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# **Seasonal changes of non-structural carbohydrates related to the growth and development of gentians**

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## **Abstract**

The growth and development of perennial gentians (*Gentiana* L.) for cut flowers are seasonally controlled. It was hypothesized that in these plants the availability of carbohydrates is a limiting factor influencing the development of crown buds, winter survival, spring re-growth, and their development to flowering; this in turn influences the yield and quality of flowering shoots. By focussing on the seasonal changes of non-structural carbohydrates (NSCs) in various organs, and the effect of differential carbohydrate supply on their growth and development, the current thesis aimed to understand the physiological function of NSCs and their potential influence on the commercial production of flowering shoots.

In addition to sucrose, fructose and glucose, the unique carbohydrates, gentianose, gentiobiose and L-bornesitol were found in the gentian hybrids investigated. Gentianose was the main storage carbohydrate, accounting for 50% - 70% total NSCs in storage roots year round, likely playing similar roles as starch and/or fructan do in other plant species. The concentration of these NSCs in various organs fluctuated with seasonal changes, reflecting periods of storage and utilization of NSCs, i.e. the transition between sink and source, in the growth and development of plants during an annual growth cycle.

When harvesting flowering shoots, commercial growers of gentian leave a set number of leaves to maintain carbohydrate levels; however, optimization of leaf number/area has never been assessed in terms of carbohydrate reserves. Utilizing varying levels of defoliation, a positive correlation was determined between the resulting concentration of NSCs in crowns (crown buds, rhizomes and storage roots) and both the number and size of new crown buds, supporting the hypothesis that the availability of carbohydrates is a limiting factor influencing the development of crown buds. Experiments *in vitro* both verified and extended the level of understanding, indicating that the morphogenesis of crown buds, including form, dormancy, and differentiation, was influenced by multiple factors including, carbohydrates, photoperiod and ethylene.

Reserves of NSCs in crowns in autumn were positively correlated with both winter survival and spring re-growth. Consistent with these results, the dynamics of NSC concentration and the activity of relevant glycoside hydrolases (GHs) indicated that a large amount of gentianose, up to 106 mg g<sup>-1</sup> fresh weight (FW) accumulated in storage roots

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before winter was subsequently hydrolysed and remobilized to the crown buds to impart cold tolerance in winter and be used during their subsequent re-growth in spring.

Substantial gentiobiose, up to 38 mg g<sup>-1</sup> FW, accumulated in stem tissue of the shoots before anthesis commenced, while gentianose increased by up to 26 mg g<sup>-1</sup> FW in petals before floret opening. These two forms of carbohydrates were considered likely candidates facilitating the fast development of florets. During the development and senescence of florets, the activity of relevant GHs controlling the hydrolysis of gentianose, gentiobiose, and sucrose, was associated with increase of pressure potential driving the opening of floret buds. The stage-specific and dramatic changes of accumulation, conversion and mobilization of NSCs in association with the activity of relevant enzymes, also provided a useful experimental model system to study the metabolism and physiological function of these unique NSCs in gentians.

The current study has provided increased understanding of the roles of specific NSCs, particularly gentianose and gentiobiose, in the growth and development of gentians. The thesis also offers a framework of information to improve the balance between carbohydrate storage in underground crowns and harvest of flowering shoots, and therefore benefits the sustainability of yield and quality of flowering shoots in commercial production.

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## List of Symbols and Abbreviations

ANCOVA	Analysis of covariance
BM	Basic medium
C	Concentration of osmolality
CAZy	the Carbohydrate-Active Enzyme
CBNC	Number of crown buds per cluster
CN	Cluster number of crown buds
DCB	Diameter of crown buds
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detector
FW	Fresh weight
GDD	Growing-degree-days
GH(s)	Glycoside hydrolase(s)
GLM	General linear model
h	Hour
HPLC	High performance liquid chromatography
<i>i</i>	van 't Hoff factor
IAA	indole-3-acetic acid
LCB	Length of crown buds
MS	Murashige & Skoog
MWCO	Molecular weight cut off
NAA	1-naphthaleneacetic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
NSC(s)	Non-structural carbohydrate(s)
NZ	New Zealand
PAGE	Polyacrylamide gel electrophoresis
PPFD	Photosynthetic photon flux density
PVPP	polyvinylpyrrolidone
<i>R</i>	Gas constant
SN	Shoot number
SSP	Proportion of crown buds sprouting in Spring
T	Thermodynamic (absolute) temperature
TCBN	Total number of crown buds per plant
TDZ	Thidiazuron
TNC	Total non-structural carbohydrates
WSP	Proportion of plants surviving winter
$\Psi$	Water potential
$\Psi_m$	Matric potential
$\Psi_p$	Pressure potential
$\Psi_s$	Osmotic potential

## Chapter 1 General introduction

### 1.1 Commercial production

Gentian (*Gentiana* L.) is composed of approximately 400 species that are widely distributed in alpine and temperate climatic regions throughout the world (Kohlein, 1991; Ho & Liu, 2001). The majority of these species are perennial herbaceous plants, with a small number being annuals and biennials (Ohkawa, 1989). In addition to the predominant species with blue-coloured flowers, there are those with white, yellow, pink, purple, violet, red, and green coloured flowers (Kohlein, 1991). For the current thesis, when referring to gentian(s), it is with reference to the perennial species and hybrids.

Gentians are widely used in horticulture, medicine, and beverages. Within ornamental horticulture, gentians were initially grown to be used for rock gardens in Europe (Bartlett, 1975). The commercial production of gentians as cut flowers and potted flowering plants began in Japan from the 1940s to 1960s (Ohkawa, 1989; Yoshiike, 1994). Gentian cultivars used for cut flower production have been primarily derived from the perennial *G. triflora* Pallas, *G. scabra* Bunge, and *G. makinoi* Kusnetz (Ohkawa, 1989). Cut flowers of gentians are traditionally very popular in Japan. For example, the Japanese market consumes approximately 130 million flowering shoots of gentians each year (Anonymous, 2007), while recently gentians for cut flowers have also been becoming known in the markets of Europe, North America and Asia.

### 1.2 Development in New Zealand

In New Zealand (NZ), gentians are developing into a new promising cut flower crop for export, with growers in both the North and South Island areas. Being located in the southern hemisphere, NZ possesses the potential marketing advantage of being “out of season” in Japan and other northern hemisphere markets, for exporting the flowering shoots of gentians as cut flowers. A relatively long vase life of around two weeks, a high tolerance to transport stress (Eason et al., 2004), together with the relatively low cost of production in their natural flowering season (Anonymous, 1984), make gentians an ideal export crop.

To enable the sustainable development of the gentian industry in NZ, some research and development projects have been conducted as collaboration between growers and The New Zealand Institute for Plant & Food Research Limited (Plant & Food Research). This has included breeding of new cultivars, performance evaluation, improvement of the growing system, post-harvest treatments, etc. (Anonymous, 2006). Distinct from most blue-flowered cultivars of gentians, novel red-flowered gentians have been developed in NZ for the industry, such as gentian ‘Showtime Diva’ and ‘Showtime Spotlight’ (Figure 1.1) (Eason et al., 2007), and some un-named yellow and purple-flowered genotypes (E. Morgan, personal communication, 2008). The postharvest technology for gentian as cut flowers has also been developed for supporting the export to long distance international destinations (Zhang & Leung, 2001; Eason et al., 2004; Eason et al., 2007). These cultivars, with novel colours, long and productive flowering shoots, and established postharvest performance, are believed to have substantial potential in the international cut flower markets.



**Figure 1.1 The gentian cultivars ‘Showtime Spotlight’ (A), ‘Showtime Diva’ (B) and ‘Showtime Starlet’ (C). Photos courtesy of Plant & Food Research.**

### **1.3 Research background**

As a typical geophyte, the growth and development of perennial gentians are controlled seasonally (refer to Section 2.3.2). In annual growth cycles, gentian plants rely on underground crowns (comprising storage roots, rhizomes, and crown buds; refer to Section 2.2.1) as a perennating organ to over-winter and initiate re-growth in spring (Ohkawa, 1989). Anecdotally, it has been reported that during production for cut flowers, significant plant mortality has sometimes occurred, due to the lack of crown bud formation and/or the failure of overwintering. It is these overwintering crown buds that give rise to the flowering shoots, which are harvested and marketed as cut flowers and, therefore, are considered critical to both the survival and horticultural productivity of this crop.

Adequate non-structural carbohydrate (Klages et al.) reserves in underground storage organs are especially important in geophytes for winter survival, supporting initiation of early spring growth when photosynthesis is not able to provide sufficient photo-assimilate to meet demand (White, 1973). In other perennial plant species, it was reported that the negative effects of defoliation on the ability to overwinter (cold tolerance) and regrow (carbon remobilization) in spring, was related to the reduction of non-structural carbohydrate (Klages et al., 2001) accumulation in underground organs before winter (Dhont et al., 2002; Uleberg et al., 2009). In gentians, flowering shoots (spike-type inflorescences with leaves; refer to 2.3.2), are the organ harvested for cut flowers. Anecdotal comments amongst growers of gentians for cut flower production suggest that removing a large quantity of leaves, as occurs during harvesting of flowering shoots, has a negative effect on the yield of flowering stems in the following growth season. As a consequence of this logic, the hypothesis emerged that the loss of leaves, i.e. the main source of synthesis of new carbohydrates, may adversely affect the development of crown buds, winter survival, and re-growth. This in turn reduces the yield and quality of flowering shoots produced the following season. To test this hypothesis in gentian, experiments are required to quantify what, if any, negative effects of defoliation occur and any relationships evident between carbohydrate supply, crown bud formation, overwintering and re-growth. This, therefore, formed the rationale behind the thesis presented here.

For perennial gentians, all flowering shoots originate from crown buds developed in the preceding year's growing season, and the size of crown buds may influence the length of flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon, 2012). In addition, recent preliminary research using a new cultivar 'Showtime Spotlight' indicated that not all the crown buds emerged in a typical growing season (Samarakoon et al., 2012b). In commercial production, a gentian plant, once planted, is normally cultivated for between eight and ten years and, newly planted young plants usually take around three years to establish adequate crown buds to reach their full commercial production (Ohkawa, 1989). During the long period of cultivation, poor development of crown buds, low winter survival and spring re-growth are recognised as limiting factors for increasing both yield and quality of cut flowers in gentians (Ohkawa, 1989; Samarakoon et al., 2012a). Given the above facts, crown buds, therefore, are not only an organ for vegetative regeneration, but also a determinant influencing the yield and quality of flowering shoots in commercial

production. In order to optimize the production of cut flowers therefore, knowledge of factors influencing crown-bud formation, winter survival, re-growth and continued development as floral shoots in gentians is essential.

The organogenesis and development of plants are complex processes regulated by multiple factors, including environmental factors (such as light and temperature), hormones and carbohydrate metabolism (Thorpe, 1974; Gemas & Bessa, 2006; Iglesias et al., 2006). While Samarakoon's research (2012) provided valuable information to understand the effect of light, temperature, and hormones, on crown bud initiation and development in gentian, to date the physiological mechanism of the morphogenesis and development for crown buds is still not fully understood. For plant growth and development, in addition to their use as energy and carbon sources (Lewis, 1984b), NSCs are also involved in physiological processes and functions, such as morphogenesis (Wyrzykowska et al., 2002), bud dormancy (Anderson et al., 2005), flowering (Corbesier et al., 1998; El-Lithy et al., 2010) signal transduction (Rolland et al., 2006), osmotic regulation (Evans & Reid, 1988), cold tolerance (Palonen, 1999; Patton et al., 2007), and defence against other adverse conditions (Patton *et al.*, 2007). While many studies have reported the physiological roles of carbohydrates in other plant genera, little published information on carbohydrate metabolism and physiological roles is available for gentians. Within this thesis, therefore, the physiological function of NSCs in terms of crown bud morphogenesis, overwintering, and re-growth of gentian will be the main focus.

In commercial production of gentian, as a continuation of crown bud development, the growth and development of floral shoots directly determines yield, quality and postharvest performance. It has been recognized that in other plant species, stems are capable of storing substantial NSCs, enabling fast reproductive growth, and as a buffer for demand for NSCs under adverse situations when photosynthetic capacity is reduced (Slewinski, 2012). Non-structural carbohydrates also act as an osmoticum, regulating water potential, and promoting water influx for cell expansion and flower opening (Reid, 2005; Kumar et al., 2008b). After harvest of cut flowers, NSCs are also known to be important for ensuring the longevity of flowering stems by providing a carbon source for respiration, continued development of immature flowers (Adachi et al., 2000; Monteiro et al., 2002), decreasing responsiveness to ethylene (Verlinden & Garcia, 2004) and maintaining water uptake to retard wilting (Kumar et al., 2008a). In gentians, Eason et al. (2004) reported

that pulsing the cut flowering shoots of gentian with sucrose (5%), improved their postharvest vase-life and pigmentation of petals on developing florets. However, the actual metabolism of NSCs and their physiological role, related to the development and senescence of gentian florets, still need to be determined. In addition, for the current thesis the tightly defined and rapidly changing stages of floret development were expected to provide a valuable experimental system for studying carbohydrate metabolism and, therefore, potential to explore their physiological functions in gentians.

It has been found that unique sugars, i.e. gentianose and gentiobiose are present in substantial amounts in *G. lutea*, a medicinal crop cultivated for its storage-root (Keller & Wiemken, 1982; Rossetti et al., 1984). While Bridel (1911) monitored the concentration changes of NSCs in storage-roots of *G. lutea* from Summer (July) to Autumn (November) and, indicated that gentianose was the main NSC with its highest concentration in autumn, the changes during winter and spring, however, have not previously been reported. Additionally, for gentian species and hybrids used for cut flowers, there is no information published on metabolism of NSCs, nor their physiological function. The process progressing from crown bud formation, dormancy in winter, re-growth in spring, through to floral shoot development and final harvest of flowering shoots, runs through the whole annual growth cycle of gentian plants. At the outset of this thesis therefore, it was considered that monitoring the seasonal changes of NSCs in various organs would potentially assist improvement in understanding of the physiological function of NSCs in the growth and development of gentian plants.

As with other metabolic reactions in plants, carbohydrate metabolism involves enzyme-catalysed biochemical reactions (Turner & Turner, 1975). Carbohydrate-related enzyme activity, therefore, can potentially also be directly related to these same physiological functions in gentians. Due to the existence of unique carbohydrates, i.e., gentianose and gentiobiose, it seems reasonable to assume that there are different enzymes related to the metabolism of the unique carbohydrates in gentians. Carbohydrates are generally stored as oligosaccharides or polysaccharides in many forms and, sucrose and starch are the most common temporary or long-term stored carbohydrates utilized in plants (Lewis, 1984b). During plant development and growth, these oligosaccharide and polysaccharides can be broken into monosaccharides or short-chain NSCs when needed, by glycoside hydrolases (GHs) including glycosidase and transglycosidases (Minic, 2008). The current study, therefore, planned several preliminary experiments to detect the activity of GHs related to

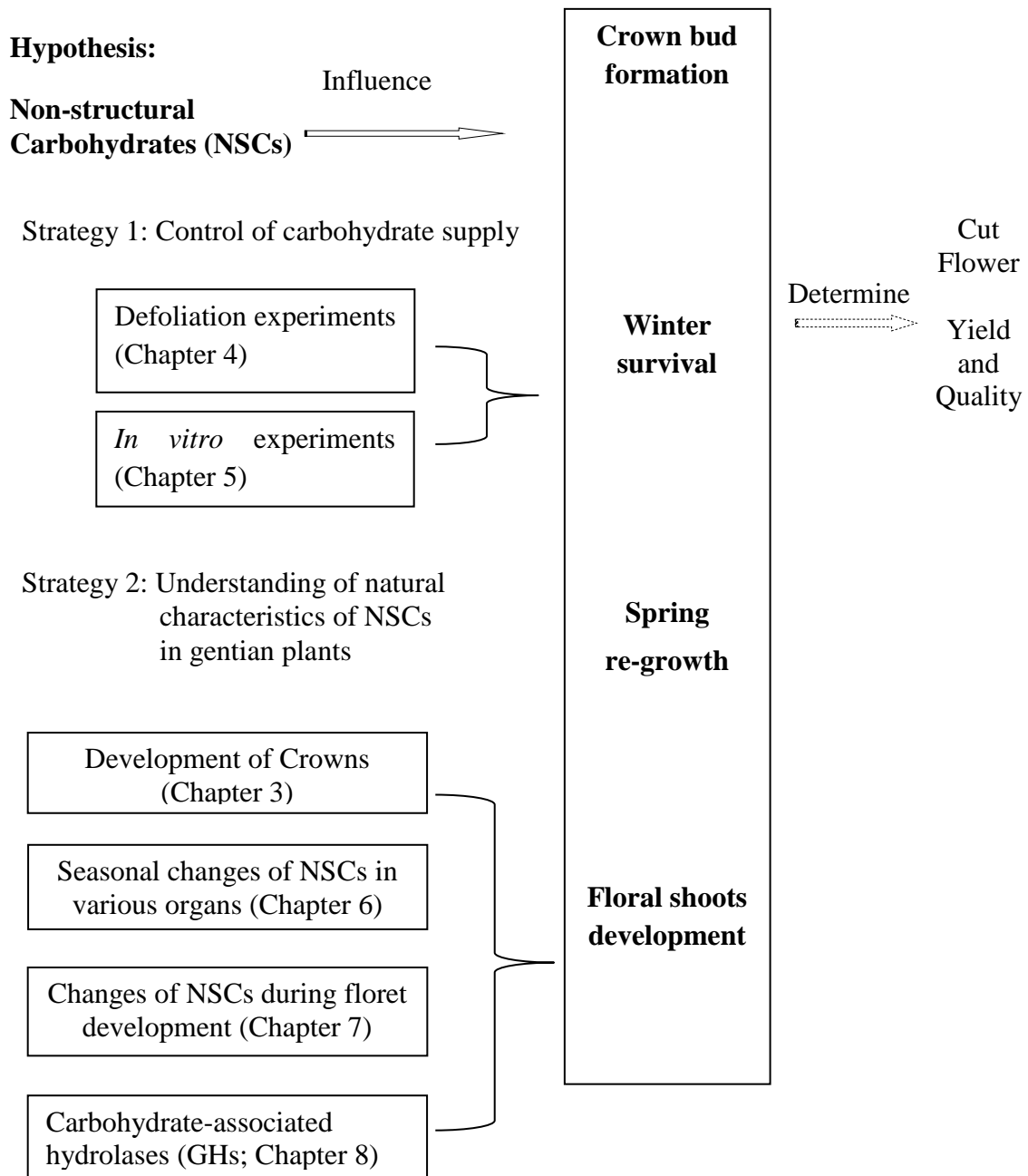
gentianose, gentiobiose utilization in gentian. A comparison between the fluctuation in concentration of NSCs and their related GHs activity was expected to be valuable for a better understanding the physiological roles of NSCs and their regulation mechanism.

## **1.4 Objectives**

As with all other crops, knowledge of plant development and physiology specific to gentian plants will enhance the development of technology to improve them as a sustainable production system. Given the growth habit and commercial cultivation of gentians, it was hypothesized that availability of carbohydrate is a limiting factor influencing the development of crown buds, winter survival, spring re-growth, and the development of floral shoots; this in turn will influence the yield and quality of flowering shoots in commercial production. The goal of this study was, therefore, to: understand the physiological function of the storage and utilization of these NSCs during the annual growth cycle; and explore their potential influence on the commercial production of flowering shoots. The proposed research strategies were: quantify the seasonal changes of NSCs in various organs of gentian plants; determine the effect of carbohydrate supply (experimentally induced *in vivo* and *in vitro*) on development of gentian plants (Figure 1.2). The specific objectives of this study, therefore, included:

- investigate the form, structure and development of the perennating storage organ, i.e. crowns (Chapter 3);
- identify the unique carbohydrate system in gentians, including the existence of unique carbohydrates, and monitor their seasonal changes of distribution in the whole plant, over the entire growth cycle (Chapters 3, 6 and 7);
- manipulate carbohydrate acquisition, both *in vivo* and *in vitro*, so as to explore what, if any, relationships exist between carbohydrate supply and the development of crown buds, winter survival, and spring re-growth (Chapters 4 and 5);
- utilise the process of floret development and senescence as a model to investigate the metabolism of NSCs in gentian and, explore their potential physiological function (Chapters 7 and 8);
- investigate the properties of GHs and detect their seasonal changes in activity (Chapter 8).

