambient and elevated CO_2 treatments ($F_{1,9} = 1.43$, $p = 0.25$) or between time points ($F_{1,9} = 0.26$, p 0.62). There was no significant interaction between time and treatment ($F_{1,9} = 0.26$, $p = 0.62$). The number of active leaves was the same over time in ambient CO_2 and rose slightly over time in elevated CO_2 (Figure 2-7B).

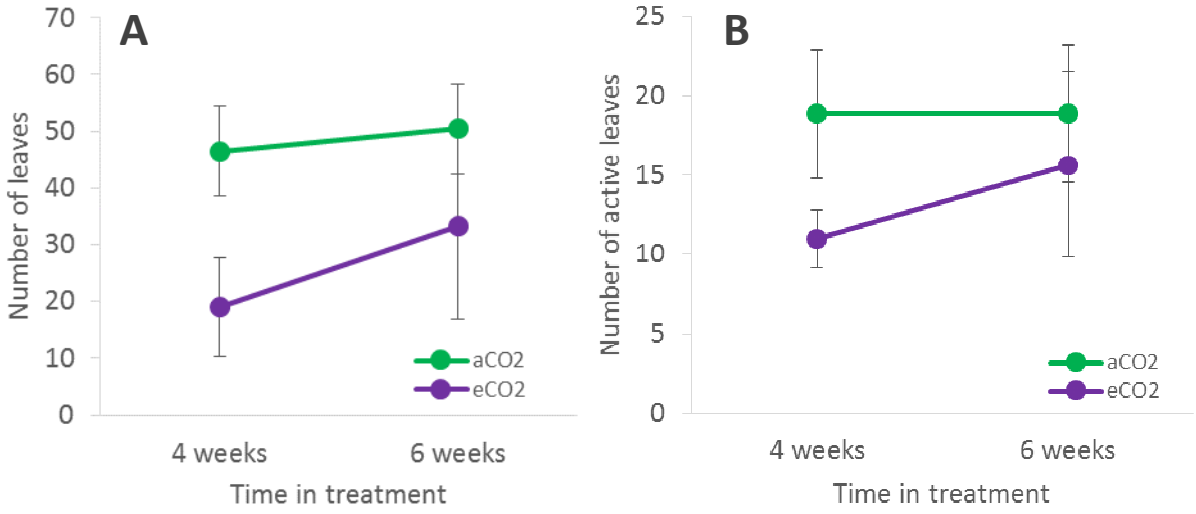

Figure 2-7. (A) Total number of leaves; and (B) Number of active leaves per *G. australe* plant under ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the CO_2 treatment. Replicates: aCO_2 n = 8 at both time points; four weeks eCO_2 n = 6; six weeks eCO_2 n = 3. Vertical bars represent ± SE of the mean.

There was a significant difference in the proportion of active leaves between treatments ($F_{1,9} = 9.52$, $p < 0.007$), but not over time ($F_{1,9} = 3.37$, $p = 0.10$). There was no significant interaction between time and treatment ($F_{1,9} = 2.69$, $p = 0.14$). The proportion of active leaves was always higher under elevated CO_2 compared with ambient CO_2 (Figure 2-8).

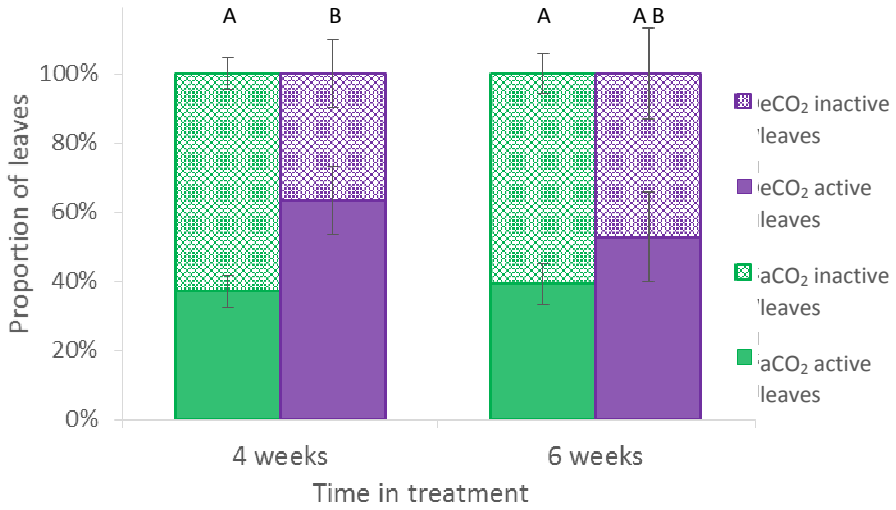

Figure 2-8. Proportion of *G. australe* leaves actively producing nectar under ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the CO_2 treatment. Replicates: aCO_2 n = 8 at both time points; four weeks eCO_2 n = 6; six weeks eCO_2 n = 3. Vertical bars represent ± SE of the mean; columns that do not share a letter are significantly different (Tukey's HSD).

There was no significant difference in the total foliar nectar volume between treatments ($F_{1,9} = 3.13$, $p = 0.10$) or over time ($F_{1,9} = 0.04$, $p = 0.84$). There was no interaction between time and treatment ($F_{1,9} = 0.02$, $p = 0.90$). Total foliar nectar volume was higher in ambient CO_2 compared with elevated CO_2 at both time points and there was a slight increase in total foliar nectar volume in both treatments over time (Figure 2-9A).

There was no significant difference in the nectar volume per active leaf between treatments ($F_{1,9} = 4.29$ $p = 0.06$) or over time ($F_{1,9} = 0.79$, $p = 0.40$). There was no interaction between time and treatment ($F_{1,9} = 0.47$ $p = 0.51$). The mean nectar volume per active leaf was higher in ambient CO_2 compared with elevated CO_2 at both time points and there was a slight decrease in nectar volume per active leaf in both treatments over time (Figure 2-9B).