## Seasonal changes of non-structural carbohydrates related to the growth and development of gentians



**Figure 1.2 Research strategies and programmes within this PhD thesis.**

## **Chapter 2 Literature review**

As each experimental chapter presents its own literature review, and thereby integrating more recent literature, this chapter is presented primarily to provide the reader with some background to the research strategies explored within the thesis.

### **2.1 Cultivars for ornamental crops**

The main commercial cultivars of gentian (*Gentiana* L.) used for cut flowers originate from three species and their interspecific hybrids, i.e. *G. scabra* Bunge. var. *buergeri* Maxim. subvar. *orinetalis* Toykuni, *G. triflora* Pall. var. *japonica* Hara, and *G. makinoi* Kusnetz (Ohkawa, 1989). All these species are perennial plants with long (20-100 cm) and erect flowering shoots. *G. triflora* is native to Eastern Siberia, Korea, Japan; *G. scabra* is native to Korea, Manchuria, northern China and Japan; with *G. makinoi* from the mountains of Hunshu, Japan (Ohwi, 1965). With the climatic conditions at the centre of origin of these species described as “*alpine and cool climate regions*” (Ohwi, 1965; Ohkawa, 1989; Ho & Liu, 2001), as discussed in subsequent sections, commercial cultivars are typically noted as being adapted to a temperate climate.

Many of the current commercial cultivars for production as cut flowers and potted plants were developed in Japan and, to date, more than 100 commercial cultivars have been registered in Japan (Kodama, 2006). The cut flower cultivars *G. makinoi* ‘Royal Blue’ and *G. triflora* ‘Japonica’, grown in Europe, are Japanese derived cultivars (Yoshiike, 1994). The large majority of these cultivars in commercial production have blue-coloured florets, with a few being white or pink coloured. Based on the flowering time within the growing season, gentians are also categorised into early, middle, and late-season cultivars. Genetic transformation has been used in breeding to extend the diversity of cultivars, particularly in terms of new floret colour, floret shape, and disease resistance (Mishiba et al., 2005; Nakatsuka et al., 2008; Nishihara et al., 2008). To protect breeder’s rights, DNA fingerprinting technology has also been applied to verify the identity of the numerous gentian cultivars (Shimada et al., 2009).

Recently, a gentian breeding programme has been conducted in New Zealand (NZ), utilizing techniques such as embryo rescue (Morgan, 2004) and polyploidization (Morgan et al., 2003). As a result, some novel cultivars for cut flower production with red and pink-

coloured florets have been introduced into the market, such as ‘Showtime Spotlight’, ‘Showtime Starlet’, and ‘Showtime Diva’ (Eason et al., 2007), and some un-named yellow and purple-flowered genotypes (E. Morgan, personal communication, 2008). These new cultivars are strongly supporting the gentian industry in NZ. To continuously improve the production system of gentians in NZ, some of these new cultivars and genotypes were used as experimental material within this thesis.

## **2.2 Growth habit**

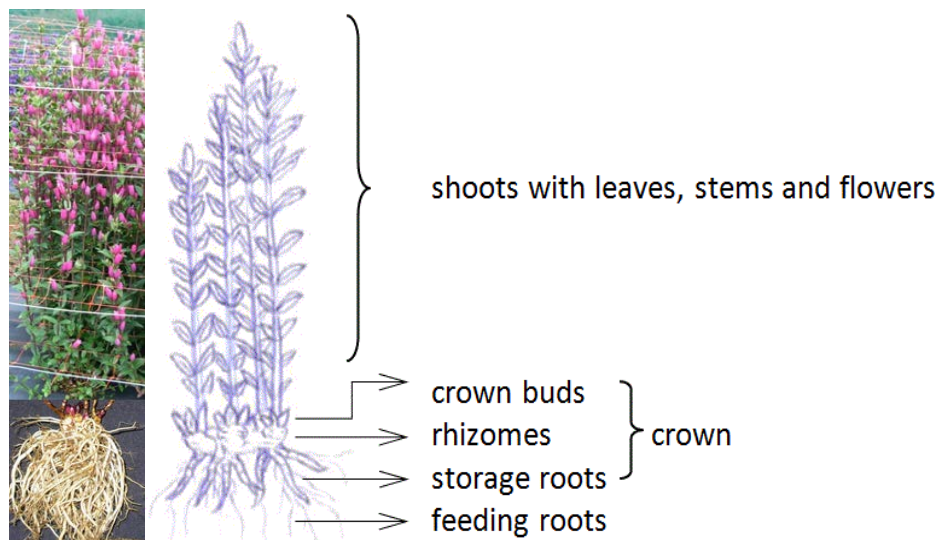
### **2.2.1 Morphological structure**

A mature perennial gentian plant is composed of erect flowering shoots, swollen crown buds, fleshy rhizomes, thick storage roots and fine feeding roots (Figure 2.1). These organs can be grouped into two parts, i.e. above-ground shoots and underground crowns. In horticulture, the crown of a gentian plant, comprising crown buds, rhizomes, storage roots, and feeding roots, is the perennating structure supporting re-growth in the next growth season (Ohkawa, 1989). Crowns of gentians are able to be lifted, divided, traded and replanted.

Crown buds are located at the top of crowns, typically being presented as covered with purple scale leaves. Crown buds commonly develop in clusters of axillary buds, and the first crown bud within a cluster is able to be initiated as an adventitious bud (Samarakoon, 2012). The fleshy rhizomes of the crown are the compressed stems, being vertical or horizontal. Within the cultivars used in this thesis, the rhizomes are normally vertical. Storage roots are stout taproots or linear-cylindrical roots arising from the rhizomes. Shoots originate from crown buds and typically develop to form an inflorescence. Although the underground crowns are an important structure for the perennial lifecycle of gentians, knowledge of the function and features of the form and structure of a crown is very limited. One aspect proposed for this thesis was, therefore, to detail the features pertaining to the form, structure and development of crowns.

Shoots are normally erect and, composed of stems and leaves. Leaves are sessile in an arrangement of decussate opposite leaves, with lower leaves of shoots being scale-like (Ohwi, 1965; Ohkawa, 1989; Ho & Liu, 2001). The spike-type inflorescence with leaves is a cyme, comprising few to many florets which are terminal and/or axillary. The corolla

is sympetalous and usually funnel-form, campanulate, tubular, or salverform, with five lobes. In this thesis, with the shoots being able to develop inflorescences, they are termed “floral shoots” and, when the floral shoots achieve anthesis, are termed “flowering shoots”.



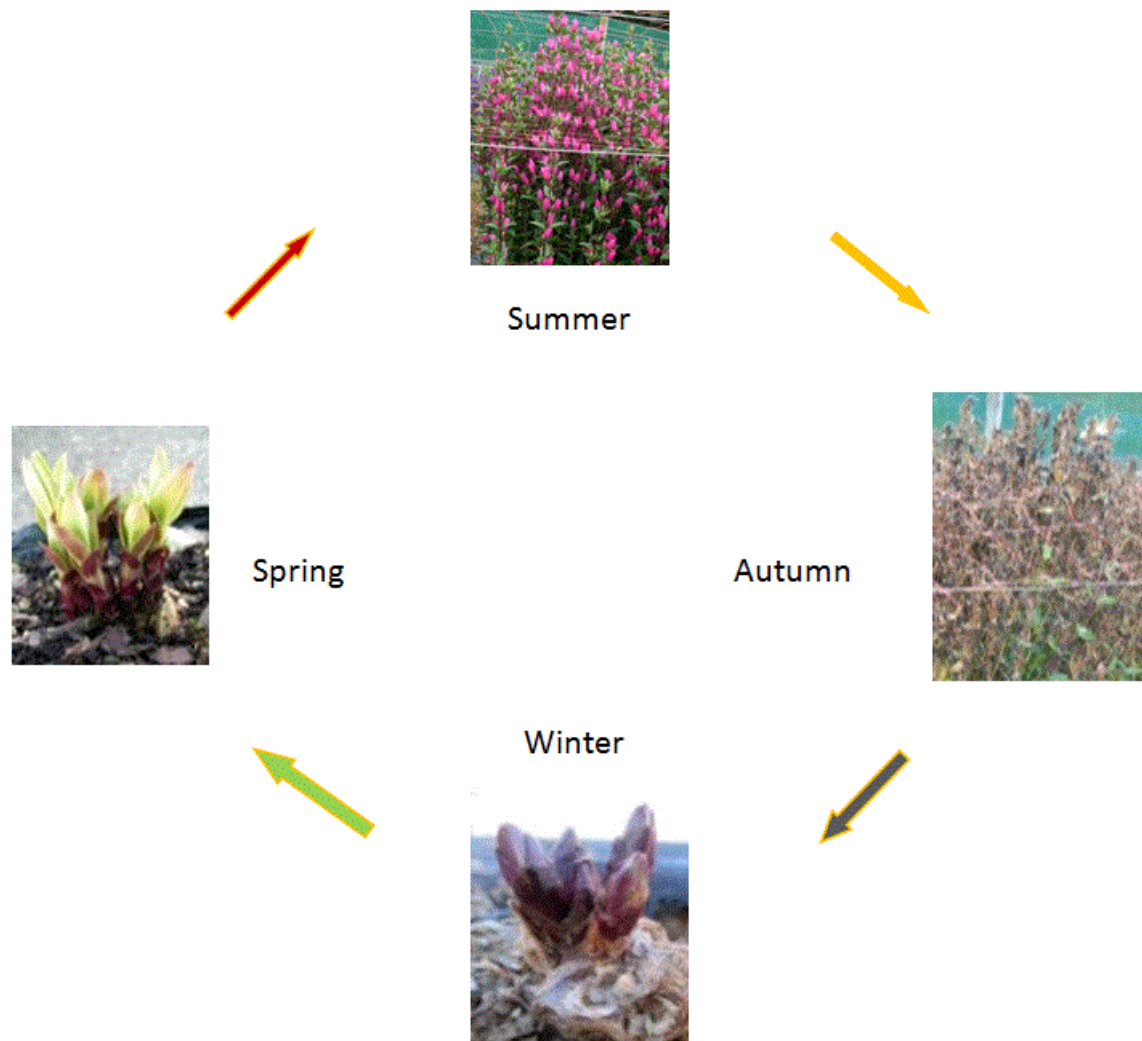
**Figure 2.1 Photograph and comparative schematic diagram presenting the morphological structure of a mature perennial gentian plant.**

### 2.2.2 Growth cycle

Perennial gentians are typical geophytes, and their growth is seasonally controlled (Figure 2.2). In annual growth cycles, gentian plants sprout in spring, flower in summer/autumn, with above-ground floral shoots typically senescing in winter; relying therefore on the underground crowns (storage roots, rhizomes and crown buds) to survive winter and initiate re-growth in the next growth season (Ohkawa, 1989).

Floral differentiation of gentians cultivars used for cut flower production, occurs after sprouting in spring (Ohkawa, 1989). In Japan, floral differentiation of early-season flowering cultivars commenced from late May to early June (seasonally being equivalent to late November to early December in the southern hemisphere) when shoots had reached 30 to 40 cm in length, and 15 to 17 pairs of extended leaves. In contrast, with late-season flowering cultivars, floral differentiation started when shoots were 60 to 70 cm long and with 24 to 28 pairs of fully expended leaves. Warm temperature (20-25 °C) and long-day conditions accelerated floral development of early-season cultivars, while late-season cultivars were promoted by low temperature and short-day conditions (Ohkawa, 1989). In Japan, the timing of anthesis of early-season flowering cultivars is late July (being

equivalent to January in NZ), mid-season flowering cultivars from August to September (February to March in NZ), and late-season flowering cultivars in late October (April in NZ). In NZ, Japanese-bred cultivars flower naturally between January and April, depending on the cultivars and weather. The timing of anthesis of the new cultivars and genotypes used in this thesis such as; ‘03/04-114’, ‘Showtime Diva’ and ‘Showtime Spotlight’, is approximately between February (late summer) and April (middle autumn), depending on weather, under the natural condition in Palmerston North (40.38° S 175.60° E), NZ (E. Morgan, personal communication, 2009).



**Figure 2.2 Diagram illustrating the annual growth cycle of perennial gentians, with focus on the seasonal changes associated with dormant crown buds in winter, shoot emergence in spring, flowering in summer/autumn, and progressing through to shoot/foilage senescence in autumn/winter.**

The new cultivars developed in NZ are derived primarily from *G. triflora* and *G. scabra* (Morgan et al., 2003). The distribution of latitude for the parent species *G. triflora* range

from approximately 34° N through to 54° N (Ohwi, 1965), with photoperiods of 14:25 h to 17 h in June and between 7:22 h to 10 h in December (Lammi, 2005). The other dominant parent, *G. scabra*, is naturally distributed approximately between latitudes 30° N through to 54° N, with photoperiods of 14 h to 17 h in June and between 7 h to 10 h in December (Lammi, 2005). Since the critical photoperiods of short and long photoperiods for any physiological response in gentians have not previously been reported, the shortest and longest day length for the centres of origin, i.e. 7 h and 17 h were referenced as the basis of short and long photoperiods within this thesis, respectively (refer to Chapter 5).

## **2.3 Horticultural Production**

### **2.3.1 Propagation**

Gentian can be propagated by seeds, cutting, division, and tissue culture. Their seeds need chilling to break dormancy. For autumn sowing, seed dormancy can be broken by the natural cold of winter; however, for spring sowing, seeds need exposure to 0-5 °C for two weeks, a treatment with 50 ppm GA<sub>3</sub> for three days, or a combination of these two treatments (Ohkawa, 1989).

Recently hybrid cultivars of gentians have become more important for supply to commercial cut flower and potted flowering plant production, so vegetative propagation has been widely used to deliver genetically uniform plants. One method of vegetative propagation is dividing the crowns and replanting, but low rates of multiplication and disease infection are noted problems (Anonymous, 1984). Cuttings are a highly efficient method for multiplication, but can only be applied to some cultivars in which it is relatively easy to form crown buds. It is anecdotally reported that difficulty forming crown buds before winter limits the wide application of this method, within Japan at least.

Plant tissue culture provides an efficient method of propagation to produce disease-free plants (Jomori et al., 1995; Hosokawa, 1996 ; Morgan et al., 1997). However, these young clonal-plants of some cultivars, also encounter a similarly low number and rate of crown bud formation after being de-flasked and planted in the field, as well as a relatively long period (approximately 3 years) to reach a commercial level of productivity of flowering shoots (Sato, 1988a). To achieve uniformity of experimental material, gentian plants used in the series of experiments within this thesis, were clonally propagated by tissue culture.

### 2.3.2 Cultivation

Since gentian cultivars used for commercial cut flower production are native of alpine and cool-climate regions, cool summer conditions are considered favourable to their growth. The optimum temperature is reputed to be between 15-18 °C (Ohkawa, 1989), although no scientific literature is available to verify this. If temperatures exceed 30 °C, shade may be used to reduce temperatures (Yamanaka & Kujii, 1978; Anonymous, 1984), but commercially in NZ shaded structures may be used so as to eliminate pollinating insects more than for temperature control. Using Japanese cultivars, the higher night temperature (15 °C) and longer day length (16 h) has been found to promote the timing of flowering, while shorter day length (8 h) and lower night temperature (11 °C and 7 °C) increased the floral shoot length and the number of nodes (Tsukada, 1984).

Acid soil (pH 5.0-5.5) with good drainage and high organic matter is considered preferable for growing gentian plants. Irrigation is required, especially for young plants that may dry out easily, and poor drainage often leads to root rot. Young plants appear to need extra care, in order to reduce mortality.

Gentian plants in cultivation are usually planted in two rows on a raised bed (100 cm wide and 6 to 15 cm high), with a row distance of 40 cm and between plant distance of 20 cm. To prevent floral shoots from bending, support-netting needs to be provided as the plants become tall and are prone to collapse. The first net is at around 30 cm high, with a second or third net raised up as the floral shoots grow. After the third year, the number of floral shoots per plant increases to such an extent that thinning is normally carried out from the 4th year to prevent over-crowding, and maintain a high quality of flowering shoots. When thinning, the common method followed is, as the floral shoots reach around 30 cm height, eight to ten of the best shoots are left to produce a harvestable crop of flowering shoots, and the rest are pinched out to remove their florets, but leaving foliage at their bases. Under this regime the harvest of flowering shoots can continue for 4-5 years from the third year after planting (Ohteki, 1982; Anonymous, 1984).

### 2.3.3 Harvesting of flowering shoots

Commonly, the harvest of flowering shoots in gentians is carried out when the floral buds at the top of the floral shoot is fully coloured but, still tightly closed. Harvesting flowering shoots at less mature stages usually results in failure of undeveloped florets to open or

light colouration of petals (Ohteki, 1982; Eason et al., 2004). Over-maturity decreases the vase life, and may require removal of the old senescing florets from the base of shoots before being marketable.

Leaf wilting is another factor affecting the vase life of gentians. Cultivars with larger leaves (e.g. ‘Late Blue’) are more prone to wilt compared with those which have smaller leaves (e.g. ‘Ashiro No-Ake’; Eason et al. 2004). Sucrose pulses can reduce leaf wilting and enhance pigmentation of the apical florets when harvesting at less mature stages. Pollination also accelerates petal senescence. Moreover, cross pollinated florets senesce more rapidly than self-pollinated or un-pollinated flowers. Growers and florists are recommended to avoid using fluoridated water for handling flowering shoots after harvest, because fluoride in water can lead to leaf softening and browning (Eason et al., 2004).

The new cultivars developed by Plant & Food Research (Palmerston North) are considered suitable as a cut flower for export from NZ, in part because they have a long vase life. For example, ‘Showtime Diva’, ‘Showtime Spotlight’, and ‘Showtime Starlet’ were reported to have a vase life of 15 days (after 8 days in cool storage), 10 days (after 3 days cool storage), and 8 days (after 5 days cool storage), respectively (Eason et al., 2006). Also these cultivars are sterile (the mechanism is still not clear) and have small leaves, which are characteristics believed to be beneficial to increase vase life.

## **2.4 Yield and quality of flowering shoots**

### **2.4.1 Yield and quality**

The yield of flowering shoots for cut flowers, in a gentian crop, is measured by the quantity of marketable flowering shoots produced from a unit area. Flowering shoots for international markets usually are required to meet higher quality standards than for the domestic markets. The parameters for assessing the quality of flowering shoots include; length of flowering shoots, node number, floral node number, shoot diameter, floret number, fresh weight of flowering shoot, vase life and health (freedom from diseases and pests). In Japan, a marketable flowering shoot usually must be a minimum length of 70 cm, and more than 10 g fresh weight (Yamanaka, 1978).

### 2.4.2 Factors influencing the yield and quality

The yield and quality of flowering shoots is affected by the entire production and handling process, including pre-harvest, harvest and post-harvest. However, for perennial gentian, as a typical geophyte, the following aspects are considered of particular importance:

- Crown bud formation

As all flowering shoots are developed from crown buds formed in the preceding year's growing season and, at least the size of crown buds influences the length of flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon, 2012), producing sufficient quantity of well-developed crown buds is a prerequisite for the high yield of flowering shoots. While the first commercial harvest of gentian flowering shoots commonly occurs in year three after adequate crown buds have been established, full production is reached in years five to six, continuing for a further four to five years (Ohkawa, 1989). In order to increase profit, it is considered desirable for gentian plants to reach full commercial harvest in a shorter number of years. Promoting the increase of crown buds in the early years of gentian is, therefore, something that is sought after. On the other hand, over the full period of cultivation (8 to 10 years), maintaining the stability of the number of crown buds is also important for sustainable production of flowering shoots of high yield and quality. Crown bud formation was clearly a topic of critical relevance for further examination within this thesis.

- Winter survival and re-growth

Gentian plants rely on underground crowns with resting crown buds to survive the cold winter and initiate new growth in spring. Although it is recognised that compared with the cold temperatures experienced in winter in the primary region of gentian production in Japan, NZ experiences a milder winter climate in areas in NZ where commercial production occurs (Anonymous, 2010b, 2010a), it has anecdotally been reported that in the production of flowering shoots of gentians, significant death rates of plants has sometimes occurred, due to failure of overwintering. In addition, using the interspecific hybrid 'Showtime Spotlight', Samarakoon and colleagues recently reported that some crown buds did not emerge in spring, albeit these crown buds had survived the winter (Samarakoon et al., 2012b). While the existence of paradormancy was hypothesised as the reason why all crown buds did not necessarily emerge, this was not validated. All these facts indicate winter survival and spring re-growth are important limiting factors to the

yield of flowering shoots and, therefore, were deemed important to examine further within this thesis.

- Floral development

For gentians, the spike-type flowering shoots are the part of the plant harvested as cut flowers and, therefore, the growth and development of floral shoots directly determines the yield and quality of flowering shoots such as their length, floral node number, fresh weight, etc (Yamanaka, 1978). In addition, the suitable harvest stage of flowering shoots of gentians was when the apical florets were fully pigmented. At that stage, a substantial number of the florets on the spike-type inflorescence have not fully developed, and the continued development and opening of these undeveloped florets will directly influence their post-harvest performance in markets. As explored further within the following sections, floral development was considered worthy of further examination within this thesis.

### **2.4.3 Hypothesis: carbohydrates influencing yield and quality**

As introduced in Chapter One, it was hypothesized that the availability of carbohydrate is a limiting factor influencing the development of crown buds, winter survival, spring re-growth, and the development of floral shoots. As a result therefore, carbohydrates are considered likely to be an important factor determining the yield and quality of the flowering shoots in commercial production. As explored further in this thesis, the likelihood of their importance is firstly based on, the physiological function of carbohydrates in regulating plant growth, development, and responses to environmental factors (Lewis, 1984b; Smeekens, 2000) that may affect crown bud development, their ability to survive winter, re-growth in the following growth season and, their development into floral shoots. Secondly, carbohydrates are the main energy reserve and source of carbon for building the structural components of the flowering shoots. Thirdly, the vase life of harvested flowering shoots, a key quality parameter, is influenced by the content of non-structural carbohydrates accumulated in the flowering shoot (Eason et al., 2004). The organogenesis and development of crown buds through to anthesis of the floral shoot is a complex series of processes regulated by multiple factors including environmental factors, hormones, etc (Thorpe, 1974; Gemas & Bessa, 2006; Iglesias et al., 2006). However, in this thesis the physiological roles of non-structural carbohydrate were specifically focused on.

## 2.5 Function and metabolism of carbohydrates

### 2.5.1 Categories and types of carbohydrates

Vascular plants contain a wide range of carbohydrates with specific functions. Also their distribution and concentration vary with species, organs, tissues, season, as well as diurnally (Lewis, 1984b).

Function-based carbohydrates are generally classified into two groups, i.e. structural carbohydrates and non-structural carbohydrates (NSCs). Structural carbohydrates, including cellulose, lignin, pectin, etc., are located within plant cell walls providing structural support. Depending on the plant species under examination, the forms of NSCs that exist in various organs, tissues, and cells, include glucose, fructose, sucrose, starch and fructans, etc., as well as alditols (also called sugar alcohols) such as mannitol or sorbitol, and cyclitols such as inositol; in addition, some genera such as *Gentiana*, have unique carbohydrates, i.e. gentianose and gentiobiose. NSCs are the main carbohydrates directly involved in metabolism within plants, as well as providing the primary forms for transport and storage.

According to the number of individual simple monosaccharide units, carbohydrates may be classified as monosaccharides (glucose, fructose, mannose), oligosaccharides (sucrose, maltose, gentiobiose, cellubiose, lactose, raffinose, gentianose) containing anywhere between two to ten monosaccharide units, termed by their degree of polymerization (DP), polysaccharides (starch, fructan, cellulose), and ill-defined complex carbohydrates (glycoproteins and glycolipids).

In gentians, most published knowledge about carbohydrates has focused on those present within the storage-roots of *Gentiana lutea* L. (i.e. ‘Great Yellow Gentian’), a perennial alpine plant with storage-roots used for medicinal purposes (Bridel, 1911; Keller & Wiemken, 1982; Rossetti et al., 1984). There is, however, little if any, literature reporting the content of carbohydrates in above-ground organs of gentian plants. Gentianose ( $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  6)- $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  2)- $\beta$ -D-fructofuranoside) is a non-reducing trisaccharide, freely soluble in water, and confined to the vegetative parts of species of *Gentiana* and *Swertia* (both in Gentianaceae) (Kandler & Hopf, 1980; Lewis, 1984b). Gentiobiose ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose), a reducing

disaccharide (Lewis, 1984b), has been found in the vegetative parts of gentian plants containing gentianose (J. Eason, personal communication, 2008), in the seed coat of the ripening seeds of ivy (*Hedera*) and, the xylem sap of birch (*Betula*) (Lewis, 1984b).

Because of the limited information on the physiological function of NSCs in the growth and development of gentian plants, in this research programme, it was considered important to first determine the types, distribution and seasonal changes of NSCs in gentian, so as to aid better understanding of the potential effect of the acquisition, storage and utilization of carbohydrates on the yield and quality of flowering shoots.

### **2.5.2 Partitioning**

Carbohydrate partitioning in plants is the distribution of carbohydrates in various organs or tissues as the results of carbohydrate transport and metabolic processes, controlling the flow of assimilates from source organs to sink organs (Marcelis, 1996). A source can be defined as an organ or tissue that is a net exporter of carbon assimilates. In contrast, a sink is a net importer. The definitions of source and sink strength of organs are usually used to discuss the capability of plant organs to export or import photoassimilates, specifically carbohydrates (Wilson, 1972; Wareing & Patrick, 1975; Marcelis, 1996; Herbers & Sonnewald, 1998; McCormick et al., 2006; Mohapatra et al., 2009); however, the validation of the definition/calculation/measurement of source and sink strength has also been argued by some authors (Farrar, 1993; Minchin & Thorpe, 1993; Herbers & Sonnewald, 1998). Source strength was early defined as source size  $\times$  source activity, i.e. leaf area ( $\text{m}^2$ )  $\times$  net assimilation ( $\text{g m}^{-2} \text{d}^{-1}$ ) (Wilson, 1967, 1972). This calculation apparently refers to the net rate at which carbon assimilates are produced rather than exported. For example, while young developing leaves may have a positive net assimilation rate, and export some photosynthate, they are initially net importers of photosynthate due to growth, usually until about 50% expanded. Similarly, sink strength was defined as sink size  $\times$  sink activity, i.e. dry weight (g)  $\times$  relative growth rate ( $\text{g g}^{-1} \text{d}^{-1}$ ) (Wilson, 1967, 1972). This calculation actually refers to the net accumulation of dry matter and does not account for the imported assimilates used for respiration and remobilization. This definition of sink strength is also questioned as the import of assimilates is not determined by the sink strength alone, but also source strength and transport in phloem (Farrar, 1993; Minchin & Thorpe, 1993; Marcelis, 1996). Parameters that are independent of the rest of plant are, therefore, needed to describe sink strength

more precisely. Biochemical and molecular studies have found the correlation between sink strength and enzyme activities such as invertases, sucrose synthase and hexokinases, and these findings have brought new promising approaches to determine the sink strength (Black, 1993; Herbers & Sonnewald, 1998).

The complex processes of carbohydrate partitioning are associated with carbohydrate synthesis, transfer, compartmentalisation, storage, hydrolysis, remobilization and, finally, utilization (Wardlaw, 1990). Plant growth and development during the growth cycle are accompanied by sink-source transitions influenced by source strength, sink strength, and transport between sources and sinks (Roitsch, 1999; Blanke, 2009). Within this thesis therefore, quantifying the distribution and seasonal fluctuation of the NSCs aimed to provide potential insight to understanding the physiological function of carbohydrates in terms of crop yield and quality.

### **2.5.2.1 Production**

The initial source of NSCs entering a plant system is photosynthesis in leaves (Farrar, 1999). Some NSCs, for example starch and fructan, are synthesized indirectly in storage organs after translocation and metabolism of assimilate from leaves or elsewhere in the plant. The biochemistry of NSCs based on sucrose and starch has been well characterized, including localization of key enzymes for synthesis and degradation (Winter & Huber, 2000; Koch, 2004). However, other NSCs of interest in this thesis, such as gentianose and gentiobiose, have not been studied extensively. In contrast to the basic biochemistry noted above, the mechanism of regulation for production of NSCs is not completely understood (Roitsch, 1999).

It is accepted that photosynthesis is influenced by environmental and physical factors like light-intensity, temperature, CO<sub>2</sub> concentration, chlorophyll content, etc. Photosynthesis occurring within the leaves determines the availability of carbohydrates for export to other organs (i.e. source strength). In addition, however, evidence increasingly supports the concept that the photosynthetic rate, and rate of carbohydrate export in leaves, is not only controlled by environmental factors, but also carbohydrate metabolism in sinks; even remote organs such as roots (McCormick et al., 2006; Cheng et al., 2008; Kaschuk et al., 2009). Given that gentian utilise an under-ground crown for storage of carbohydrates, i.e. a sink, it was considered plausible that this sink might also regulate the photosynthetic rate

and carbohydrate export in leaves. Despite the obvious importance of photosynthesis on carbohydrate acquisition in gentians, limited time resources did not permit development of experiments to investigate within this thesis.

### **2.5.2.2 Transportation**

The phloem is the primary pathway for carbohydrate transport, particularly long distance transport from the site of synthesis in the leaves to the sites of utilization or storage (Thorne & Giaquinta, 1984). The process of sugar transport between source and sink regions of plants includes the movement of sugars to the phloem, loading into the phloem, transport within the phloem, and unloading to the sink tissue and consuming cells (Thorne & Giaquinta, 1984).

Sucrose is the main carbohydrate transported in most plants, but other oligosaccharides and polyalcohols (sugar alcohols) may also be transported (Reidel et al., 2009; Rennie & Turgeon, 2009). Conventionally in most plant species, the carbohydrates transported in the phloem are non-reducing carbohydrates, such as sucrose, raffinose or polyalcohol; however, van Bel (2008), using EDTA exudation, found a widespread occurrence of hexoses, i.e. a reducing monosaccharide, in the phloem sap. This may indicate that hexose translocation may also be a normal mode of transfer of carbohydrate by the phloem, being equivalent to that of sucrose, raffinose-family-carbohydrates, or polyalcohol. While this result was later denied by Chao and Turgeon (2012), as using EDTA exudation they detected only sucrose in phloem of the same plant species used by van Bel.

To investigate phloem transport, it is essential to analyse the phloem sap to know the chemical composition and solute concentrations, so as to understand the partitioning of NSCs and sink-source interactions. However, research into phloem transport is currently restricted by the methodologies available to obtain pure phloem sap. Phloem sap may be collected by incision and cutting (Hall & Baker, 1972; Kallarackal & Komom, 1989), EDTA-induced exudation (King & Zeevaart, 1974; Urquhart & Joy, 1981; van Bel & Hess, 2008) and, via the stylets of insects (Hayashi & Chino, 1986; Oshima et al., 1990; Gould et al., 2005). For differing plant species each method has advantages and disadvantages. Despite the presence of unusual carbohydrates, i.e. gentianose and gentiobiose, the forms of carbohydrate(s) transported in the phloem of gentians have not been reported. While time consuming and requiring special equipment, either the stylet technique or EDTA-induced exudation were considered worthy of exploration within this thesis.

### 2.5.2.3 Storage and remobilization

Depending on the ability to export or import carbohydrates, plant organs can be divided into sources (mature leaves and exporting storage organs) and sinks (immature leaves, stems, seeds, rhizome/stem, storage roots, tubers, etc.). Typically each plant species stores one type of carbohydrate (for instance, sucrose, starch, raffinose, etc.) over others (Brocklebank & Hendry, 1989). Any one species, however, often contains different types of carbohydrates, so providing a flexible means of regulating carbon flux and mobilization (Farrar, 1999).

The changes in concentration and nature of pools of NSCs in various plant organs and tissues, or during different seasons, e.g. the turnover of NSCs in storage pools, can provide important information as to the dynamics of supply and demand for carbohydrates (Boldingh et al., 2000; Gesch et al., 2007), and the possible activities of regulating enzymes such as invertase, sucrose synthase, hexokinase, fructokinase and sucrose-phosphate synthase (Klann et al., 1993; Irving et al., 1997). In storage roots of *G. lutea*, the changes of gentianose, gentiobiose and sucrose in the storage roots of *G. lutea* were measured, and showed a similar trend of accumulation of these NSCs in autumn as evident in other perennial plants (Bridel, 1911), however, data covering winter and early spring, or for other organs, was not presented. Hence, in the current study, it was proposed that the seasonal changes of the concentration of NSCs should be quantified, so as to explore the physiological function of the storage and utilization of NSCs in the growth cycle of gentians, and their importance to the commercial production of their flowering shoots.

### 2.5.3 Factors influencing partitioning of NSCs

Environmental factors such as photoperiod (Grange, 1985) and temperature (Prud'homme et al., 1993), can influence the partitioning of carbohydrates. In perennial plants, storage carbohydrates in underground organs allow the plant to avoid periods of adverse seasonal conditions and remobilize these carbohydrates for rapid re-growth in the next growth season (Pressman et al., 1993; Gesch et al., 2007). In *Asparagus officinalis* L, a shorter photoperiod resulted in greater biomass and higher concentration of soluble carbohydrates to the crowns (Woolley et al., 1999). As introduced in Chapter 1, it was hypothesised that the partitioning of NSCs may influence the yield and quality of flowering shoots of gentians, hence in this thesis it was proposed that the seasonal changes of carbohydrates in

various organs should be monitored, so as to understand the pattern of carbohydrate partitioning in gentians.

Sink strength varies in different tissues and organs of plants. Generally, the growth of fruit and seed have priority to obtain carbohydrates in comparison with the vegetative growth; shoot growth dominates over the roots; and underground storage organs have the same sink strength as fruits (Wardlaw, 1990). This suggests that the timing of organ initiation and development may change the carbon demand, and then lead to the switches of carbon partitioning between various tissues and/or organs. If the same dynamics occur in relation to the seasonal changes in organ development in gentians, this may mean we could expect that the partitioning of carbohydrates is similarly related to their organogenesis and development.

It has been reported that enzyme activity related to the synthesis and degradation of carbohydrate potentially contributes to sink strength, for example, invertase, sucrose synthase, amylase and ADPglucose pyrophosphorylase, etc (Sung et al., 1994; Mohapatra et al., 2009; Kaur et al., 2012). The biochemistry of enzymatic metabolism of carbohydrates in gentian may reveal their mechanism of partitioning and physiological function. In the current research programme therefore, a series of preliminary experiments were proposed to determine the possible relationship between enzyme activity and carbohydrate accumulation and degradation, in various organs, with the development of gentian plants.

#### **2.5.4 Function of NSCs**

As noted in the preceding sections, NSCs are the primary energy and substrate resource for metabolic activities in plants, and these contribute to determining the yield and quality of crops. Simultaneously, they also play significant physiological functions in regulating plant growth, development, and responses to environmental factors. Acting as osmotic agents, some sugars convey resistance against periods of adverse environment, such as drought, salinity and cold (Jacobsen et al., 2007; Martinelli, 2008; Perez-Lopez et al., 2010). Evidence indicates an additional role of sugars as signalling molecules in plants (Smeekens, 2000; Teng et al., 2005). As detailed in the following sections, NSCs and their physiological function and metabolism are documented.

#### 2.5.4.1 Energy and metabolites

In higher plants, there are two fates for the metabolism of NSCs: one is incorporation into newly synthesized carbon compounds including amino acids, phytohormones, cell wall, more complex carbohydrates (e.g. starch) and lipids; the other is to produce energy for plant metabolism by respiration (i.e. the tricarboxylic acid (TCA) cycle, glycolysis and the oxidative pentose phosphate pathway), in which energy is released as chemical energy in the form of ATP from sugar (glucose) by a series of chemical reactions controlled by enzymes (Farrar, 1999). During respiration, glucose is the favoured substrate for respiration. Other carbohydrates are usually first converted into glucose by various enzymes before they are used for respiration (Lewis, 1984b). Hence in the current research programme, the properties and activity of carbohydrate-associated hydrolases were proposed for preliminary investigation.

#### 2.5.4.2 Osmoregulation

Carbohydrates can also be related to the tolerance of adverse conditions, such as drought, coldness and salinity due to their roles as osmotic protectants (Jacobsen et al., 2007; Patton et al., 2007; Martinelli, 2008; Perez-Lopez et al., 2010). For example, the frost resistance of Quinoa (*Chenopodium quinoa* Willd.) was correlated to a high soluble sugar content, which may result in a lowering of the freezing point of plant tissue. The content of sugars has, therefore, been suggested as a useful indicator in future breeding of Quinoa (Jacobsen et al., 2007), indicating the potential value of such a research strategy. Similarly in gentian, Keller et al (1982) suggested that the high concentration of carbohydrates in storage-roots may serve as a cryoprotectant, allowing the crown to tolerate the cold winter. Considerable variation exists in the ability of different gentian genotypes to survive winter in Japan (J. Eason, personal communication, 2008). For instance, gentian ‘Showtime Spotlight’ survived the cold winter conditions well in Japan, but ‘Showtime Starlet’ did not. It has been hypothesised that the poor tolerance to winter by ‘Showtime Starlet’, may be attributed to the lack of an alpha/beta-hydrolase fold-protein (Hikage et al., 2007), however, whether the carbohydrates in the storage-roots, rhizome and crown buds of gentian are responsible for cold tolerance deserves further study.

The content of various carbohydrates may change in response to differing strengths of stress. For example, under mid-range stress in the leaves of *Sporobolus stapfianus* Gand.,

i.e. 95% and 56% relative water content (RWC) of plants, significant increases in glucose, fructose and sucrose were observed; when severe desiccation stress was applied, i.e. lower than 56% RWC, glucose and fructose content decreased to very low levels, but sucrose content increased to the maximum level (Martinelli, 2008). It was possible therefore that seasonal changes of carbohydrate composition in gentian might also be able to be interpreted similarly.

Carbohydrates not only provided carbon and energy, but also acted as an osmotic agent influencing morphogenesis via affecting cell pressure potential and expansion (Brown et al., 1979; Milani et al., 2013). With gentian treated *in vitro* to increasing sucrose concentration of the medium (i.e. from 1.5% to 6%), when subsequently grown *in vivo*, the increasing sucrose concentration resulted in an increased frequency of initiation of crown buds (i.e. 30% to 95%), and more crown buds per plant (i.e. 1.3 to 2.1) (Sato, 1988a). Within this thesis, it was proposed that the role of carbohydrates in the formation of crown buds of gentian would be further examined via control of the carbohydrate supply *in vitro* and *in vivo*.