Bracteal nectar – *G. sturtianum*

There was no significant difference in the total bracteal nectar volume over time ($F_{2,18} = 1.20$, $p = 0.32$) or between treatments ($F_{1,18} = 3.46$, $p = 0.07$). At each time point, the total bracteal nectar volume in the elevated CO_2 treatment was greater than in ambient CO_2 (Figure 2-10A). There was a large range of bracteal nectar volumes in both the ambient and elevated CO_2 treatments (Figure 2-10B).

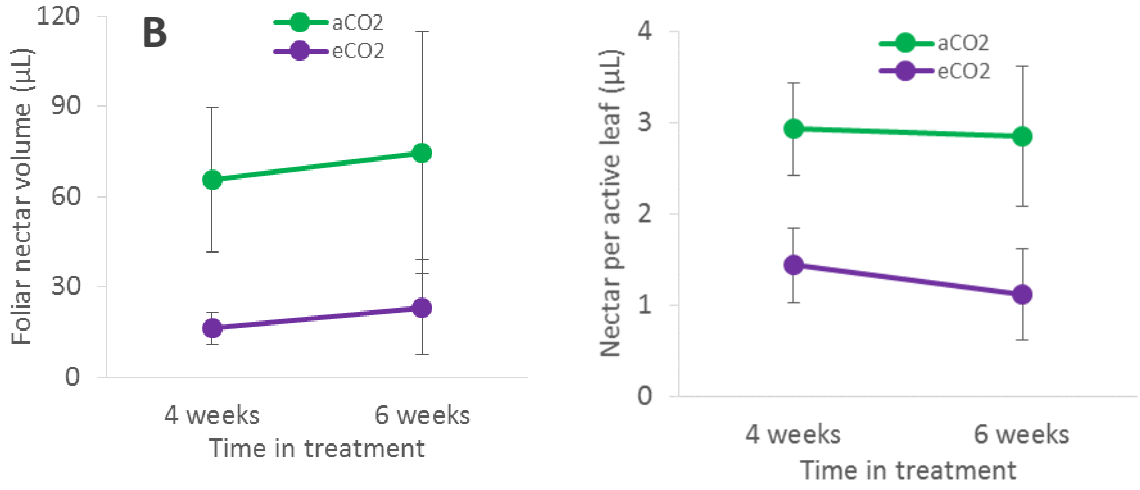

Figure 2-9. *G. australe* (A) Total foliar nectar volume per plant; and (B) Nectar volume per active leaf under ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the CO_2 treatment. Replicates: aCO_2 n = 8 at both time points; four weeks eCO_2 n = 6; six weeks eCO_2 n = 3. Vertical bars represent ± SE of the mean.

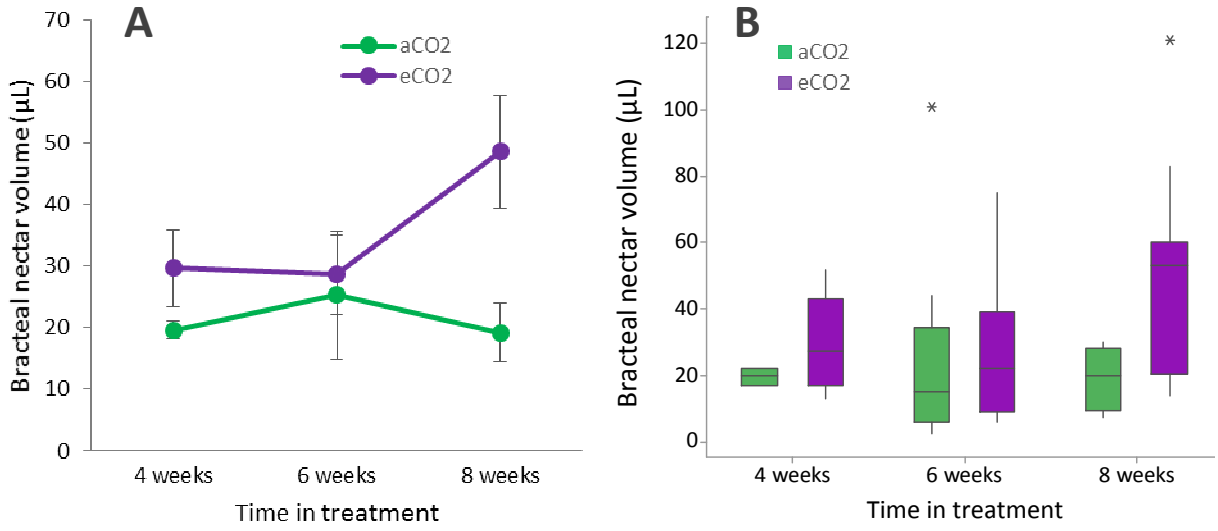

Figure 2-10. *G. sturtianum* (A) Total bracteal nectar volume per plant; and (B) Range of bracteal nectar volumes in ambient (aCO_2) and elevated (eCO_2) conditions at each stage of the treatment. Replicates: four weeks aCO_2 n = 3, eCO_2 n = 6; six weeks aCO_2 n = 9, eCO_2 n = 12; eight weeks aCO_2 n = 4, eCO_2 n = 12. Vertical bars represent ± SE of the mean.

2.4.3 Sugar composition

Foliar and bracteal nectar – *G. sturtianum*

There was no significant difference in concentration between the ambient and elevated CO_2 treatments for fructose ($T_{12} = 0.57$, $p = 0.57$), glucose ($T_{12} = 0.39$, $p = 0.70$) or sucrose ($T_8 = 1.73$, $p = 0.12$). The variation in fructose concentrations was much higher than the variances in glucose and sucrose concentrations (Figure S-3). There was no significant difference in the hexose to sucrose ratio of *G. sturtianum* foliar nectar under ambient and elevated CO_2 treatments ($T_9 = 1.99$, $df = 9$, $p = 0.08$; Table 2-3).