#### **2.5.4.3 Signalling function**

It has been identified that carbohydrates (mainly small molecular sugars) play a signalling function (Smeekens, 2000). For example, different carbohydrates play different roles in the shoot development of tobacco (*Nicotiana tabacum* L.) *in vitro*, wherein the presence of maltose increased the number of developed shoots, whereas, fructose enhanced shoot length (Gemas & Bessa, 2006). Many studies indicated that sugars, as signalling compounds, have a phytohormone-like function involving all stages of the plant's life cycle (Smeekens, 2000; Rolland et al., 2006; Cho & Yoo, 2011). Recent research in sugar signalling in plants has found that a wide variety of genes are sugar-regulated at the transcriptional level (Sheen, 2001).

Carbohydrates are the products of photosynthesis and, simultaneously, carbohydrates regulate photosynthesis and the source-sink relationship. This regulation ultimately determines the pattern of carbon partitioning among the various plant organs, tissue and cells (Roitsch, 1999). Feedback inhibition is a long-known mechanism in photosynthetic regulation (Abdin et al., 1998), in which sugars can play a key role by inhibiting enzymatic activity or repressing the expression of photosynthetic genes (Koch, 1996;

Roitsch, 1999; Blanke, 2009). Sugars as a link between source and sink also induce a number of sink-related enzymes involved in sugar degradation and synthesis of storage products (Roitsch & Gonzalez, 2004). Extracellular invertase has been suggested to be a key enzyme in source-sink regulation, with the function of supplying carbohydrates to sink tissue, regulation of source-sink transition, amplification of signals, and integration of signals (Roitsch, 1999).

In gentians, little information has been published about the effects of sugars on crown bud development. As a potential parallel, it has been suggested that sugar-signalling may play an important role in regulating the growth and development in underground adventitious buds in Leafy Spurge (*Euphorbia esula* L.), by enhancing the gene expression of hexokinase and sucrose synthase, which increase the influx of sugar into underground buds (Anderson et al., 2005). However, whether or how the sugars in gentian influence crown bud initiation and development and, in turn, affect the yield and quality of flowering shoots, needs to be investigated. In the current study, it was the first intention to investigate the effect of carbohydrate supply on crown bud formation, winter survival, and re-growth, before any future investigations could be undertaken to understand whether carbohydrates act as a signal and regulate relevant gene expression.

### **2.5.5 Metabolism of gentiobiose and gentianose**

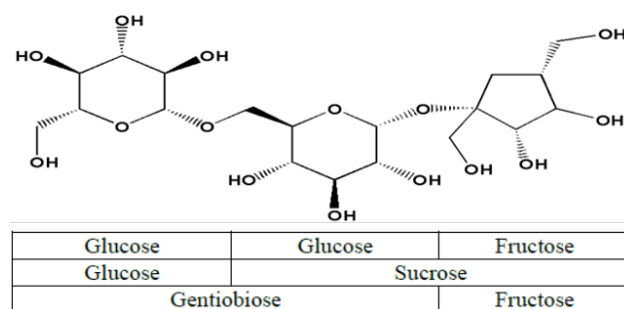
The diversity of mono-saccharides and their linkages (refer to 2.5.1) greatly increases the structural diversity of carbohydrates, and there is a vast spectrum of enzymes with either high or low specificities involved in carbohydrate biosynthesis, breakdown and modification (Cantarel et al., 2009). Currently, the Carbohydrate-Active Enzyme (CAZy) database has described up to 132 families of glycoside hydrolases (GHs), based on amino-acid sequence similarity (Henrissat et al., 2013) and, in *Arabidopsis* for example, GHs are represented by 379 genes/proteins, classed in 29 families (Henrissat et al., 2001). Unlike the widely studied enzymes related to carbohydrates such as sucrose, raffinose, starch, and fructan, in other plant species (Winter & Huber, 2000; Chalmers et al., 2005; Li & Wang, 2008; Streb & Zeeman, 2012), there is very little information available for enzymes in gentians that involves the metabolism of the unique carbohydrates, i.e. gentianose and gentiobiose. The limited information available in gentian comes from a study focussing on compartmentalisation within the storage-roots, which showed that gentianose is the predominant carbohydrate reserve, with 100% of both gentianose and gentiobiose

compartmentalised in the vacuoles. In contrast, 80% of fructose and glucose, and 50% of sucrose was compartmentalised in the vacuole (Keller & Wiemken, 1982). However, which enzymes are involved in the metabolism of gentianose and gentiobiose in gentians has not been reported.

The physiological function and metabolism of gentianose and gentiobiose have not been studied as extensively as has been done for sucrose and starch (Kandler & Hopf, 1980; Lewis, 1984a). Gentianose is confined to the vegetative parts of species of *Gentiana* and *Swertia* (both members of Gentianaceae) (Kandler & Hopf, 1980; Lewis, 1984b). Gentiobiose is accumulated in large quantities in *Gentiana* (J. Eason, personal communication, 2008), but is only found in small amounts in other plants (Dumville & Fry, 2003).

### 2.5.5.1 Molecular structure

Gentiobiose (( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose) is a reducing disaccharide composed of two units of D-glucose joined with a  $\beta$  (1 $\rightarrow$ 6) linkage. It is a structural sub-component of the trisaccharide gentianose (Figure 2.3). Gentianose ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside) is a non-reducing trisaccharide, isolated from storage-roots of *G. lutea* (Meyer, 1882), and is the only sucrosyl-glucoside isolated from plants. In structure, gentianose can be broken down to glucose and sucrose or gentiobiose and fructose (Figure 2.3) but, *in vivo*, it is still not clear what the products of the hydrolysis of gentianose are.



**Figure 2.3 Molecular structure of gentianose (Sensonet, 2008). One molecule of gentianose is composed of two molecules of glucose and one molecule of fructose. In structure, gentianose can be broken down to one molecule of glucose and one of sucrose, or to one molecule gentiobiose and one fructose, or two molecules of glucose and one molecule of fructose.**

### 2.5.5.2 Synthesis and hydrolysis

To date synthesis of gentianose has not been elucidated. The intracellular distribution of  $^{14}\text{C}$  in gentianose showed the glucosyl residue bound to the glucose moiety of sucrose exhibited a higher specific activity after a short feeding of  $^{14}\text{CO}_2$  to the leaves of *G. lutea* (Heiniger & Franz, 1977). This indicates that biosynthesis of gentianose might involve the transfer of a glucosyl residue to sucrose. Attempts to demonstrate synthesis *in vitro*, using nucleotide-activated glucose or D-glucose-1-phosphate as a donor, have not succeeded (Kandler & Hopf, 1984). Hopf and Kandler (Kandler & Hopf, 1980) also found that the glucinol was not significantly labelled during photosynthesis by feeding  $^{14}\text{CO}_2$  to the leaves, with labelled gentianose formed, suggesting that the glucinol might not serve as a glucosyl donor. Gentianose and gentiobiose are strictly localized in vacuoles of parenchyma cells in storage-roots of *G. lutea* (Keller & Wiemken, 1982), which suggests that the location of the synthesis of gentianose and gentiobiose is in the vacuoles, and relevant enzymes or substrates also may be co-located there.

In plants, the metabolism of gentiobiose is still not clear, although the metabolism of gentiobiose has been investigated in detail in some bacteria (Palmer & Anderson, 1972; Kawano et al., 2008). Gentiobiose is commonly known as a breakdown product of gentianose, for example gentiobiose was isolated from storage-roots of gentian (Badenhuizen et al., 1964) and obtained from gentianose treated with invertase (Bourquelot & Herissey, 1901). Gentiobiose also occurred in the products of the commercial hydrolysis of corn starch (Berlin, 1926). Considerable quantities of gentiobiose has been found in the seed coat of the ripening seeds of ivy (*Hedera*) and in the xylem sap of birch (*Betula*), while no gentianose was detected in these plants (Kandler & Hopf, 1984). Therefore, whether gentiobiose is the product of the hydrolysis of gentianose in gentian still needs to be investigated.

Gentiobiose could also be synthesized using UDP-D- $^{14}\text{C}$  glucose as a substrate, with a dialysed enzyme preparation from ripening seeds of ivy (Kandler & Hopf, 1984). The optimum activity of this enzyme preparation was at pH 6.0 and  $2 \text{ mmol l}^{-1} \text{ Na}_2\text{S}_2\text{O}_4$ . It is needed to be noted that only UDP-D-glucose, not glucose or D-glucose-1-phosphate, could serve as donor or acceptor. This indicates that the substrate needs to possess an energy-rich phosphate bond. However, the enzymes for gentiobiose synthesis in gentian plants have not been reported.

Due to the existence of unique carbohydrates, i.e., gentianose and gentiobiose, it seems reasonable to assume that there must be a different enzyme system, controlling carbohydrate metabolism in gentian plants. Given that little information has been published regarding the enzymes related to the metabolism of either gentianose or gentiobiose in gentians, a preliminary experiment was proposed in the current thesis to firstly characterize the property of enzymes related to the hydrolysis of gentianose, gentiobiose and sucrose, and secondly, to explore the relationships between the fluctuation in concentration of NSCs and their related enzyme activity, so as to help better understand the physiological function of NSCs in the growth and development of gentians (refer to Chapter 8).

## **2.6 Methods of analysis for carbohydrates and activity of related enzymes**

As proposed above, the physiological function of NSCs is the focus of this thesis; therefore, the potential methods for the analysis of carbohydrates and associated enzymes are briefly introduced and compared for their relevance within this thesis.

### **2.6.1 Carbohydrate analysis**

#### **2.6.1.1 Comparison of methods**

A very large number of analytical techniques have been employed to analyze the concentration of both total and individual carbohydrates, such as colorimetric methods (e.g. Anthrone method and Phenol - Sulfuric Acid method); and Chromatographic methods, e.g. gas chromatography (GC) and high-performance liquid chromatography (HPLC), etc. (Shaw, 1988; Chaplin & Kennedy, 1994). The colorimetric methods are widely used to determine the total concentration of carbohydrates, while the disadvantage of this type of analysis is not being able to distinguish individual carbohydrates. Given the presence in gentians of specific and unique carbohydrates, such general methods are not considered applicable within the current thesis.

Chromatographic methods are very powerful analytical techniques for separation, identification, and quantification of soluble carbohydrates. As volatile samples are required for GC analysis, a derivatization step is needed to prepare volatile carbohydrate derivatives, whereas the samples for HPLC analysis are more easily prepared. Due to

simple, specific, sensitive and precise measurements, HPLC is currently the most powerful method for analyzing carbohydrates (Wannet et al., 2000; de Sa et al., 2011).

### **2.6.1.2 High Performance Liquid chromatography (HPLC)**

In HPLC analysis, carbohydrates can be separated on the basis of their partition coefficients, polarities or sizes, depending on the type of column used. For insoluble polysaccharides such as starch, most methods hydrolyze starch to glucose, which is subsequently analysed by HPLC or other methods mentioned above. Starch can be hydrolysed by perchloric acid, sulphuric acid, and enzymes (amylase and amyloglucosidase). Enzyme digestion is considered as the most accurate method. In present therefore, within the current thesis starch would be first broken down to glucose by enzyme digestion (Amylase/Amyloglucosidase), and then analysed by the above methods.

As a convenient and sensitive method, HPLC is also used for detecting and/or monitoring the substrates and products in bioprocesses of carbohydrate metabolism (Wannet et al., 2000; de Sa et al., 2011). As part of such investigations, HPLC is commonly used in conjunction with nuclear magnetic resonance (NMR) or mass spectrometry (MS) to identify the chemical structure of any unknown molecules (Sommer et al., 2003). For example, within this thesis gentianose, gentiobiose and the unknown component (i.e. L-bornesitol) were identified using HPLC-NMR (Appendix I) and, in conjunction with an evaporative light scattering detector (ELSD), HPLC was used for quantifying and monitoring carbohydrate concentration in a series of experiments (Appendix II) and, analyzing the products of carbohydrate hydrolysis so as to assay the activity of GHs (refer to Chapter 8).

## **2.6.2 Isolation and detection of enzymes**

### **2.6.2.1 Protein isolation and detection**

Isolation of one particular enzyme from all other proteins in a complex mixture of plant materials requires the methods for both separating and detecting the specific enzyme. Various methods are used in enzyme isolation and purification such as ion exchange chromatography (IEX), Gel electrophoresis, and HPLC (Lodish et al., 2000; Price & Nairn, 2009). Polyacrylamide gel electrophoresis (PAGE) is most widely used for proteins

separation under either denaturing-conditions or non-denaturing-conditions; however, this method is required to detect the enzyme of interest in the gel band. Many methods of enzyme detection on electrophoretic gels have been developed, such as chromogenic reactions, fluorogenic reactions, autoradiography, immunoblotting and two-dimensional gel spectroscopy (Lundblad & Macdonald, 2010). Immunoblotting (i.e. Western blotting) is one of the most powerful methods for detecting and quantifying a particular protein separated by electrophoretic gels; however, antibodies specific to the proteins of interest, are required for this method. Another common method for enzyme detection on gels utilises chromogenic (staining) reactions that result in formation of chromophore at the sites where active enzymes are located. In most cases, the primary product(s) of the reaction catalysed by an enzyme of interest in a gel is not readily detectable and, a supplementary reagent(s) and/or an additional enzyme(s) are needed to obtain a visible product(s), i.e. the coupling of enzymatic reactions. For this method, due to the specificity of enzymes for their substrates, coloured products will only form where the activity of enzymes of interest presents. In addition, a detecting procedure must be simple and fast in order to minimize the possibility that the enzymes of interest is denatured or degraded while the detection is being performed. More detail of the potential staining methods are explored in the following section.

#### **2.6.2.2 Staining methods for detecting enzymes involved in carbohydrate metabolism**

Staining methods for detecting a range of enzymes involved in carbohydrate metabolism, were mainly derived from histochemical or colorimetric enzyme assay methods (Sergeeva & Vreugdenhil, 2002; Polit & Ciereszko, 2009), and have been successfully employed on electrophoretic gels (Finlayson et al., 1990; Trudel et al., 1998; Obroucheva & Lityagina, 2009). For the detection of activity of GHs in gel, one common staining method is using the reaction of the produced reducing carbohydrates with 2, 3, 5-triphenyltetrazolium chloride (TTC) to generate red-coloured zones (Trudel et al., 1998; Obroucheva & Lityagina, 2009). This method, however, may not be able to detect the GHs of gentiobiose, as both gentiobiose and its likely product of hydrolysis (i.e. glucose) are reducing carbohydrates (Lewis, 1984b).

Another powerful method is based on the coupling of the enzymatic reaction to the reduction of nicotinamide adenine dinucleotide (NAD) to NADH (reduced form of NAD), and subsequent reduction of nitroblue tetrazolium (NBT) to an insoluble precipitate with a

dark blue colour (Sergeeva & Vreugdenhil, 2002). Dahlqvist and Brun (1962) have developed a similar staining method for detecting the activity of glycoside hydrolases (GHs) such as invertase and trehalase, which produce glucose on the hydrolysis of their substrates. In the coupling enzymatic reactions of this staining method, glucose was oxidized by glucose oxidase resulting in the formation of gluconic acid lactone, which subsequently reduced nitroblue tetrazolium (NBT) to an insoluble dark-blue coloured precipitate. This method has been used for detecting enzymes involved in carbohydrate metabolism in electrophoresis gels (Finlayson et al., 1990). In the current study, glucose is assumed to be the product of hydrolysis of gentianose, gentiobiose and sucrose. It was therefore proposed that a staining method based on Dahlqvist and Brun's method, for detecting GHs of sucrose, gentianose and gentiobiose on electrophoresis gels, would be developed (refer to Chapter 8).

## **2.7 Summary**

As discussed above, carbohydrates play an important function in the life cycle of plants as energy reserves, carbon building blocks, protectants against adverse environmental factors and, as signalling compounds regulating growth and development. In terms of commercial production of flowering shoots of gentians, the research presented in this thesis is based on the hypothesis that carbohydrate availability (particularly the unique carbohydrate, gentianose and gentiobiose), influences crown bud formation, winter survival, re-growth and floral development. All of these key factors directly relate to the yield and quality of flowering shoots. To test this hypothesis, it was proposed a series of experiments to; determine the effect of carbohydrate supply (experimentally induced via defoliation or *in vitro*) on development of gentian plants and, quantify seasonal (natural) changes of non-structural carbohydrates (NSCs) in various organs, as well as activity of relevant glycoside hydrolases (GHs). Within this thesis, HPLC, supplemented with NMR, was used as the main analytical technique for: identification of the unique carbohydrates, gentianose, gentiobiose, and L-bornesitol; quantification of carbohydrates present in gentian plants; and determination and quantification of the products of carbohydrate hydrolysis.

## **Chapter 3 Form, structure and development of crowns in established plants of gentian 03/04-114**

### **3.1 Introduction**

As a typical geophyte, gentians rely on perennating crowns to survive adverse environmental conditions during winter and re-grow in spring (Ohkawa, 1989). The structure of a crown comprises storage roots, vertical rhizomes and regenerative crown buds (Figure 2.1) (Ho & Liu, 2001). Although the underground crowns are an important structure for the perennial life cycle of gentians, detailed knowledge of the functions and features of the form and structure of a crown is very limited.

In geophytes, underground storage organs store various nutrients (e.g. carbohydrates, protein, mineral salt, and even water) for overwintering and initiating re-growth (Le Nard & De Hertogh, 1993a). Within the topic of this thesis, however, the storage and utilization of carbohydrates will be the main target for study. Based on the features of the form and structure of crowns as previously described, i.e. thick storage roots, fleshy rhizomes, and swollen crown buds (Ho & Liu, 2001), any of them could be the main storage organs; however, the distribution of non-structural carbohydrates in the three parts of crowns is still not clear. To address this lack of knowledge, biomass proportions of various parts of crowns, i.e. storage roots, rhizomes and crown buds, and the concentration of non-structural carbohydrates (NSCs) in these parts, need to be determined. In this chapter the biomass proportion was quantified, with the carbohydrate concentration quantified in later chapters (refer Chapters 4 and 6).

In the life cycle of gentians for cut-flower production, from one year to the next, crown buds sprout in spring, flower in summer/autumn, and all shoots die back, leaving an underground storage organ (i.e. the crown) before winter (Figure 2.2). Vegetative crown buds on top of crowns undergo dormancy during the cold of winter and, after dormancy has been broken by low temperatures in winter, crown buds then re-grow again in spring (Ohkawa, 1989). All flowering shoots originate from crown buds developed in the previous year's growing season, and the bud size may influence the length of flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon et al., 2012b). Recent preliminary research using the interspecific hybrid 'Showtime Spotlight' indicates that not all the crown buds sprout in spring (Samarakoon et al., 2012b). In addition, young plants of

gentian usually take around three years to establish enough crown buds to reach their full commercial production (Ohkawa, 1989). All these facts imply that crown buds are a key factor influencing the yield and quality of flowering shoots.

Knowledge, in terms of the morphological and developmental features of crown buds of gentians, is necessary for studying the effect of carbohydrate supply on the development of crown buds (in later chapters of this thesis). However, such information in previous reports is very limited. Crown buds were commonly found in clusters (Sato et al., 1988; Samarakoon, 2012). Samarakoon's research (2012), using young seedlings and newly deflasked plants from tissue culture with various species and interspecific hybrids of gentians (primarily *G. triflora* and *G. scabra*), indicated that the first bud initial of a cluster of crown buds arises adventitiously on the transition zone (vertical rhizomes) between shoots and roots. In contrast Sato et al. (1988) (NB, non-peered reviewed report), using seedling and shoot cuttings of gentians, indicated that clusters of crown buds developed from axillary buds initiating from the axils of nodes at the base of shoots. However, both authors reported that the crown buds that subsequently developed within each cluster were co-associated by being axillary buds in a spiral arrangement. To detail the feature of the development of crown buds and potentially clarify inconsistencies between previous reports, the developmental pattern of crown buds were further investigated in this chapter.