The osmolality (amount of dissolved solutes) of *G. sturtianum* foliar nectar was significantly higher under the elevated CO_2 treatment, compared with ambient CO_2 ($F_{1,14} = 7.94$, $p = 0.01$), but there was no significant difference in the osmolality of *G. sturtianum* bracteal nectar between the two treatments ($F_{1,6} = 2.18$, $p = 0.19$; Table 2-3). The % sucrose-equivalents of *G. sturtianum* foliar nectar was higher under the elevated CO_2 treatment, compared with ambient CO_2 ($F_{1,14} = 6.68$, $p = 0.02$), but there was no significant difference in the % sucrose-equivalents of *G. sturtianum* bracteal nectar between the two treatments ($F_{1,6} = 3.23$, $p = 0.12$; Table 2-3).

Table 2-3. *G. sturtianum* foliar and bracteal nectar composition after four weeks of ambient and elevated CO_2 treatment. ± SE of the mean; sugar concentration in µg/µL.

Measure of composition	Foliar nectar (n = 8)			Bracteal nectar (n = 4)		
	Ambient CO_2	Elevated CO_2	Sig	Ambient CO_2	Elevated CO_2	Sig
Fructose	398 ± 22	420 ± 33	NS	nd	nd	nd
Glucose	201 ± 9	197 ± 6	NS	nd	nd	nd
Sucrose	73 ± 13	96 ± 4	NS	nd	nd	nd
Hexose : Sucrose	10 ± 2	7 ± 1	NS	nd	nd	nd
Osmolality (Osm)	5.6 ± 0.3	7.8 ± 0.8	*	6.0 ± 0.7	8.1 ± 1.3	NS
% Sucrose-equivalents (g per 100 g water)	105 ± 6	131 ± 8	*	91 ± 12	134 ± 21	NS

Sig = significance; * = significant at $p < 0.05$; NS = not significant at $p < 0.05$; nd = not determined

Total sugar production at four weeks was calculated by multiplying the EF nectar sugar concentration by the total foliar nectar volume per plant. There was a significant difference between sugar species in *G. sturtianum* foliar nectar sugar production ($F_{2,42}$ = 47.77, p < 0.001) and between the ambient and elevated CO_2 treatments ($F_{1,42}$ = 46.47, p < 0.001; Figure 2-11). There was no significant interaction between sugar species and treatment ($F_{2,42}$ = 0.18, p = 0.83). Sugar production was higher in the elevated CO_2 treatment compared with ambient CO_2 and in each treatment fructose had the highest production followed by glucose and then sucrose.

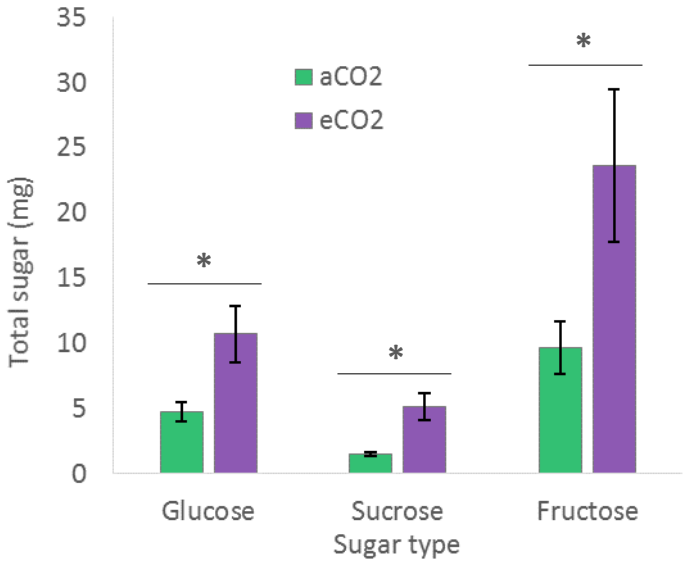

Figure 2-11. Sugar production by *G. sturtianum* foliar nectaries after four weeks of ambient (aCO_2) and elevated (eCO_2) treatment (n = 8). Sugar production calculated by multiplying the EF nectar sugar concentration by the total foliar nectar volume per plant. Vertical bars represent ± SE of the mean; * = significant at p < 0.05.

Foliar nectar – *G. australe*

There was a significant difference between sugar species in *G. australe* foliar nectar sugar concentrations ($F_{2,18}$ = 101.63, p < 0.001) and between the ambient and elevated CO_2 treatments ($F_{1,18}$ = 8.16, p = 0.01; Table 2-4). There was no significant interaction between sugar species and treatment ($F_{2,18}$ = 0.86, p = 0.44). The concentration of each sugar was greater in elevated CO_2 than in ambient CO_2, but the differences were not significant (Tukey's HSD). There was no significant difference in the hexose to sucrose ratio ($F_{1,6}$ = 2.34, p = 0.18), the osmolality ($F_{1,6}$ = 3.94, p = 0.09), or the % sucrose-equivalents ($F_{1,6}$ = 1.85, p = 0.22) of *G. australe* foliar nectar under ambient and elevated CO_2 treatments (Table 2-4).

Table 2-4. *G. australe* foliar nectar composition after four weeks of ambient (aCO_2) and elevated (eCO_2) treatment (n = 8). ± SE of the mean; sugar concentrations in µg/µL.

Composition measure	**aCO_2**	**eCO_2**	**Significance**
Fructose	304 ± 37	384 ± 22	NS
Glucose	157 ± 14	196 ± 18	NS
Sucrose	34 ± 11	61 ± 9	NS
Hexose : Sucrose	17 ± 4	10 ± 2	NS
Osmolality (Osm)	5 ± 1	7 ± 1	NS
Sucrose-equivalents (g per 100 g water)	88 ± 17	116 ± 12	NS

NS = not significant at $p < 0.05$

Total sugar production at four weeks was calculated by multiplying the EF nectar sugar concentration by the total foliar nectar volume per plant. There was a significant difference in *G. australe* foliar nectar sugar production between the three sugar species ($F_{2,18} = 70.07$, $p < 0.001$) and overall between ambient and elevated CO_2 ($F_{1,18} = 5.61$, $p = 0.03$; Figure 2-12). There was no significant interaction between sugar species and treatment ($F_{2,18} = 1.11$, $p = 0.35$). The total production for each sugar was greater in the ambient CO_2 treatment than in elevated CO_2, but the differences were not significant (Tukey's HSD).