Given the importance of crowns in the life cycle and the very limited published information in terms of the development of crowns of gentians, further knowledge is required to detail the features pertaining to the form, structure and development of crowns. The specific objectives of the current study were to:

- 1) characterize the form and structure of the crown of a new selection of hybrid gentian, including crown buds, rhizomes and storage roots;
- 2) determine the position and developmental pattern of crown buds;
- 3) determine what, if any, relationships exist between shoot number in the current growing season and the resulting number of new crown buds and crown bud clusters;
- 4) quantify the biomass distribution between crown buds, rhizomes and storage roots within the crown.

## 3.2 Materials and methods

### 3.2.1 Plant material

The gentian hybrid known as 03/04-114, with parents primary including *G. triflora* and *G. scabra*, was used in the current investigation. Plants were propagated *in vitro* and initially grown at The New Zealand Institute for Plant & Food Research, Palmerston North.

The plants had been deflasked and grown outside for between six and nine months in 60-cell trays (45 ml cell volume) containing a bark: pumice medium, and had passed through their first winter when acquired in October 2008. Hence, for the purposes of comparative description with other investigations presented in this thesis, the plant material was considered established. The growing medium used was Dalton's Base growing medium (CAN Fines A Grade 50%; Fibre 30%; Pacific Pumice 7 mm 20%; 0.5 kg/m<sup>3</sup> superphosphate (9.1P–11S–20Ca), 2.0 kg/m<sup>3</sup> each of agricultural lime and dolomite (21Ca–10Mg; Prebbles Seeds, Christchurch, New Zealand), and 1 kg/m<sup>3</sup> Gypsum), with 4.3 kg/m<sup>3</sup> of 8–9-month Osmocote<sup>®</sup> 16N–3.5P–10K (Grace-Sierra International, The Netherlands), 0.4 kg/m<sup>3</sup> calcium ammonium nitrate (27N–6Ca–4Mg), 0.5 kg/m<sup>3</sup> potassium sulphate (42K–18S), and 3 kg/m<sup>3</sup> Osmocote<sup>®</sup> (23N).

At the commencement of the experiment 16 plants, each of which comprised a crown with between 3 and 17 emerging shoots per plant, were used as individual replicates. Plants were transplanted into polythene bags (1.7 L) of growing media in spring (October 2008), and grown on two raised planting beds, outside at the Plant Growth Unit, Massey University (40.38° S 175.60° E). Cultivation utilised a capillary irrigation system with one overhead watering per week, so as to minimise periods of water or nutrient salinity stress.

### 3.2.2 Methods

The position and developmental pattern of crown buds was monitored throughout the whole growing season (i.e. October 2008 through to May 2009). The total number of new crown buds per plant (TCBN) of 16 plants and the flowering percentage (number of flowering plants/total number of plants, expressed as a percentage), were recorded every 2-3 weeks till the end of autumn (20<sup>th</sup> May 2009); with flowering defined as when the apical floret was fully pigmented and tightly closed, but not yet open (Eason et al., 2004). The number of new crown bud clusters per plant (CN), number of new crown buds per

cluster (CBNC) and the maximum shoot number (SN) of each of these plants were also recorded at the end of autumn (20<sup>th</sup> May 2009), for determining the relationships between SN in the current growing season and each of TCBN, CN, and CBNC at the end of that season.

At the end of autumn (20<sup>th</sup> May 2009), crowns of plants were removed from growing bags for observation and recording of the form and structure of crowns. The biomass of the components of the crowns of four plants (with similar shoot number across the replicates), i.e. crown buds, rhizome and storage roots, was determined by destructive sampling with each component measured separately. Fresh weights were recorded after plants were washed with tap water and dried with paper towels; dry weights were recorded after drying at 60 °C for one week.

### **3.2.3 Statistical analysis**

Regression analysis was used to identify what, if any, relationships existed between TCBN, CN, CBNC and SN, using Minitab 16 for Windows 7. All other data were analysed using an analysis of variance using GLM (Mintab 16). When significant differences between treatments occurred, mean values were separated using the Tukey method ( $P < 0.05$ ).

## **3.3 Results**

### **3.3.1 Form and structure of crowns**

It was evident that the crowns (in autumn) were comprised of three parts, i.e. crown buds, rhizomes and storage roots. Crown buds were normally attached to the tops of rhizomes, swollen, and covered with purple coloured scale leaves; rhizomes were thick and fleshy, and attached to the crown buds above and storage roots below; linear-cylindrical storage roots, attached to rhizomes, were also thick and fleshy (Figure 3.1). In addition, some storage roots showed visual evidence of being contractile roots, as evident by their wrinkled surface, attached to the rhizomes (Figure 3.2).

### **3.3.2 Biomass distribution**

At the end of autumn, storage roots accounted for the majority of the biomass in the crowns of a plant (i.e. 79% fresh weight; 80% dry weight), which was more than four-fold

that accounted for by rhizomes (i.e. 16% as either fresh weight or dry weight;  $P < 0.001$ ). In contrast, crown buds accounted for the least biomass proportion, at only approximately 4% as either fresh weight or dry weight ( $P < 0.001$ ; Table 3.1).



**Figure 3.1** Structure of a crown of gentian 03/04-114. Samples were collected from potted plants in autumn (20<sup>th</sup> May 2009).



**Figure 3.2** Contractile roots attached to rhizomes of gentian 03/04-114. Samples were collected from potted plants in autumn (20<sup>th</sup> May 2009).

**Table 3.1** Proportions of the fresh weight (FW) and dry weight (DW) of various organs, i.e. storage roots, rhizomes, and crown buds in the crowns of gentian 03/04-114. Samples were collected at the end of May 2009 with similar shoot number across the replicates.

	FW (g)	Proportion	DW (g)	Proportion (%)
<b>Storage roots</b>	49.0 ± 7.3 a <sup>Z, Y</sup>	79.2 ± 1.8 a	11.6 ± 1.5 a	80.2 ± 1.0 a
<b>Rhizomes</b>	9.9 ± 0.5 b	16.5 ± 1.1 b	2.3 ± 0.2 b	16.1 ± 0.5 b
<b>Crown buds</b>	2.5 ± 0.4 c	4.3 ± 0.9 c	0.5 ± 0.1 c	3.7 ± 0.8 c
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

<sup>Z</sup> Mean values (± SE). n = 4

<sup>Y</sup> Within the same column, mean values followed by different letters were significantly different (ANOVA,  $P < 0.05$ ).

### 3.3.3 Development of crown buds

In autumn (May 2009), the total population of crown buds on each plant of 03/04-114 comprised several clusters of crown buds (CCB; Figure 3.3B). These clusters were most commonly localized toward the base of shoots (Figure 3.3A and B). Not all shoots formed CCB, and usually each shoot only developed one cluster. An individual cluster originated from an initial single bud, which developed from one of three sources:

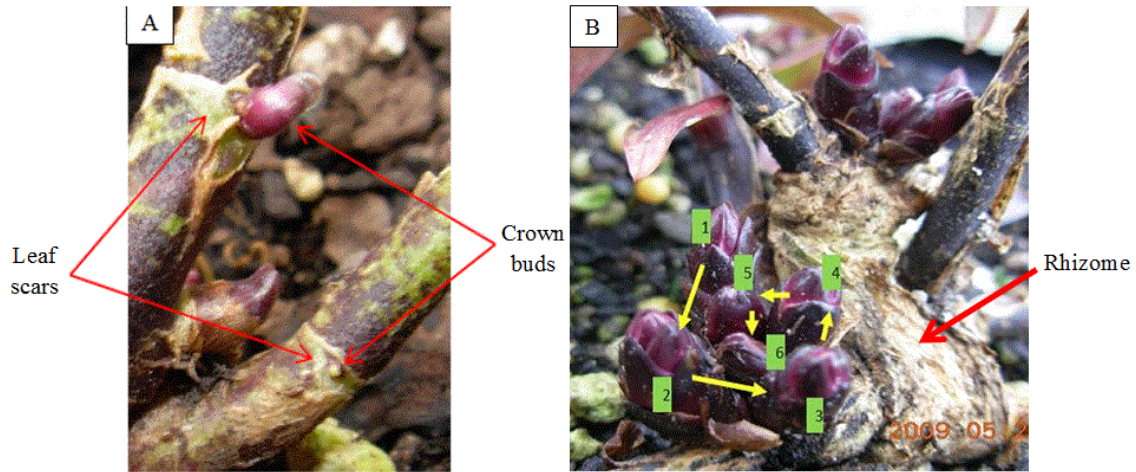
- commonly the leaf axils at the basal nodes of a shoot (Figure 3.3A). With the development of a cluster of crown buds, the stem below the node where the cluster formed, became thick and also formed a thickened rhizome (Figure 3.3B).
- an existing bud within the cluster of crown buds that had formed in the last growth season but had not developed into a shoot (Figure 3.4A and B).
- less frequently, however, some crown buds occasionally developed directly on the thickened rhizomes.

Within an individual cluster, crown buds appeared sequentially from early summer (December) through to the end of autumn (May; Figure 3.5). Each bud appeared by breaking through an enveloping scale leaf. The crown bud that formed subsequently was positioned approximately at a right-angle to the previous crown bud and only developed from one of a pair of leaf axils, rather than arising within a pair of leaf axils. This resulted in a spiral pattern of crown buds within a cluster, arranged in either a clockwise (Figure 3.6A) or anticlockwise spiral pattern (Figure 3.6B).

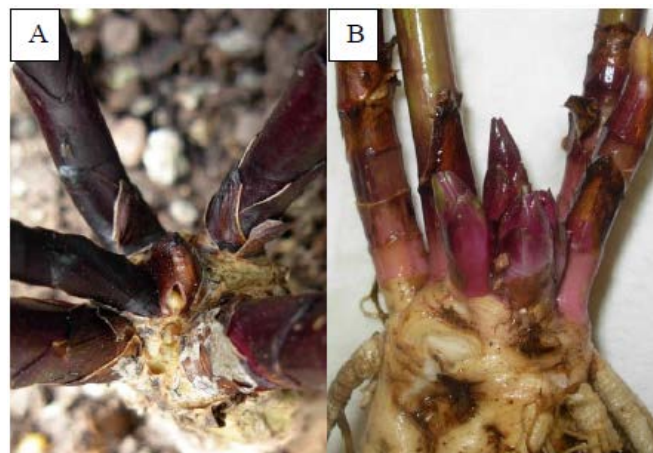
Some of the crown buds differentiated roots at the interface of their base and the rhizome to which they were attached and, if these roots were in contact with the growing media, they elongated. In contrast, if the roots were not in contact with the media, their development would terminate (Figure 3.7A and B). Within each cluster of crown buds, the crown buds that developed or enlarged early in the growth season commonly developed more purple-coloured scale leaves, and became bigger in size (Figure 3.8).

New crown buds were macroscopically visible from December (i.e. the beginning of summer) with the total number of crown buds per plant (TCBN) significantly ( $P \leq 0.001$ ) increasing through to the end of recording in autumn (May 2009; Figure 3.9). Once most plants had flowered (i.e. February), the rate of increase in TCBN increased, and reached

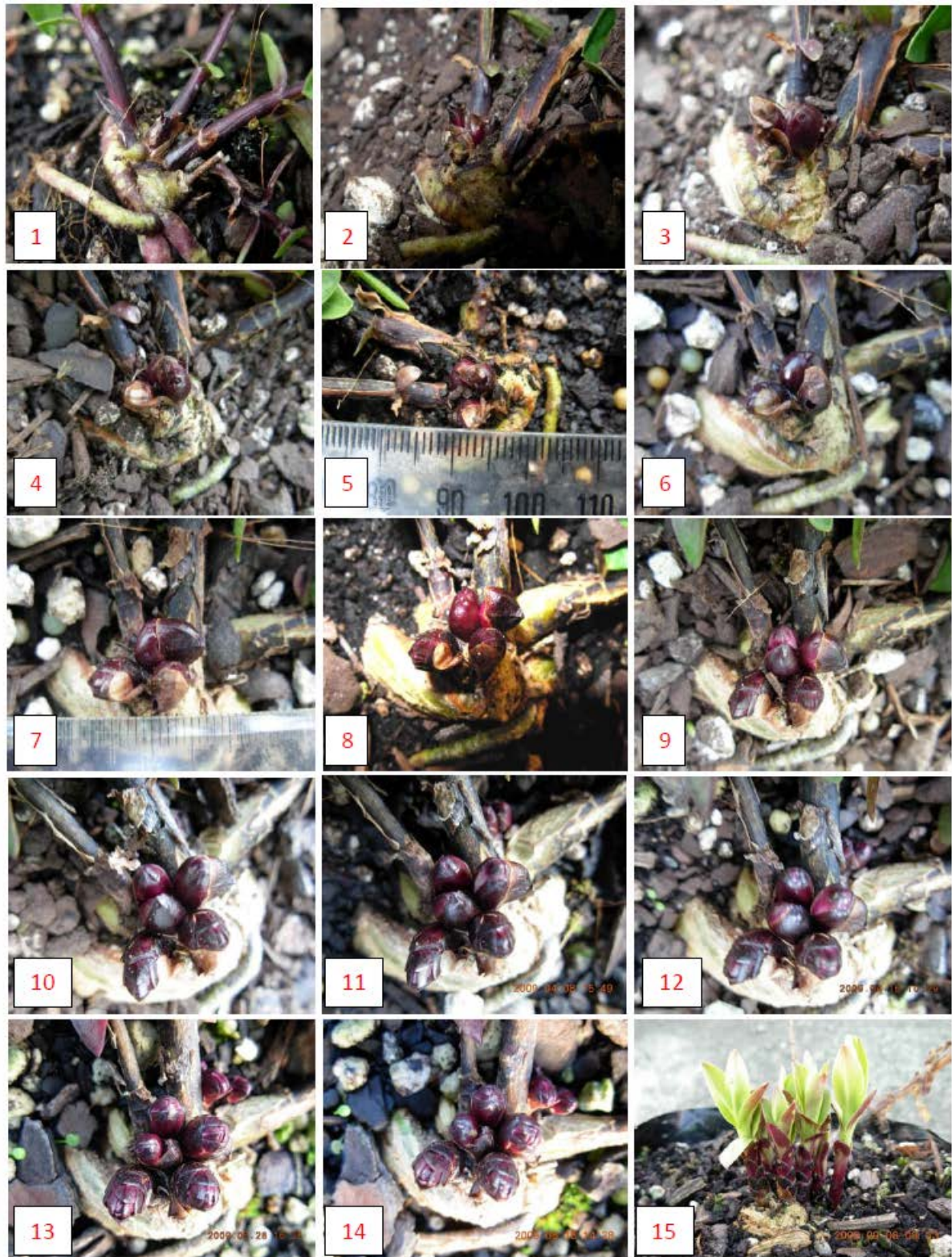
its maximum at the end of March, before decreasing gradually to a similar rate in May as January/February (Figure 3.9).



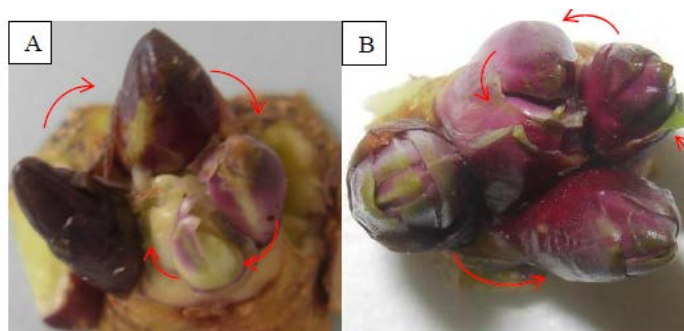
**Figure 3.3** Crown buds initiated at leaf axils at the base of shoots of 03/04-114 (A). The total population of crown buds on an individual plant were composed of several clusters of crown buds (B). Within (B) the yellow arrows and associated numbers show the spiral arrangement and progressive order of appearance of crown buds within one such cluster, and the red arrow points to the thickened rhizome to which this cluster of crown buds was attached.



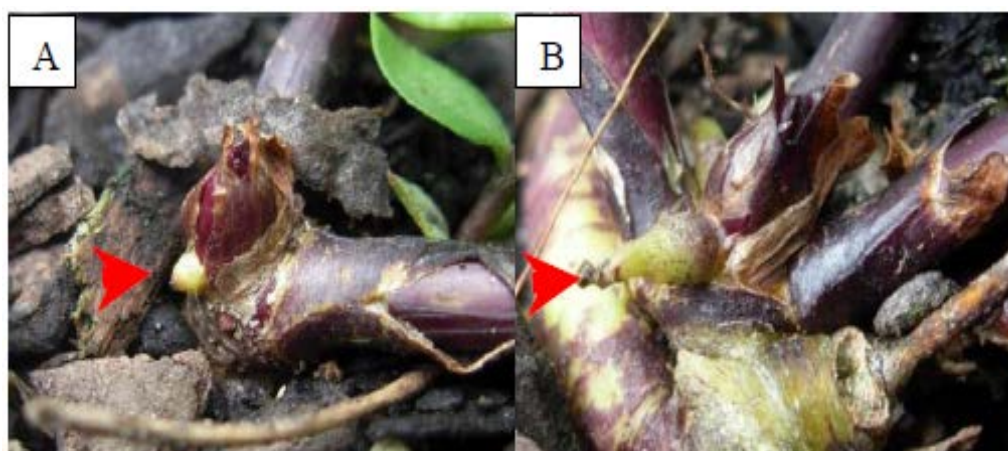
**Figure 3.4** A cluster of crown buds (B) developed from the bud developed in the previous growing season (A), in the same plant of 03/04-114. Photo A was taken in December 2008 and photo B in March 2009.



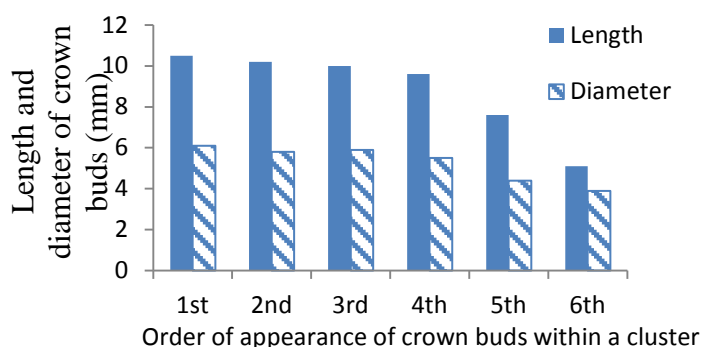
**Figure 3.5** A progressive sequence of the development of crown buds (photos 1 to 15) within the same cluster of crown buds in a plant of 03/04-114. Photos were taken from summer (photo 1-6) through autumn (photo 7-13), winter (photo 14) to the following spring (photo 15) (i.e. December 2008 through to September 2009).



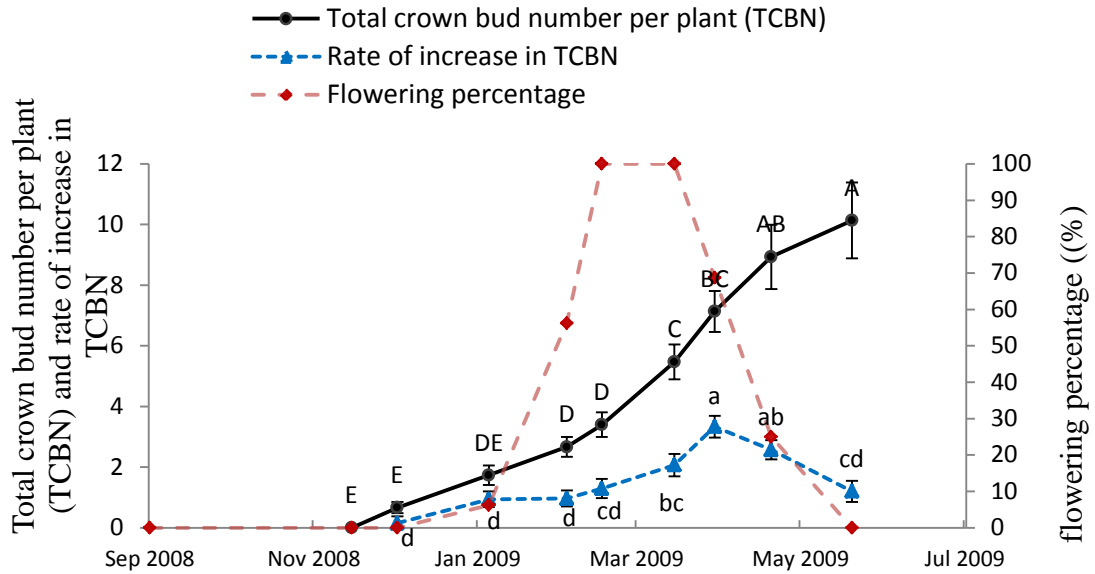
**Figure 3.6** Spiral patterns of crown buds within a cluster of 03/04-114 could be; (A) clockwise or, (B) anticlockwise. Arrows indicate the progressive order of appearance of crown buds within a cluster. Samples were collected in autumn (20<sup>th</sup> May 2009).



**Figure 3.7** A root initiated at the interface of a crown bud and the rhizome of 03/04-114 (A); and a root with terminated development (B). Red arrow-head indicates the roots. Samples were collected in summer (January 2009).



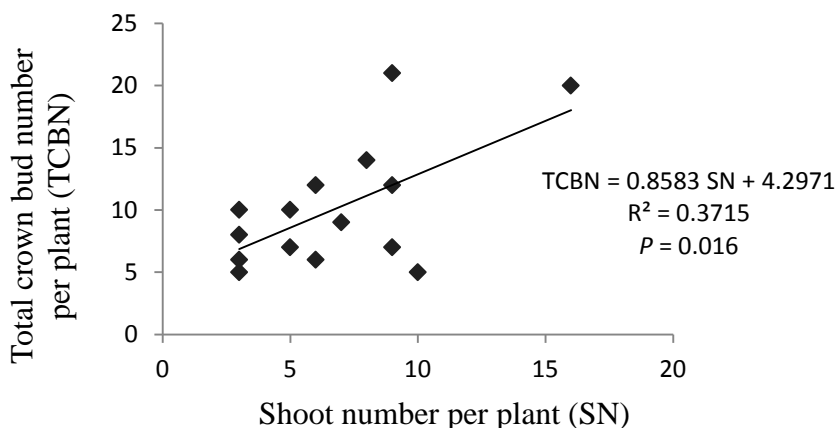
**Figure 3.8** Length and diameter of crown buds within a single cluster measured in autumn (20<sup>th</sup> May 2009). The ordinal numbers from left to right describe the order of appearance of the buds from December to May (old to young) within a plant of 03/04-114.



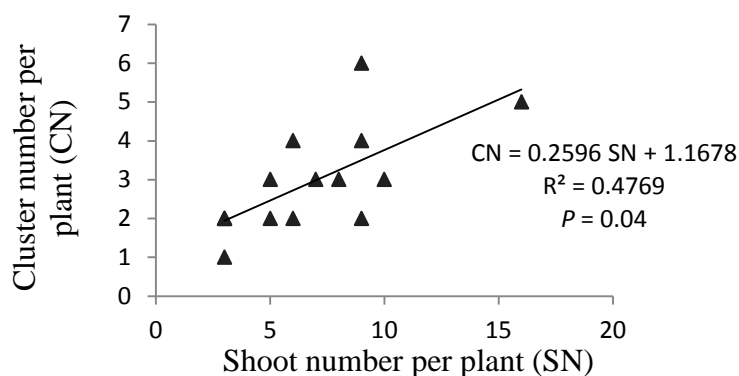
**Figure 3.9** Changes in total crown bud number per plant (TCBN), rate of increase in TCBN (number/month) and flowering percentage (number of flowering plants/total number of plants; expressed as %) of 03/04-114 during the growth season from Sep 2008 to May 2009. For each variable, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ;  $n = 16$ ). Vertical lines =  $\pm$  SE.

### 3.3.4 Relationships between TCBN, CN, CBNC and SN

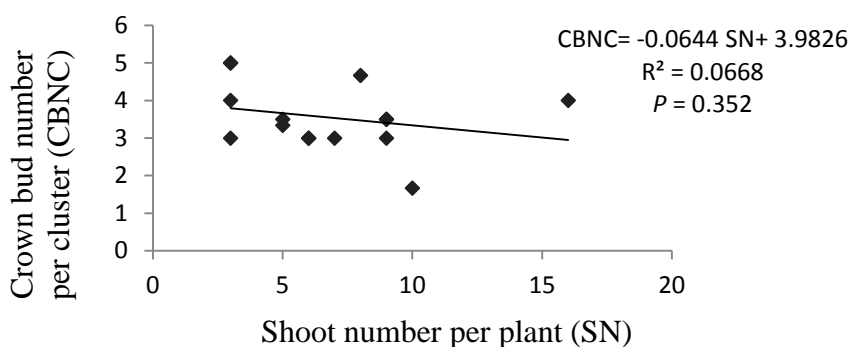
Evidently, the cluster number of crown buds per plant (CN) and number of crown buds per cluster (CBNC) determined the total number of crown buds per plant (TCBN). Linear regression analysis indicated that at the end of the growing season the maximum shoot number per plant (SN) was positively correlated with TCBN that formed in the current season (Figure 3.10;  $P = 0.016$ ) and CN (Figure 3.11;  $P = 0.04$ ), accounting for 37.2% and 47.7%, respectively; however, SN was not correlated with CBNC (Figure 3.12;  $P = 0.352$ ), and CBNC was also not correlated with CN (Figure 3.13;  $P = 0.462$ ).



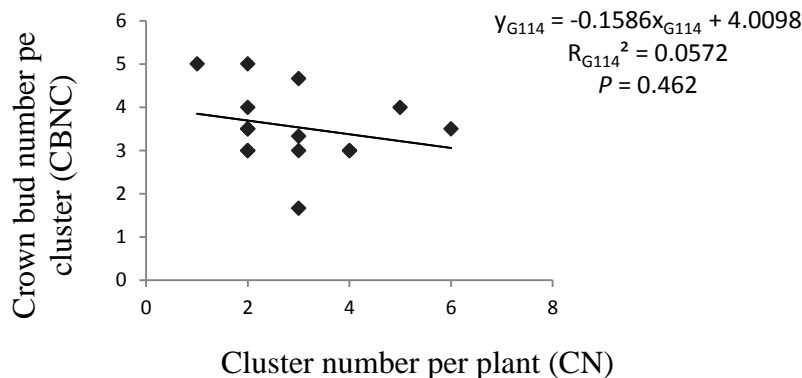
**Figure 3.10 Relationship between shoot number per plant (SN) and total number of crown buds per plant (TCBN) of 03/04-114 in autumn (May 2009; DF 1, 14;  $R^2 = 0.3715$ ;  $P = 0.016$ ).**



**Figure 3.11 Relationship between shoot number per plant and cluster number per plant (CN) of 03/04-114 in autumn (May 2009; DF 1, 14;  $R^2 = 0.4759$ ;  $P = 0.04$ ).**



**Figure 3.12 Relationship between shoot number per plant (SN) and the number of crown buds per cluster (CBNC) of 03/04-114 in autumn (May 2009; DF 1, 14;  $R^2 = 0.0668$ ;  $P = 0.352$ ).**



**Figure 3.13 Relationship between the cluster number per plant (CN) and the number of crown buds per cluster (CBNC) of 03/04-114 in autumn (May 2009; DF 1, 14;  $R^2 = 0.0572$ ;  $P = 0.462$ ).**

### 3.4 Discussion

In the current study, plants of gentian 03/04-114 presented similar features in the form and structure of crowns as previously reported for *G. triflora*, i.e., swollen axillary crown buds, thick and fleshy rhizomes, and thick and linear-cylindrical storage roots (Sato et al., 1988; Ho & Liu, 2001; Samarakoon et al., 2012b). As explored further in the following sections, the current study, however, also provided more detailed information with regard to the following aspects of established gentian plants:

- source of both crown buds and clusters of crown buds;
- pattern of the development of crown buds within a cluster;
- correlation between TCBN, CN, CBNC, and SN;
- potential influence of carbohydrate supply to the development of crown buds;
- contractile roots and their function;
- biomass distribution in crown buds, rhizomes and storage root.

The formation of the clusters of crown buds is an obvious feature in the development of crowns in gentians (Sato et al., 1988; Samarakoon et al., 2012b). According to their location on a plant, buds may be classified as terminal, axillary or adventitious (Walters & Keil, 1996). The results from the current study indicated that for established plants of 03/04-114 a cluster of crown buds commonly appeared as an axillary bud from a leaf axil (with an evident node and leaf scar) at the base of a shoot (Figures 3.3 to 3.5). This was consistent with a non-peer reviewed report using *G. triflora* as the plant material (Sato et al., 1988) and previous taxonomic description for the section *Gentiana* (Ho & Liu, 2001).

While crown bud clusters can form from axillary buds, anatomical investigations of seedlings illustrate that in this comparatively young material, initiation of the first bud of a cluster of crown buds on the transition zone of gentians is adventitious (Samarakoon et al., 2013). These seemingly disparate results are likely to illustrate the plasticity of growth response of gentians, due in part to the age of plant material, with juvenile seedlings and newly deflasked plants from tissue culture being used in Samarakoon's experiments; in contrast, relatively older, i.e. more established plants, were used both in the current and Sato's study. As further evidence of this plasticity of response, even within the same genotype, in the current experiment it was found that some of the new crown bud clusters developed on the thickened rhizomes itself, on which no apparent nodes were visible, i.e. potentially suggesting their origin as being adventitious (Samarakoon et al., 2013). Determining if these particular crown buds are of adventitious or axillary origin needs further anatomical investigation. Because only macroscopic observations were used in the current experiment, further investigations, such as those involving dissection and microscopic examination, may be needed to clearly confirm the initial source of crown buds. In summary therefore, accepting the concept of plasticity of response in gentian plants, the initial bud from which a crown bud cluster is derived may be adventitious or axillary.

Within each cluster of crown buds, the spiral pattern of arrangement of crown buds found in the current study was consistent with previous studies (Sato et al., 1988; Samarakoon, 2012). A right-angle between sequential crown buds within a cluster (Figures 3.3 and 3.6), was compatible with the decussate, opposite-leaf arrangement of gentians, i.e. progressing along the stem, the point of attachment of each successive pair of leaves is rotated 90° (Ho & Liu, 2001). It was interesting that only the axillary bud from one of the pair of leaf axils developed into crown buds (Samarakoon, 2012), either clockwise or anticlockwise, rather than the pair of leaf axils (Figure 3.6). This feature resulted in a spiral pattern of crown buds within a cluster. The same developmental pattern of axillary buds was also reported in *Michelia fuscata* (Tucker, 1963) and *Gossypium* (Mauney & Ball, 1959). The regular spiral arrangement in the position of crown buds may be related to the spatial arrangement of shoots in the canopy of gentian plants, and also strongly supports the hypothesis that crown buds were derived from axillary buds within the clusters.

It has been recognised that the size of crown buds might influence the quality of flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon et al., 2012b). In the current study the sequential development of crown buds within a cluster resulted in different sizes (i.e. length and diameter) among crown buds within a cluster, i.e. macroscopically visible crown buds that appeared early, became larger in both length and diameter than buds that formed later (Figure 3.8). This may be due to the different duration of growth among the crown buds within each cluster. If size correlates to maturity of crown buds, then the size of crown buds may affect the node number, length, or flowering time, of flowering shoots. In contrast it was reported using gentian ‘Showtime Spotlight’ that the diameter of crown buds and flowering time were not correlated with the time of bud appearance for the population of crown buds within a plant (Samarakoon et al., 2012b), although within a cluster the pattern of the size of crown buds has not been reported. The same study also reported that crown buds of greater diameter produced longer flowering shoots at harvest maturity (Samarakoon, 2012). Considering the result in the current study, i.e. within a cluster the earlier the visible crown buds appeared, the bigger the size they achieve, further experiments are therefore, required to investigate the correlation between the size (maturity) of crown buds within a cluster and the development of floral shoots, i.e. length, number of nodes and flowering time. This research may help to determine if the early arising larger buds always produce flowering shoots before the smaller buds; also whether smaller buds always produce weaker flowering shoots.

Multiple regression analysis indicated that both the number of clusters per plant (CN) and the number of crown buds per cluster (CBNC) were correlated with the total number of crown buds per plant (TCBN; Eq 1). The fact that the number of shoots (SN) during the current season was positively correlated with CN, but not correlated with CBNC at the end of the growing season, implies that SN may, via influencing CBNC, therefore influence TCBN. There being no correlation between CBNC and CN implies that CN does not influence the CBNC, i.e. the increase of CN does not reduce the CBNC. In addition, the positive correlation between SN during the growing season and CN at the end of the season also was compatible with the finding that the cluster of crown buds originated from the leaf axils at the base of shoots. Because in established plants all floral shoots come from the crown buds developed in the previous growing season, the number of crown buds was suggested as being a key factor limiting the number of floral shoots of gentians (Yamanaka, 1978; Ohkawa, 1989; Samarakoon, 2012). In the commercial practice of

producing flowering shoots, newly planted young plants of gentian usually take around three years to establish enough crown buds to achieve their full commercial production (Ohkawa, 1989). This long period required for the establishment of crown buds, potentially limits the yield and increases the cost for production of flowering shoots and, therefore, methods promoting the increase of crown buds are required in commercial production. Following this logic therefore, the effect of potential factors (e.g. carbohydrates, plant growth regulators, temperature and photoperiod) on the development of crown buds, and/or clusters of crown buds, were deemed worthy of further investigation (refer to Chapters 4, and 5).

The growth and development of geophytes are largely affected by the distance of the geophytic organ (i.e. bulb, corm etc.) from the soil surface (Galil, 1958). Contractile roots have been found widely in geophytes, producing a downward pulling force which pulls the geophytic organ to an appropriate level in the ground (Putz et al., 1995). In the current investigation, thick contractile roots characterised by their wrinkled surface developed in crowns, arising from rhizomes or the base of crown buds (Figure 3.2). As discussed above, if crown buds are formed at the base of shoots, theoretically the position of crown buds and rhizomes will be lifted up higher and higher with each perennial generation. As the new roots initiating from the base of crown buds or rhizomes would therefore not physically touch the soil, these new roots would terminate (Figure 3.7). The existence of contractile roots in gentian may, as occurs in other geophytes, play an important role in pulling down the rhizomes and crown buds on crowns to the appropriate ground level for maintaining normal growth.

It has been recognized that bud development required carbon supply (Davidson & Remphrey, 1994; Vesik & Westoby, 2004). In the current results there was a peak in the rate of increase in crown bud number, soon after the peak of flowering (Figure 3.9B). It is reasonable to suggest therefore, that the increased rate of appearance of crown buds after peak flowering may be related to the change of partitioning of carbohydrates (photo-assimilate) at this time (refer Chapter 4), i.e. the main sink of carbohydrates transfers from the floral shoots to underground organs (i.e. crown buds, rhizome and/or storage roots). It is worth noting that 03/04-114 is sterile (Ed Morgan, personal communication), hence carbohydrates are not required for seed development. The continuous increase in the size of crown buds through winter (data not shown), when natural senescence of above ground

shoots had already completed (end of May 2009), additionally suggests that the carbohydrate reserves in the rhizome and/or storage roots, may be mobilized to support crown bud development. Further investigation in terms of the possible relationship between carbohydrate metabolism and the development of crown buds would seem to be worthy of subsequent investigation, as was conducted in the following chapters of this thesis.

Various geophytes have different modified underground organs providing storage function (Le Nard & De Hertogh, 1993a), such as corms (modified stems) in *Gladiolus* (Shillo & Halevy, 1981); bulbs (modified leaves) in *Tulipa* (Kamenetsky et al., 2003), or fleshy roots in *Paeonia* (Walton et al., 2007), etc. Given the morphological features of the crown of gentian 03/04-114 (Figure 3.1), all parts of the crown, i.e. the swollen crown buds, thickened rhizomes and fleshy storage roots, could function as a storage organ. However, quantification of biomass indicated that storage roots were the main storage organ accounting for 80% of the biomass of the crown (Table 3.1). In geophytes, the accumulation of NSCs in crowns prior to winter is considered a determinant both for winter survival via their action as cold protectants (Patton et al., 2007), and as energy and carbon reserves for initiation of early re-growth in spring (Pressman et al., 1993; Woolley et al., 1999; Uleberg et al., 2009). In gentians, however, little published information is available as to the NSCs in crowns and their physiological function. The knowledge in terms of the NSCs in crowns, including the type, distribution and dynamics during the growth and development of gentian plants will be addressed in Chapters 4 and 6 of this thesis.

### 3.5 Conclusion

- 1) The crown of the new hybrid gentian 03/04-114 was, similar to that described in previous reports for *G. triflora*, being comprised of swollen crown buds, thickened rhizomes, and fleshy storage roots and, based on biomass, storage roots were the main storage organ in crowns.
- 2) The formation of clusters of crown buds with individual buds arranged in a spiral pattern, and clusters initiating from the leaf axils, were evident features of the development of crown buds.
- 3) The total number of crown buds was determined by the number of clusters and the number of crown buds per cluster and,

### Chapter 3 – Form and Structure

- 4) the number of shoots might, via influencing the number of clusters, influence the total number of crown buds.
- 5) Given that the rate of increase in TCBN increased just after flowering, and that crown buds continued to increase in size through winter, the supply of carbohydrates may be a potential factor influencing the development of crown buds.

## **Chapter 4 Effect of defoliation on carbohydrate reserves, crown-bud development, and spring re-growth in gentian**

**03/04-114**

### **4.1 Introduction**

The investigation presented in Chapter 3 indicated the significance of crown development as a storage and perennating organ in the perennial life-cycle of gentians. Development of vegetative crown buds and the swollen storage organs, comprising vertical rhizomes and storage roots, enable survival under adverse winter conditions and initiation of re-growth in spring. In the commercial practice of producing flowering shoots of gentians, once planted, a gentian crown is cultivated for between eight and ten years. During the succeeding seasons following planting, poor development of crown buds, low winter survival and spring re-growth, are suggested as being key factors limiting both the commercial yield and quality of cut flowers in gentians (Yamanaka, 1978; Ohkawa, 1989; Samarakoon et al., 2012a). In order to optimize the production of cut flowers therefore, knowledge of factors influencing crown-bud development, winter survival, and re-growth in gentians is essential.

Anecdotal comments amongst growers of gentians for cut flower production, suggest that removing too large a quantity of leaves, as occurs during over-harvesting of flowering stems, has a negative effect on the yield of flowering stems in the following growth season. As a consequence of this logic, growers are recommended to retain as many leaves as possible on plants, so as to maintain sustainable yield and quality in the following growth season. In practice, growers may either only harvest two thirds of the total number of flowering shoots produced by each plant in any one year or, when harvesting each flowering shoot, as many of the lower leaves are left on the plant as long as the length of the flowering shoot meets commercial requirements (i.e. 90 cm to 100 cm in length) (Ohteki, 1982; Anonymous, 2009). Within the context of the current discussion, the logic is that the loss of leaves, the main source of synthesis of new carbohydrates, may adversely affect the development of crown buds, winter survival, and re-growth. This in turn reduces the yield and quality of flowering shoots produced the following season. Despite the current commercial recommendations, no quantitative analysis is available about how the loss of leaves influences the accumulation of non-structural carbohydrates

(NSCs), crown-bud development, winter survival, and re-growth in the following season. While the negative effects of defoliation on the ability to overwinter, and spring re-growth, have been reported in other perennial plant species (Dhont et al., 2002; Uleberg et al., 2009), no such information exists for gentians. As a result of this current lack of information, providing specific recommendations of horticultural practice to minimise any potential negative effects is, therefore, not possible. Within the experiment presented in this chapter, the timing of artificial defoliation was used as a research tool to determine the effect of variation in carbohydrate acquisition. It was assumed that the later the defoliation commenced, the longer duration plants had to acquire carbohydrates.