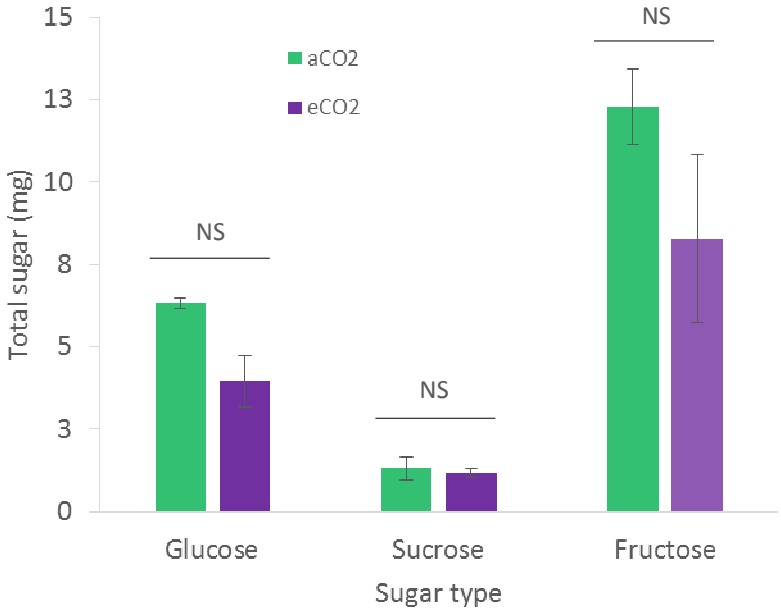

Figure 2-12. Sugar production by *G. australe* foliar extrafloral nectaries after four weeks of ambient (aCO_2) and elevated (eCO_2) treatment (n = 4). Sugar production calculated by multiplying the sugar concentration by the total foliar nectar volume per plant. Vertical bars represent ± SE of the mean; NS = not significant at $p < 0.05$.

2.5 Discussion

This study shows that elevated CO_2 affects some aspects of EF nectar production and composition. Under elevated CO_2, the total number of leaves and total foliar nectar volume of *G. sturtianum* remained constant over time. The proportion of leaves with nectar present was higher in elevated CO_2 than in ambient CO_2 while the nectar per active leaf declined over time. The number of active leaves was greater in elevated CO_2, but there was a discrepancy between the two elevated CO_2 growth cabinets at eight weeks, so these results should be interpreted with caution. There were a greater number of plants flowering at each time point in the elevated CO_2 treatment, although this did not influence foliar nectar production. In contrast, under ambient CO_2 the total number of leaves and nectar volume per active leaf remained constant with time, the number and proportion of active leaves was lower and the total foliar nectar volume dropped over time. The responses of *G. australe* EF nectar production to elevated CO_2 were less pronounced than for *G. sturtianum* and no comparisons showed a significant difference. All measures of *G. australe* foliar nectar sugar concentration remained the same under ambient and elevated CO_2 treatments.

Overall, there was no change in the total resources that *G. sturtianum* plants allocated to EF nectar under elevated CO_2. This response of EF nectar production to elevated CO_2 contrasts with the findings of the majority of floral nectar studies examining responses to elevated CO_2. For example, Erhardt et al. (2005), Lake and Hughes (1999) and Dag and Eisikowitch (2000) all found a significant increase in floral nectar volume under elevated CO_2, although Osborne et al. (1997) did not find a change in floral nectar volume and Rusterholz and Erhardt (1998) found lower nectar volumes in three species, but not in two others. As photosynthetic rate increases under elevated CO_2, there is an expectation of more available carbon-based resources (Ainsworth & Rogers 2007), but if this occurred in this study these resources must have been allocated to processes other than increasing investment in EF nectar, such as growth, reproduction or other types of defences.

The total nectar volume under ambient CO_2 increased steadily over the course of the experiment, whereas the total nectar volume in the elevated CO_2 treatment reached the equivalent level of nectar production by the first time point and remained constant. This suggests there is a fixed nectar volume that each plant produces and the plants in the

elevated CO_2 treatment are achieving this level of production faster. Physiological effects of elevated CO_2 are often seen in the short term, but over the longer term these effects level off (Eamus 1991; Smith & Dukes 2013). This is supported here by the differences in total nectar volume over time for each treatment. Because the real world increase in atmospheric CO_2 is happening gradually, and not rapidly as in this study, it is possible that plants will acclimate over time (Lee et al. 2011), resulting in only a slight, if any, effect of rising CO_2 on EF nectar in the field.

Even though there was no overall difference in the total foliar nectar volume between treatments, there was a change in how the total nectar pool was allocated within individual plants. The increase in the proportion of active leaves without an increase in total nectar volume in the elevated CO_2 treatment means there was a decrease in average nectar volume per active leaf. Ant activity on plants with EF nectaries varies as nectar volume changes (Bentley 1977b), so any change in nectar volume could affect the number of ants present. For example, O'Dowd (1979) found a positive correlation between petiolar nectar production and ant presence. As the total nectar volume is the same under ambient and elevated CO_2, the number of ants each plant can support would remain the same. A reduction in the volume of nectar per active leaf means the ant density across each plant could be lower. This could be detrimental for the plant if the ant defence is too sparsely distributed to be effective.

The flowering pattern and the number of active leaves in the elevated CO_2 treatment suggests plant development is speeding up under elevated CO_2. This trend to earlier flowering was also observed by Erhardt et al. (2005) in their study of floral nectar under elevated CO_2. Increased atmospheric CO_2 is known to have physiological effects on plants (changing growth and chemistry; Cotrufo et al. 1998) as well as accelerated phenology and ontogeny (DeLucia et al. 2012). The greater occurrence of flowering under elevated CO_2 in this study is consistent with this general principle. The production of EF nectar is generally associated with younger leaves (Heil et al. 2000) so the increase in the number of leaves with nectar present suggests that the average age of the leaves in the elevated CO_2 treatment is lower. This indicates that the rate of leaf formation is increased under elevated CO_2 (and the rate of senescence is also increased as the total number of leaves does not vary with treatment).