The accumulation of NSCs in crowns prior to winter is considered a determinant in other perennial plant species, both for winter survival as cold protectants (Patton et al., 2007), and as energy reserves for initiation of early re-growth in spring (Pressman et al., 1993; Woolley et al., 1999; Uleberg et al., 2009). For example, the winter survival of the perennial *Trifolium repens* L. (white clover) was dependent on the concentration and/or amount of NSCs reserves in the roots and stolons at the onset of winter (Harris et al., 1983; Turner & Pollock, 1998; Dhont et al., 2002), and the yield of spears from *Asparagus officinalis* L. (asparagus) in spring, was largely determined by the NSCs stored in the storage roots from the previous growth season (Wilson et al., 2001). In addition, as illustrated in vitro, NSCs have also been shown to be directly involved in plant organogenesis (Brown et al., 1979; Gemas & Bessa, 2006; Luo et al., 2009; de Santana et al., 2011). As such, some carbohydrates (e.g. sucrose, glucose and fructose) have been identified as signalling compounds, offering phytohormone-like function in all stages of the plant's life cycle (Smeekens, 2000; Rolland et al., 2002; Rolland et al., 2006). Extending upon this logic therefore, exploring the potential relationship between NSCs and crown-bud development, winter survival, and re-growth in gentians, was considered worthy of investigation. The objectives of the current study were to:

- quantify how defoliation during the growing season affects; the reserves of NSCs in crowns, the development of crown buds, the winter survival of crowns and, re-growth in the following spring;
- determine what if any relationships exist between reserves of NSCs, winter survival, spring re-growth, and the development of crown buds.

## 4.2 Materials and methods

### 4.2.1 Plant material

The gentian hybrid known as 03/04-114 (refer Section 3.2) was propagated *in vitro* and initially grown at The New Zealand Institute for Plant & Food Research, Palmerston North. The plants had been deflasked and grown outside for between six and nine months in 60-cell trays (45 ml cell volume) containing a bark: pumice medium, and had passed through their first winter when acquired in October 2008. The growing medium used was Dalton's Base growing medium (CAN Fines A Grade 50%; Fibre 30%; Pacific Pumice 7 mm 20%; 0.5 kg/m<sup>3</sup> superphosphate (9.1P–11S–20Ca), 2.0 kg/m<sup>3</sup> each of agricultural lime and dolomite (21Ca–10Mg; Prebbles Seeds, Christchurch, New Zealand), and 1 kg/m<sup>3</sup> Gypsum), with 4.3 kg/m<sup>3</sup> of 8–9-month Osmocote<sup>®</sup> 16N–3.5P–10K (Grace-Sierra International, The Netherlands), 0.4 kg/m<sup>3</sup> calcium ammonium nitrate (27N–6Ca–4Mg), 0.5 kg/m<sup>3</sup> potassium sulphate (42K–18S), and 3 kg/m<sup>3</sup> Osmocote<sup>®</sup> (23N).

At the commencement of the experiment plants comprised a crown presenting anywhere between 3 and 17 emerging shoots per plant (Figure 4.1).



**Figure 4.1** Plants of 03/04-114 at the commencement of the experiment (October 2008).

At the beginning of the experiment in spring (October 2008), plants were transplanted into polythene bags (1.7 L) of the same growing medium as described above. Plants were grown on two raised planting beds outside at the Plant Growth Unit, Massey University (40.38° S 175.60° W), utilising a capillary irrigation system with one overhead watering per week, so as to minimise periods of water or nutrient salinity stress.

### 4.2.2 Experimental design and defoliation treatments

To observe the effects of defoliation, the timing of complete and on-going defoliation was used as a treatment during the growth season. The experiment was conducted as a randomized block design, with two blocks (i.e. two planting beds). Five defoliation treatments were applied commencing from either:

- 1 6<sup>th</sup> October 2008,
- 2 8<sup>th</sup> November 2008,
- 3 6<sup>th</sup> January 2009,
- 4 10<sup>th</sup> March 2009, or
- 5 without defoliation (i.e. control).

Plants of each treatment were randomly allocated, with initially 8 single-plant replicates, in each block, i.e. 40 plants in total within each block. At the end of the first growth season in autumn (20<sup>th</sup> May 2009), four plants of each treatment per block were destructively sampled for measurement of biomass and carbohydrate concentration. The remaining four plants of each treatment per block were used for determining the proportion of plants surviving winter, as well as the proportion of crown buds sprouting in spring.

**Table 4.1 List of variables, abbreviations, and units of measurement.**

Abbreviation	Description of variable	Unit
DW	Dry weight	g
CBNC	Number of crown buds per cluster	
CN	Cluster number of crown buds	
DCB	Diameter of crown buds	mm
FW	Fresh weight	g
LCB	Length of crown buds	mm
NSC	Non-structural carbohydrate	
SSP	Proportion of crown buds sprouting in Spring	%
TCBN	Total number of crown buds per plant	
TNC	Total non-structural carbohydrate concentration	mg g <sup>-1</sup> FW
WSP	Proportion of plants surviving winter	%

When defoliation treatments were applied, all leaves were removed using tweezers, with only the unexpanded tips of shoots retained (Figure 4.2). Once defoliations had

commenced in any one treatment, it was repeated at weekly intervals until no new leaves emerged, i.e. autumn.



**Figure 4.2 Defoliation treatment applied to plants of 03/04-114. When defoliated, all leaves were removed, with only the tips of shoots retained. Left: non-defoliated control; right: defoliated plant.**

#### 4.2.3 Growing degree-day monitoring

Throughout the duration of the experiment accumulated growing-degree-days (GDD) were calculated on a daily basis, utilising meteorological data from AgResearch, Palmerton North (-40.38° N 175.61° W). GDD between the date of emergence,  $t$  (1<sup>st</sup> September, 2008) and the commencement dates of defoliation for each treatment  $a$  through  $e$  (refer Section 4.2.2), were calculated utilising Eq. 4.1. In the absence of any specific information, the assumed base temperature was 0 °C (McMaster & Wilhelm, 1997).

$$\text{GDD} = \sum_t^{(a)-(e)} \left[ \frac{T_{\max} + T_{\min}}{2} - T_{\text{BASE}} \right] \quad \text{Eq. 4.1}$$

Where  $T_{\max}$  and  $T_{\min}$  were the daily maximum and minimum temperatures and, where if  $T_{\max}$  or  $T_{\min} < T_{\text{BASE}}$ , then  $T_{\max}$  or  $T_{\min} = T_{\text{BASE}}$ .

For the purposes of calculation of GDD, for plants in the non-defoliated control, the date of destructive harvest at the end of the first growing season, i.e. 20th May 2009, was used for calculating the GDD for treatment. According to the timing of defoliation, the accumulated GDD for each treatment was:

- a.* 400 GDD (Oct 2008),
- b.* 800 GDD (Nov 2008),

- c. 1766 GDD (Jan 2009),
- d. 2884 GDD (Mar 2009),
- e. 3786 GDD (control).

#### **4.2.4 Assessment of crown buds**

At the time of harvest in autumn of the first growth season (20<sup>th</sup> May, 2009) the following was recorded for each plant: the total number of crown buds (TCBN), and number of clusters (CN) (refer Chapter 3). Crown bud size was also determined at this time, using a vernier calliper. Size of crown buds was defined by both the length (measured from tip to base) and diameter (measured at the base).

#### **4.2.5 Winter survival and spring re-growth**

Plants were examined weekly during spring, from 1st September (2009) through until 10th October (2009), after which date no new dead plants became apparent and no further crown buds emerged as shoots. Within each treatment the total number of plants alive in autumn (20th May, 2009) and spring (10th October 2009) were used to calculate winter survival utilising Eq. 4.2. The number of sprouted and un-sprouted crown buds of each plant in spring (10th October 2009), were used to calculate the proportion of crown buds which sprouted utilising Eq. 4.3.

$$\text{Winter survival proportion (WSP)} = \frac{\text{total number of plants alive in spring}}{\text{total number of plants in autumn}} \times 100\% \quad \text{Eq. 4.2}$$

$$\text{Spring sprouting proportion (SSP)} = \frac{\text{number of sprouted crown buds per plant}}{\text{total number of crown buds per plant}} \times 100\% \quad \text{Eq. 4.2}$$

#### **4.2.6 Biomass**

At the time of harvest in autumn of the first growth season (May 2009), the biomass of the crown, i.e. rhizome and storage roots, was destructively sampled and measured. The above-ground stem tissue and feeding roots were not included. Fresh weights were recorded after plants were washed with tap water and dried with paper towels; dry weights were recorded after drying at 60 °C for one week.

#### **4.2.7 Non-structural carbohydrate analysis**

Fresh plant tissue of crown buds, rhizomes and storage roots were collected at the end of May 2009, separately, and frozen in liquid nitrogen after quickly washing in tap water.

Frozen tissue was ground in liquid nitrogen to a fine powder, and stored at -80 °C until used for carbohydrate analysis.

The method of analysis for individual NSCs was based on that previously published (O'Donoghue et al., 2004) with slight modifications. Soluble NSCs were extracted from 0.2 mg frozen powder (fresh weight) of crown buds, or 0.1 mg frozen powder (fresh weight) of either rhizome or storage-root tissue, in 62.5% methanol at 55 °C for 1 hour with periodic vortexes for 10 second at 15-minute intervals. An internal standard (cellobiose, Sigma, final concentration of 5 mg/ml) was added to the extraction solution (62.5% methanol) to calculate the losses that occurred during the analysis. The extracts were centrifuged at 25,000 g (Eppendorf Refrigerated Microcentrifuge Model 5417 R, Germany) for 20 min at 4 °C to remove particulates, and the supernatants were analyzed by High-performance Liquid Chromatography (HPLC, Dionex Ultimate 3000). Aliquots (20 µl) were separated by HPLC on an Alltech® Prevail™ Carbohydrate ES 5µm column (250 x 4.6 mm; Grace Davidson Discovery Science), in a solvent mix of 25% water and 75% acetonitrile at a flow rate of 1 ml/min. The column temperature was kept at 30 °C. Peaks were detected using an Evaporative light scattering detector (ELSD 1000, Polymer Laboratories, Church Stretton, Shrop, UK) gas flow of 1.0 standard litre per minute, nebulizing temperature 40 °C, evaporating temperature 90 °C), and identified by their retention times against standards (fructose (Fisher Scientific) 7.8 min, glucose (Sigma) 10.4 min, sucrose (Merck KGaA) 14.7 min, cellobiose (Sigma) 16.4 min, gentiobiose (Sigma) 21.6 min, and gentianose (Extrasynthese) 28.4 min). The areas under the peaks of individual carbohydrates were integrated using Chromeleon software, version 4.6 (Dionex) and individual NSCs were quantified against standard curves produced during the analysis.

Concentration of TNCs was presented as the sum of the concentration of fructose, glucose, sucrose, gentiobiose, and gentianose. The cyclitol known as bornesitol was also identified as present (Appendix I) and, in the absence of a pure standard, the concentration of bornesitol was estimated using the standard curve of fructose, i.e., expressed as fructose equivalents. Starch was detected in the residue after soluble sugar extraction using a Starch assay Kit (Amylase/Amyloglucosidase Method, Sigma). All analyses were repeated three times.

#### **4.2.8 Statistical analysis**

Because 0% and 100% of plants survived in the defoliation treatment which commenced in January 2009 and control, respectively, the number (n) was adjusted slightly to  $n \pm 0.01$ , so as to avoid incidents of zero values. The adjusted values were then analysed using an analysis of variance utilising GLM (Minitab 16). This adjustment did not affect the statistical results.

An analysis of covariance (ANCOVA), utilising the General Linear Model (GLM) procedure (Minitab 16, Minitab Inc., State College PA, USA), was used to analyse the effect of defoliation on crown bud number and cluster number, with shoot number (a continuous variable) as the covariate. Use of the covariate was to reduce error variance, because of the significant influence of shoot number on both crown bud number and cluster number identified in Chapter Three.

The relationships between carbohydrate concentrations and crown bud number, proportion of winter survival and sprouting of crown buds in spring, were determined using correlation and stepwise regression analyses (Minitab 16). Because of the destructive sampling for the measurement of carbohydrates, the values of carbohydrate concentration, crown bud number, and their subsequent emergence, could not be obtained from the same plants for regression analysis. To solve this problem, the mean values of carbohydrate concentration and crown bud number in the same block, were used as a pair of data to conduct correlation and regression analysis of the two variables. The same method was also applied for regression analysis of other variables in this chapter. In addition, for the purposes of accuracy and precision, only the major NSCs (i.e. gentianose and sucrose) were included in the Pearson's correlation and multiple regression analysis. This was because the low concentration of other NSCs (i.e. glucose, fructose, and gentiobiose) reduced the confidence and their concentration could be determined with real accuracy utilising the protocols followed. For future experiments, to enable the measurement of these other NSCs, the sample solution could be concentrated before proceeding with HPLC analysis. Given the preliminary nature of the current experiment, this was not undertaken.

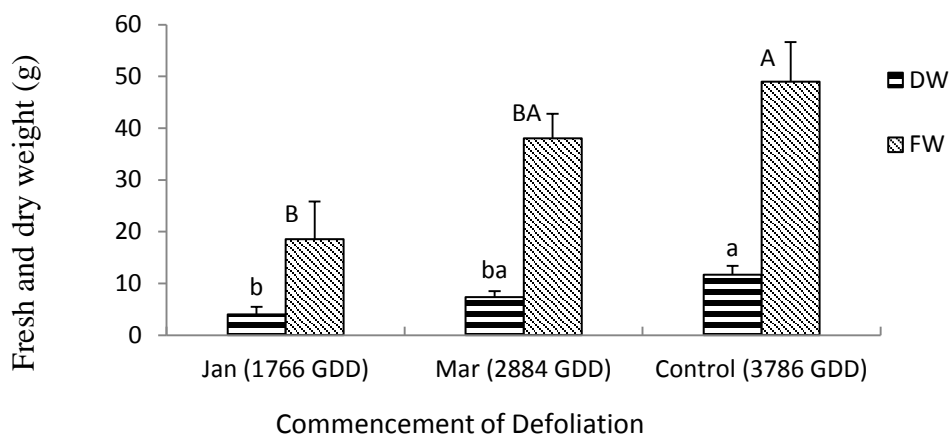
All other variables were analysed using an analysis of variance utilising GLM (Minitab 16). When significant differences between treatments occurred, mean values were separated using the Tukey method ( $P < 0.05$ ).

### 4.3 Results

The plants that were defoliated commencing from either October or November 2008, after 400 and 800 GDD respectively, started to die from January 2009 and, by the end of May 2009, most plants in these two treatments were dead. While by itself this is a treatment effect, more detailed data analysis presented in the following sections did not include these two treatments. Plants in treatments which commenced defoliation from January or March 2009 (1766 GDD and 2884 GDD, respectively) were only defoliated once, because no new leaves emerged after the first defoliation treatments.

#### 4.3.1 Biomass of underground organs

Both fresh (DF = 2, 8;  $P = 0.012$ ) and dry weight (DF=2, 8;  $P = 0.013$ ) of the underground biomass (i.e. storage root, rhizome and crown buds) declined with increased severity/duration of defoliation (Figure 4.3). However, differences between those defoliation commencing March and, both the control and those defoliated from January, were not significant ( $P > 0.05$ ; Figure 4.3).



**Figure 4.3** Fresh (FW) and dry weight (DW) of crowns (storage root, rhizome and crown buds) of 03/04-114 in autumn (May 2009), following defoliation commencing in different months or control (Growing degree-days in brackets). For each variable, mean values ( $\pm$  SE) with different letters are significantly different ( $P < 0.05$ ),  $n = 4$ .

### **4.3.2 Carbohydrates**

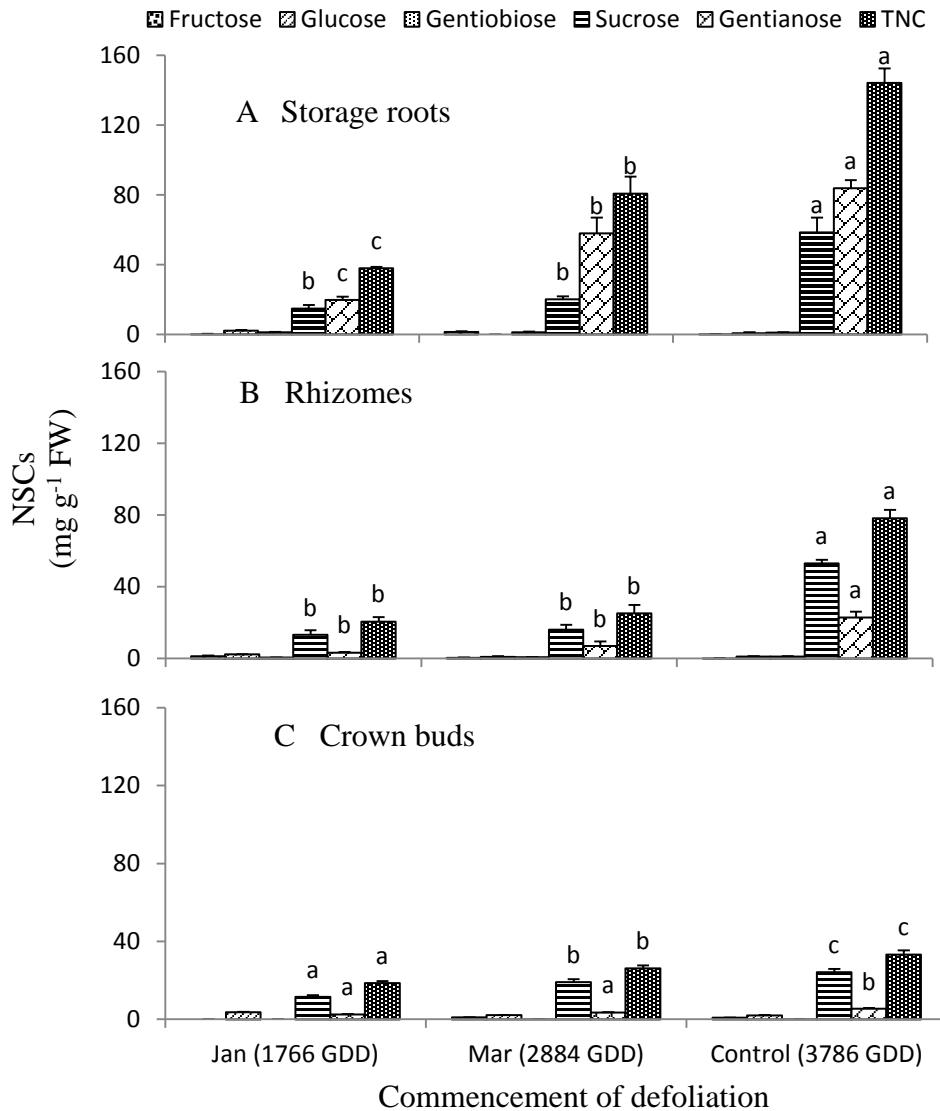
When sampled at the end of the growing season, starch was not detected in any part of the crown (i.e. crown buds, rhizomes, and storage roots) in 03/04-114, with only soluble NSCs detected (i.e. gentianose, sucrose, gentiobiose, fructose, and glucose; Figure 4.4). In addition, the cyclitol known as bornesitol was also detected (Figure 4.6; Appendix I).