The sugar concentration and hexose to sucrose ratio of *G. sturtianum* EF nectar remained the same in both CO_2 treatments. Osmolality and % sucrose-equivalents were, however, both greater under elevated CO_2 conditions. The increase in osmolality and % sucrose-equivalents, but not sugar concentration, could be due to an increase in solutes other than sugars. For example, many amino acids have a refractive index much higher than that of sugars (Inouye et al. 1980), so a small increase in amino acids could have a disproportionately large effect on the refractive index of nectar and therefore the % sucrose-equivalents. Based on the lack of changes in floral nectar sugars under elevated CO_2 (Osborne et al. 1997; Rusterholz & Erhardt 1998; Lake & Hughes 1999; Dag & Eisikowitch 2000; Erhardt et al. 2005) the lack of a difference between the nectar sugar compositions under elevated and ambient CO_2 was expected. These results further support the idea that the investment in EF nectar is not changing under elevated CO_2.

This study is the first to examine the effect of elevated CO_2 on EF nectar production and composition. The response of EF nectar production to elevated CO_2 in this study is inconsistent with studies of floral nectar production under elevated CO_2. However, the response of EF nectar sugar is consistent with studies on the impact of elevated CO_2 on floral nectar. These findings contribute to the bodies of research on the dynamics of plant indirect defences and the effect of major environmental changes on plant-insect interactions. The next step in understanding the response of EF nectar production to elevated CO_2 conditions is to expand this work into a broader range of conditions and species. This would indicate if the responses of *G. sturtianum* and *G. australe* EF nectar to elevated CO_2 in this study are broadly representative. Further work on EF nectar under elevated CO_2 could pursue questions such as the effect of herbivory on the allocation of additional carbon resources to EF nectar and the allocation of EF nectar among leaves to determine whether there is variability in leaf nectar volume and what effect this may have on defence.

Elevated CO_2 affects plant development and phenology, and that in turn can affect the interactions plants have with mutualists (DeLucia et al. 2012; Rafferty et al. 2015). As the production of EF nectar is linked to development in many cases (Heil et al. 2000; Kwok & Laird 2012; Yamawo et al. 2014), changes in the developmental rate of plants could affect

the timing of EF nectar production, especially for EF nectaries around inflorescences. The total foliar nectar per plant in this study did not change under elevated CO_2, but there was a reduction in nectar volume per leaf and the suggestion of faster development. A change in time to maturity of EF nectary bearing plants could affect the timing of nectar availability which could have flow-on effects for ant food sources and plant protection against herbivores. A closer examination of plant development under elevated CO_2 and its effects on the available nectar pool would shed light on whether mutualistic relationships with ants may be affected under elevated CO_2.

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Appendices

Appendix I: Anatomy and carbon metabolism

Table S-1. Studies of extrafloral (EF) nectaries in Australian plants.

Species	Findings	Reference
13 species from south-west Australia (including six *Acacia* spp.)	List of species with EF nectaries	Lamont (1979)
43 *Acacia* species	EF nectary structure and anatomy	Boughton (1981)
Alyogyne hakeifolia	Ant visitors to bracteal EF nectaries	Scott (1981)
Helichrysum bracteatum *Helichrysum viscosum*	Ant visitation to EF nectaries and impact on herbivore populations	O'Dowd and Catchpole (1983)
41 *Acacia* species	EF nectary structure and anatomy	Boughton (1985)
Acacia pycnantha *Acacia myrtifolia*	Composition of EF nectar (saturated fatty acids and sugars); EF nectary anatomy	Marginson *et al.* (1985)
Acacia terminalis	Composition of EF nectar (sugars and amino acids)	Knox *et al.* (1985)
Eight *Acacia* species from Victoria, Australia	Hymenoptera visitors to EF nectaries	Bernhardt (1987)
Acacia terminalis	Role of EF nectaries in pollination; EF nectar sugar content and EF nectary size	Kenrick *et al.* (1987)
Acacia pycnantha	Location of EF nectaries and pollinator behaviour	Vanstone and Paton (1988)
Adenanthos cygnorum	Hymenoptera visitors to EF nectaries	Lamont (1989)
Acacia longifolia	Location of EF nectaries and mutualist behaviour	Thorp and Sugden (1990)
Chamelaucium uncinatum	EF nectary structure and anatomy	O'Brien (1995)
Adriana tomentosa var. *tomentosa*	Ant visitation to EF nectaries and impact on herbivore populations	MacKay and Whalen (1996)
Adriana tomentosa var. *tomentosa*	Ant visitation to EF nectaries and impact on herbivore populations	Mackay and Whalen (1998)
Acacia pruinosa	EF nectar sugar concentration; nectary numbers relative to height; arthropod pollinator visitors to EF nectaries	Hunter and Hunter (1999)
29 rainforest species	Determined EF nectary structural types	Blüthgen and Reifenrath (2003)
16 rainforest species	Composition of EF nectar (sugars and amino acids)	Blüthgen *et al.* (2004)
Adriana spp.	Geographic variation in EF nectaries in relation to water availability	Whalen and Mackay (2007)
Adenanthos cuneatus	Type and location of EF nectaries	Groom and Lamont (2015)

Table S-2. Percentage germination success and number of Australian native wild cotton seeds subjected to each pre-germination treatment.

Species	Control	Soak 24 hours	Hot water, cool to RT	Sliced seed coat	Sliced seed coat + soak 24 hours
Gossypium sturtianum	8% n = 50	39% n = 80	46% n = 35	70% n = 50	67% n =15
Gossypium bickii	0% n = 10	69% n = 35	17% n = 35	NA	NA
Gossypium australe	0% n = 10	0% n = 55	1% n = 75	58% n = 50	77% n = 30
Gossypium robinsonii	0% n = 10	0% n = 20	0% n = 20	16% n = 19	NA

Control = no treatment; RT = room temperature; NA indicates method not applied.

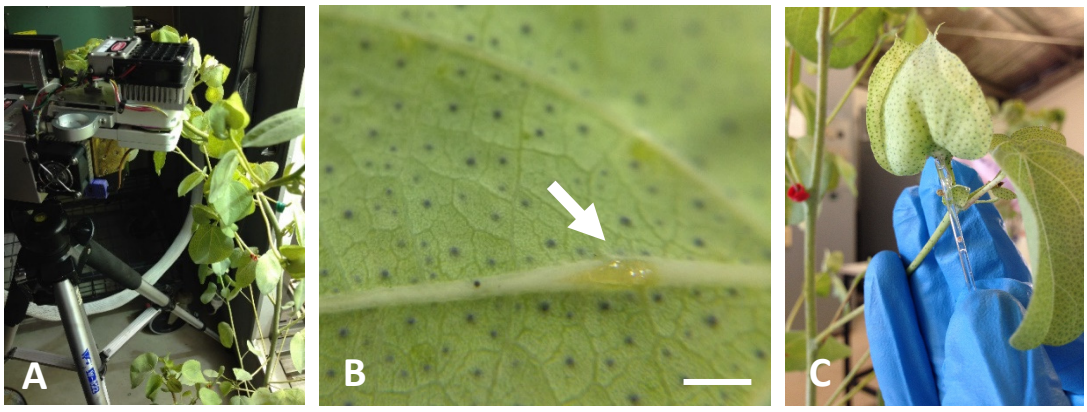

Figure S-1. Set up for measuring photosynthetic rate and collecting extrafloral nectar. (A) Measuring the photosynthetic rate of *G. sturtianum* leaves using a LiCor Li-6400XT; (B) Nectar droplet (arrow) on *G. sturtianum* foliar nectary (scale bar = 2mm); and (C) Collection of *G. sturtianum* bracteal nectar with a microcapillary.

Calculations for ^{14}C labelling experiment

Based on:

- Leaf with surface area = 8 cm^2
- Photosynthetic rate = 15 µmol CO_2 m^{-2} s^{-1}
- Plastic ziplock bag volume = 3 L
- Atmospheric carbon dioxide concentration = 400ppm

Carbon fixation per centimetre per minute (µmol CO_2 cm^{-2} min^{-1}):

= 15 µmol CO_2 m^{-2} s^{-1} x 10^{-4} x 60 x 8 cm^2

=0.72 µmol CO_2 $leaf^{-1}$ min^{-1}

Moles of carbon dioxide in 3 L plastic bag:

= 400 x 10^{-6} CO_2 x 3 L ÷ 22.4 L/mole

= 0.05 x 10^{-3} moles CO_2

= 50 µmoles CO_2

Time taken for one leaf to exhaust CO_2 in 3 L bag:

= 50 µmoles CO_2 ÷ 0.72 µmol CO_2 $leaf^{-1}$ min^{-1}

= 69.44 minutes

Table S-3. Exponential decay equations for labelled leaf sections.

Sections	**Exponential decay equations**
Leaf blade	$y = 3.8868e^{-0.538x}$
Terminal vein	$y = (6 x10^6)e^{-0.618x}$
Vein with nectary	$y = (1 x 10^7)e^{-0.795x}$
Proximal vein	$y = (5 x 10^6)e^{-0.756x}$
Petiole	$y = (3 x 10^6)e^{-0.639x}$

Appendix II: Extrafloral nectar response to elevated carbon dioxide

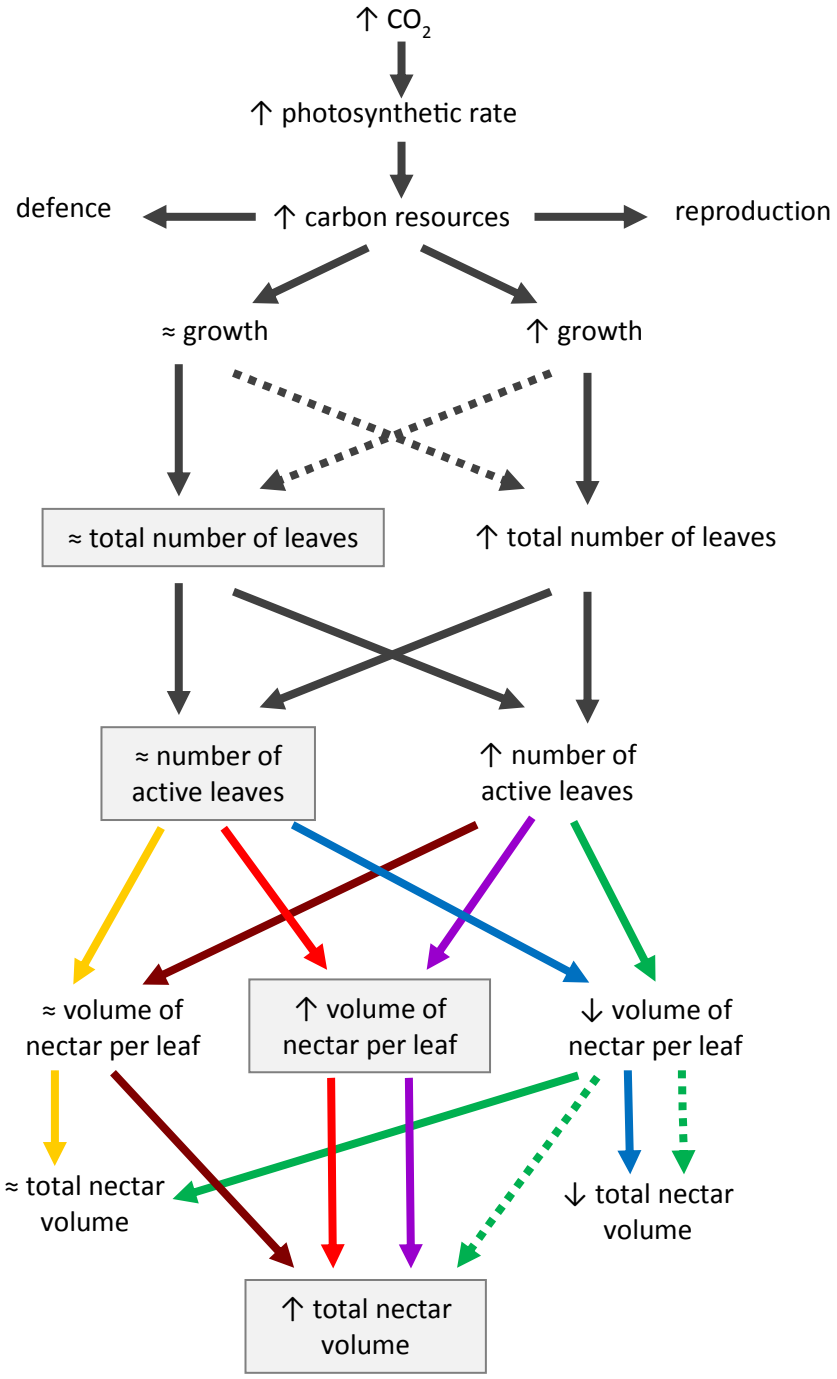

Figure S-2. Possible changes in extrafloral nectar production under elevated CO_2 conditions. Coloured arrows indicate individual response pathways and dotted lines indicate responses that are less likely. Shading indicates the predictions for this study.

Table S-4. Findings from studies manipulating CO_2 and measuring changes in floral nectar production and sugar composition.

Species	Environmental factor	Change in floral nectar	Reference
Borago officinalis *Tropaeolum majus*	Deprived plants of CO_2 25% increased CO_2	• When deprived of CO_2, nectar production diminished 13-50%. • In elevated CO_2 treatment *B. officinalis* nectar had only 33% sugar concentration & 15% sugar content of control levels and *T. majus* nectar had 58% sugar concentration & 44% sugar content of control levels.	Huber (1956) cited in Davis (2003)
Ipomoea purpurea	CO_2 at 255 and 710 µL/L	• No significant effect on floral nectar-sugar production • Nectar production increased within a day of moving plants from ambient to elevated CO_2 treatment	Rathcke (1992) cited in Davis (2003)
Vicia faba cv. Sutton	CO_2 at 350 and 700 µL/L	• No significant difference in floral nectar volume, total nectar sugar per flower or nectar solute concentration	Osborne et al. (1997)
Betonica officinalis *Centaurea jacea* *Lotus corniculatus* (Fabaceae) *Trifolium pratense* (Fabaceae) *Scabiosa columbaria*	CO_2 at 350 and 660 µL/L	• Lower nectar volumes in *B. officinalis*, *C. jacea*, and *S. columbaria*, but did not affect volumes of floral nectar secreted by the legumes. • 40–50% lower nectar sugar production in *C. jacea* and *S. columbaria*. • Nectar concentration of sugars did not differ, nor did the proportion of nectar carbohydrates (glucose, fructose, sucrose).	Rusterholz and Erhardt (1998)
Tropaeolum majus cv. Jewel	CO_2 at 380 and 750 ppm	• Major increase in nectar volume, but quantities of nectar sugar were not significantly different. The concentrations of sugar in nectar remained constant.	Lake and Hughes (1999)

Species	Environmental factor	Change in floral nectar	Reference
Cucumis melo	'Enriched' sector (CO_2 400-1,000 ppm throughout day) or 'control' sector	• Significantly greater nectar volumes but no difference in nectar-sugar concentration.	Dag and Eisikowitch (2000)
Epilobium angustifolium	CO_2 at 350 and 650 ppm	• Significantly increased nectar production per day and total sugar per flower. • No significant change in nectar sugar concentration, proportion of glucose/fructose and proportion of sucrose/(glucose + fructose)	Erhardt et al. (2005)
Cucurbita maxima Var. 'Little Cutie'	CO_2 at 360 and 700 ppm Temperature 19°C & 23°C	• Positive effect of elevated CO_2 x temperature on sugar concentration • No effect of any drivers on sucrose, however, there was significant positive effect of elevated CO_2 on concentrations of glucose and fructose although this effect was reduced by elevated temperature • CO_2 had a negative effect on the ratio of sucrose to glucose + fructose, and there was a significant temperature x CO_2 interaction	Hoover et al. (2012)

Table S-5. Results of statistical testing for cabinet effects in each comparison for *G. sturtianum* and *G. australe*.

Comparison	*G. sturtianum*	*G. australe*	Treatment and time	Affects treatment level result
Photosynthetic rate	NS	N/A		
Proportion of total leaf sugar exuded in foliar nectar	NS	N/A		
Total number of leaves per plant	*	NS	eCO_2 8 weeks	No
Number of active leaves per plant	*	NS	eCO_2 8 weeks	Yes
Proportion of active leaves	NS	NS		
Total foliar nectar volume	*	NS	eCO_2 8 weeks	No
Nectar volume per active leaf	*	NS	eCO_2 8 weeks	No
Foliar nectar volume with and without flowers	NS	N/A		
Total bracteal nectar volume	NS	N/A		
Concentration of each sugar species	*	N/A	aCO_2 4 weeks Sucrose only	No
Foliar nectar hexose : sucrose ratio	*	N/A	aCO_2 4 weeks	No
Bracteal nectar hexose : sucrose ratio	N/A	N/A		
Osmolality of foliar nectar	NS	N/A		
Osmolality of bracteal nectar	N/A	N/A		
% sucrose-equivalents in foliar nectar	NS	N/A		
% sucrose-equivalents in bracteal nectar	N/A	N/A		
Total sugar production	NS	N/A		

N/A = not applicable (comparison not performed or low number of replicates per cabinet); eCO_2 = elevated CO_2; aCO_2 = ambient CO_2; NS = non-significant; * = significant difference between cabinets.

Table S-6. Model output for multiple linear regression of photosynthetic rate and nectar volume per leaf for ambient and elevated CO_2 treatments.

Term	Coefficient	SE of coefficient	T-value	P-value	VIF
Constant	0.7378	0.0477	15.47	0.000	
Avg p/synth rate	0.0271	0.0164	1.65	0.109	2.54
Treatment eCO2	-0.2836	0.0670	-4.23	0.000	1.16
Avg p/synth rate * Treatment eCO2	-0.0527	0.0223	-2.37	0.025	2.35

Reference level is ambient CO_2.

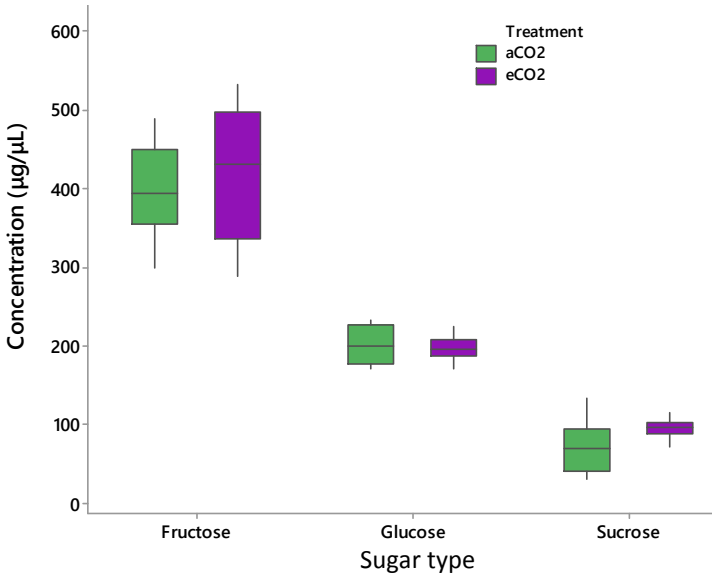

Figure S-3. Range of *G. sturtianum* foliar nectar sugar concentrations after four weeks of ambient (aCO_2) and elevated (eCO_2) treatment (n = 8). Vertical bars represent ± SE of the mean.

Appendix III: Plant, Cell & Environment author guidelines

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PC&E strongly recommends, for controls, the use of wildtype seed batches that are generated at the same time as the transgenic seed lines or, even better, wild-type plants in segregating populations derived from selfing or backcrossing of heterozygous plants (or in the case of a transgene, hemizygous plants). These seeds should be collected and stored in the same conditions as the mutant seed. PC&E recognizes that this is not always possible, but it is especially important for studies addresses phenotypic traits that are known, or are likely, to be affected by seed quality.

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Manuscripts may be submitted in the following formats: Microsoft Word, Adobe PDF, or Corel WordPerfect. Manuscripts submitted in Word or WordPerfect formats will be converted to PDF format and sent to the authors to check the accuracy of conversion. Only the PDF file will be used for peer review. Embed all tables, figures and other graphic elements into a single file wherever possible.

Manuscripts should be double-spaced with margins of at least 2.5 cm. All pages should be numbered consecutively including those containing acknowledgements, references, tables and figure legends. Line numbers are **mandatory**. English spelling should conform to The Concise Oxford Dictionary of Current English.

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Authors for whom English is a second language may choose to have their manuscript professionally edited before submission to improve the English. A list of independent suppliers of editing services can be found at www.authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

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The following format is designed for Full Papers, and should be modified appropriately for manuscripts in other categories. The manuscript should be arranged as follows, with the Introduction beginning on a separate page:

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   (i) Title: this should be concise and informative and a short running title should be provided.

(ii) List of authors' preferred names.
(iii) Institute or laboratory of origin.
(iv) The name and address of the corresponding author, including an email address.

2. *Abstract*
   This should provide a concise statement of the motivation for the work done, the scope of the work and the principal findings. The abstract should be less than 200 words.

3. *Keyword index*
   This is to contain 'keywords' or phrases to enable retrieval and indexing by modern searching techniques. The list should not exceed 10 words.

4. *Introduction*
   This should argue the case for your study, outlining only essential background, but should not include either the findings or the conclusions. It should not be a review of the subject area, but should finish with a clear statement of the question being addressed.

5. *Materials and methods*
   This should provide sufficient details of the techniques used to allow them to be repeated.

6. *Results*
   This should not include material appropriate to the Discussion section.

7. *Discussion*
   This should highlight the significance of the results and place them in the context of other work. It should not introduce new material, be over-speculative, reiterate the results, or exceed 20% of the total length. The Results and Discussion sections may be amalgamated for short papers if desired, but, in this case, the final paragraph ought to provide a resume of the main conclusions.

8. *Acknowledgments*

9. *References*
   The reference list should be in alphabetical order and include the full title with the name of the journal given in full, thus:

   Atkin O.K., Botman B. & Lambers H. (1996) The relationship between the relative growth rate and nitrogen economy of alpine and lowland Poa species. *Plant, Cell and Environment* 19, 1324-1330.

   Schjoerring J.K. (1991) Ammonia emission from the foliage of growing plants. In *Trace Gas Emissions by Plants* (eds T. D. Sharkey, E. A. Holland & H. A. Mooney), pp. 267-292. Academic Press, San Diego.

   In the references, for papers with eight or more authors, the author list should

give the first six names then an ellipsis should be inserted (…) followed by the final name.

Atkin O.K., Lombardo V.A., Osorio S., Borsani J., Lauxmann M.A., Bustamante C.A., …, Lambers H. (1996) The relationship between the relative growth rate and nitrogen economy of alpine and lowland Poa species. *Plant, Cell and Environment* 19, 1324-1330.

In the text, for papers with three or more authors, all references should be in the form "Smith et al. 2006". Regardless of whether it is the first citation or not, the format is always the first author followed by "et al". When different groups of multiple authors with the same first author occur, they should be cited as "Smith et al. 1996a, b".

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting.
EndNote reference styles can be searched for here:
http://www.endnote.com/support/enstyles.asp
Reference Manager reference styles can be searched for here:
http://www.refman.com/support/rmstyles.asp

10. *Tables*
    Each table should be on a separate page, numbered, and accompanied by a title and explanatory caption at the top. Each table must be referred to in the text, and an indication of preferred position in the text should be given. Data must not be presented in both tabular and graphical form.

11. *Figure legends*
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