The concentration of TNC, and individual NSCs in plants within the control, differed between the different parts of crowns (Figure 4.4). The concentration of TNC was highest in storage roots (144.3 mg/g FW), which was approximately 1.8 times the concentration found in rhizomes (80.8 mg/g FW) and approximately 3.8 times that found in the crown buds (38.0 mg/g FW). In storage roots, gentianose was the most abundant NSC accounting for 58.1% of TNC, followed by sucrose, accounting for 40.5%, with the total amount of fructose, glucose, and gentiobiose contributing less than 1.5% of TNC. In the rhizome, sucrose was the most abundant sugar accounting for 67.7% of the amount of TNC, followed by gentianose (29.3%). with the total amount of fructose, glucose, and gentiobiose contributing less than 3.0% of TNC. In crown buds, sucrose was also the most abundant sugar accounting for 72.8% of TNC, followed by gentianose (16.3%), glucose (6.0%), fructose (2.5%), and gentiobiose (1.0%).

Compared to the control treatment, all defoliation treatments resulted in a significant reduction in the concentration of TNC within all parts of the crown, i.e. storage roots (DF= 2, 5; F = 45.68;  $P = 0.001$ ), rhizomes (DF= 2, 5; F = 53.05;  $P < 0.001$ ), and crown buds (DF= 2, 5; F = 34.55;  $P = 0.001$ ; Figure 4.4).

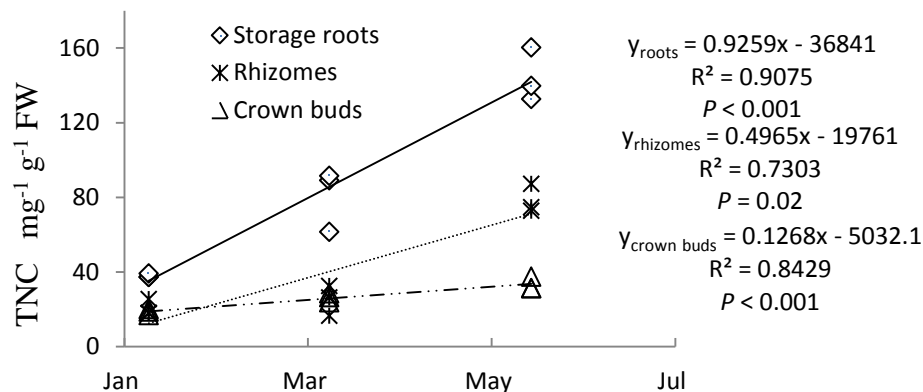
Gentianose and sucrose were the major carbohydrates in each part of the crown tissue. As described below, changes in the concentration of these two carbohydrates were most evident in response to defoliation treatments (Figure 4.4). In storage roots, compared to the control the concentration of gentianose was reduced approximately 31% and 77%, in samples defoliated commencing March and January, respectively ( $P = 0.001$ ); The concentration of sucrose in roots, being the second most abundant sugar, was reduced approximately 66% for plants defoliated commencing March, and 75% for those commencing January ( $P = 0.002$ ). In rhizomes, compared to the control the concentration of sucrose was reduced 70% and 76% for plants defoliated commencing March and January, respectively ( $P < 0.001$ ); the concentration of gentianose was reduced 70% and

86% for those plants defoliated commencing March and January, respectively, compared to the control ( $P = 0.002$ ). In crown buds, compared to the control the concentration of sucrose was reduced 21% and 53% for plants defoliated commencing March and January, respectively ( $P = 0.002$ ); the concentration of gentianose was reduced 34% and 53% for those plants defoliated commencing March and January, respectively ( $P = 0.001$ ).



**Figure 4.4** Concentration of individual soluble non-structural carbohydrates (NSCs) and total non-structural carbohydrates (TNC) per unit fresh weight (FW) in; (A) storage roots, (B) rhizomes and, (C) crown buds of 03/04-114, when sampled in autumn (May 2009), following defoliation commencing in different months or control (without defoliation; Growing degree-days in brackets). For each NSC, mean separation between dates by Tukeys at  $P \leq 0.05$ .

Furthermore, in all parts of the crown, i.e. storage roots, rhizomes and crown buds, the concentration of TNC increased linearly with delayed time of defoliation, i.e. decreasing severity/duration of defoliation (Figure 4.5).



**Figure 4.5 Relationships between concentration of total non-structural carbohydrates (TNC) and the date defoliation commenced in plants of 03/04-114.**

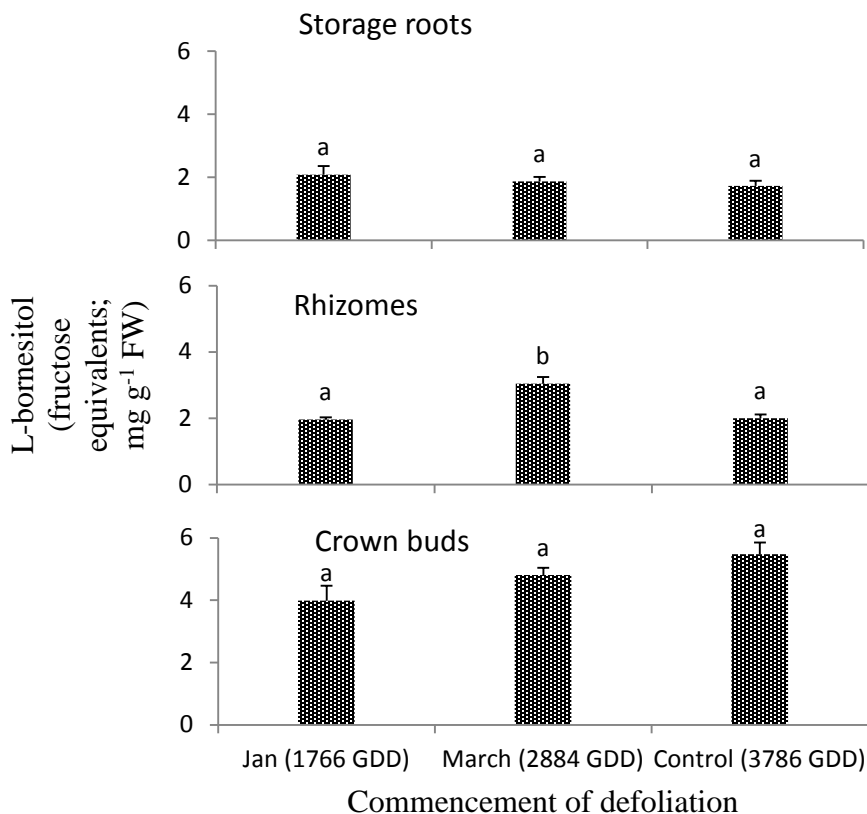
The concentration of L-bornesitol in plants within the control also differed in different parts of the crown (Figure 4.6). The concentration of L-bornesitol was highest in crown buds (5.5 mg/g FW), where it was approximately 3 times the concentration found in rhizomes (2.0 mg/g FW) and storage roots (1.7 mg/g FW) (DF = 2, 6; F = 70.8;  $P < 0.001$ ).

Compared to the control treatment, defoliation did not result in significant changes in the concentration of L-bornesitol within crown buds (DF= 2, 6; F = 3.84;  $P = 0.084$ ) or storage roots (DF= 2, 6; F = 0.8;  $P = 0.493$ ; Figure 4.6). In contrast, compared to the control treatment, the concentration of L-bornesitol in the rhizomes was significantly increased 50% by defoliation commencing in March, but did not change in January (DF= 2, 6; F = 18.93;  $P = 0.003$ ; Figure 4.6).

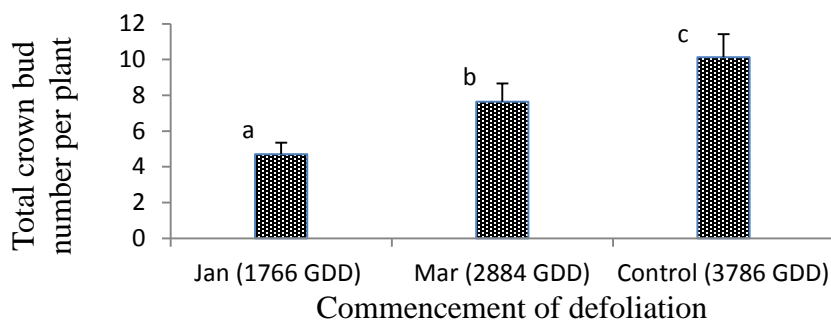
### 4.3.3 Crown buds

When recorded at the end of the growing season, all plants that survived formed crown buds, with plants within the control treatment achieving an average of 10.3 total number of crown buds per plant (TCBN) (Figure 4.7). With the increase of severity/duration of defoliation, i.e. commencement dates from March to January, the TCBN decreased approximately ¼ and ½ of that found with the control, respectively (ANCOVA: DF= 2, 34;  $P < 0.001$ ).

The size of crown buds was also influenced by the severity of defoliation, with the length of buds reduced by 25% and 58% of that achieved within the control ( $DF= 2, 114; P < 0.001$ ), for plants defoliated commencing from March and January, respectively (Figure 4.8). For those plants defoliated commencing from January, the diameter of crown buds (3.5 mm), was reduced approximately 20% of that achieved by those within the control (4.3 mm) ( $DF= 2, 114; P < 0.001$ ). In contrast, however, the diameter of buds when on plants defoliated in March (4.2 mm) was not significantly reduced compared with the control (4.2 mm) ( $DF= 2, 114; P > 0.05$ ; Figure 4.8).

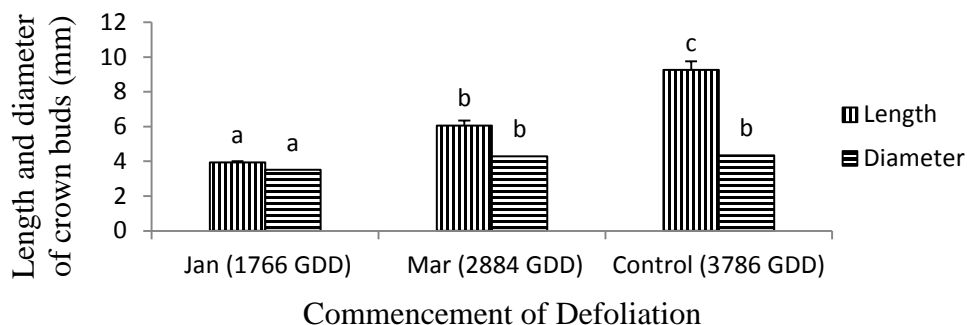


**Figure 4.6** Concentration of L-bornesitol per unit fresh weight (FW) in separate organs of 03/04-114, when sampled in autumn (May 2009), following defoliation commencing in different months or control (without defoliation; Growing degree-days in brackets). For each variable, mean values ( $\pm$  SE) with different letters are significantly different ( $P < 0.05$ ).

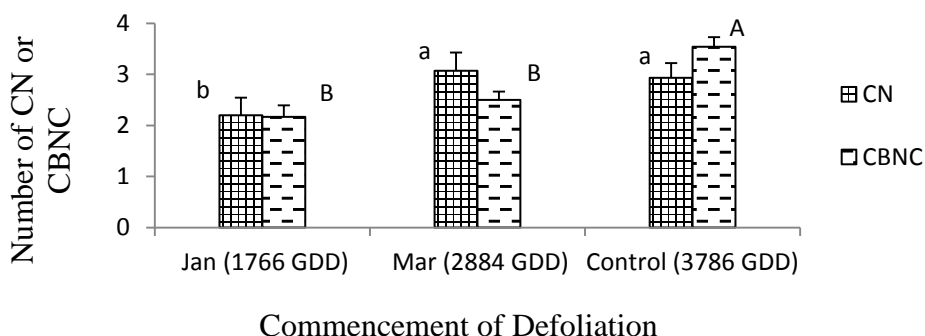


**Figure 4.7 Total number of crown buds per plant of 03/04-114, when sampled in autumn (May 2009), following defoliation commencing in different months or control. Bars (mean ± SE) with different letters are significantly different ( $P < 0.05$ ).**

Compared with the control, defoliation commencing January significantly reduced both the number of crown bud clusters per plant (CB) ( $P = 0.0271$ ) and the number of crown buds per cluster (CBNC) by 25% and 39%, respectively ( $P = 0.0003$ ; Figure 4.9). While those plants defoliated commencing March did not show a significant reduction in the number of crown-bud-clusters per plant ( $P = 0.9883$ ), there was a significant reduction by 30% in the crown bud number per cluster ( $P = 0.0018$ ; Figure 4.9).



**Figure 4.8 Length and diameter of crown buds when sampled in autumn (May 2009), following defoliation commencing in different months or control. For each variable (mean ± SE) with different letters are significantly different ( $P < 0.05$ ).**



**Figure 4.9** Number of crown bud clusters per plant (CN) and crown buds per cluster (CBNC) when sampled in autumn (May 2009), following defoliation commencing in different months or control. For each variable, mean ( $\pm$  SE) values with different letters are significantly different ( $P < 0.05$ ).

#### 4.3.4 Winter survival and sprouting in spring

The proportion of plants surviving through winter ( $P < 0.05$ ), and also the proportion of crown buds sprouting in the following spring ( $P < 0.05$ ) decreased with the increase of severity/duration of defoliation (Table 4.2). No plants defoliated commencing January (1766 GDD) did not survived through winter. In contrast, almost all plants defoliated commencing March (2884) and 100% of plants in the control treatment (3786), survived through winter. Compared with plants in the control treatment, the proportion of crown buds sprouting the following spring was reduced by approximately 20% in plants with defoliation commencing in March.

**Table 4.2** Proportion of plants of 03/04-114 surviving winter (WSP) and crown buds sprouting per plant (SSP) the next spring (2009), following differing periods of defoliation in the preceding growing season (2008-2009). Data recorded 10 October 2009.

Treatments	WSP (%)	SSP (%)
Non-defoliated (control)	100 ( $\pm$ 0) <sup>y</sup>	76.1 ( $\pm$ 2.62) <sup>b</sup> <sup>z</sup>
Defoliation from March	87.5 ( $\pm$ 12.5) <sup>b</sup>	57.9 ( $\pm$ 5.63) <sup>a</sup>
Defoliation from January	0 ( $\pm$ 0) <sup>a</sup>	-
	$P = 0.004$	$P = 0.013$

<sup>z</sup> Mean values ( $\pm$  SE). n = 8

<sup>y</sup> Within the same column, mean values followed by different letters were significantly different (WSP: ANOVA,  $P < 0.05$ ; SSP: ANOVA,  $P < 0.05$ ).

#### 4.3.5 Relationships between key variables

The dry weight biomass of the collective crown (i.e. storage roots, rhizome and crown buds), was positively correlated with TCBN, LCB, WSR, SSR, and CBNC, with coefficients of correlation ( $r$ ) ranging between 0.839 and 0.963 (Table 4.3). In contrast,

with an  $r$  value of 0.332 DCB was not correlated with this same collective DW and, despite the relatively high  $r$  value of 0.743 between CN and this same collective DW, the probability value of 0.090 did not pass the  $P \leq 0.05$  threshold for significance.

**Table 4.3 Pearson’s correlation coefficients ( $r$ ) between the dry weight (DW)<sup>Z</sup> of the collective crown (storage root, rhizome and crown buds) of 03/04-114 and variables<sup>Y</sup> describing development of crown buds, plant survival or re-growth, following differing periods of defoliation in the preceding growing season.**

	Biomass DW (g crown <sup>-1</sup> )
	$r$
TCBN	0.963 **
WSP	0.871 *
SSP	0.882 *
LCB	0.839 *
DCB	0.332 ns
CN	0.743 ns
CBNC	0.895 *

<sup>Z</sup> DW, FW, TCBN, CN, CBNC, LCB and DCB were measured in autumn (20 May 2009) at the end of the growth season. WSP and SSP were measured in spring (10 October 2009), at the start of the following growth season.

<sup>Y</sup> abbreviations for variables as described in Table 4.1

'ns', '\*', and '\*\*' indicate no significance at  $P \leq 0.05$ , significance at  $P \leq 0.05$  and  $P \leq 0.01$ , respectively.

The concentration of TNC in storage roots and crown buds was positively correlated with TCBN, LCB, WSR, SSR and CBNC, with  $r$  values ranging from 0.842 to 0.946; however it was not significant for rhizomes except for CBNC ( $r = 0.914$ ,  $P < 0.05$ ) (Table 4.4). The concentration of gentianose was positively correlated with TCBN, LCB, WSR, SSR and CBNC in storage roots and crown buds. In contrast, while the concentration of sucrose was positively correlated with TCBN, LCB, WSR, and SSR in crown buds, there was no significant correlation evident in storage roots and rhizomes except with CBNC (root:  $r = 0.931$ ,  $P < 0.01$ ; rhizome:  $r = 0.913$ ,  $P < 0.05$ ). Both CN and DCB were not correlated to the concentration of any individual carbohydrates in any of the organs examined ( $r$  values ranging from 0.136 to 0.688,  $P < 0.05$ ).

Furthermore, stepwise regression using TNC, gentianose, and sucrose in all tissues showed that the best predictors were:  $TNC_{\text{crown buds}}$  for TCBN;  $Sucrose_{\text{crown buds}}$  for WSP;  $Gentianose_{\text{storage roots}}$  for SSP;  $Gentianose_{\text{storage roots}}$  for LCB;  $Sucrose_{\text{storage roots}}$  for CBNC; and no predictor at  $\alpha \leq 0.05$  confidence interval for DCB and CN (Table 4.5).

**Table 4.4 Pearson's correlation coefficients (r) between the concentration of individual (sucrose or gentianose)<sup>Z</sup> or total non-structural carbohydrates (TNC) within each underground organ from plants of 03/04-114 in autumn, and variables<sup>Y</sup> describing development of crown buds, plant survival or re-growth, following differing periods of defoliation in the preceding growing season.**

	Concentrations of NSCs (mg g <sup>-1</sup> FW)		
	TNC r	Gentianose r	Sucrose r
	<u>storage roots</u>		
TCBN	0.844 *	0.85 *	0.714 ns
WSP	0.848 *	0.898 *	0.676 ns
SSP	0.931 **	0.971 **	0.761 ns
LCB	0.889 *	0.945 **	0.701 ns
DCB	0.467 ns	0.688 ns	0.136 ns
CN	0.562 ns	0.711 ns	0.297 ns
CBNC	0.913 *	0.795 ns	0.931 **
	<u>rhizome</u>		
TCBN	0.761 ns	0.833 *	0.722 ns
WSP	0.647 ns	0.701 ns	0.644 ns
SSP	0.762 ns	0.822 *	0.755 ns
LCB	0.808 ns	0.922 **	0.761 ns
DCB	0.269 ns	0.463 ns	0.219 ns
CN	0.371 ns	0.534 ns	0.317 ns
CBNC	0.914 *	0.874 *	0.913 *
	<u>crown buds</u>		
TCBN	0.946 **	0.899 *	0.939 **
WSP	0.907 *	0.823 *	0.932 **
SSP	0.922 **	0.899 *	0.932 **
LCB	0.842 *	0.906 *	0.819 *
DCB	0.355 ns	0.425 ns	0.353 ns
CN	0.708 ns	0.616 ns	0.72 ns
CBNC	0.921 **	0.925 **	0.909 *

<sup>Z</sup> NSCs, TCBN, LCB, CN, CBNC and DCB were measured in autumn (20 May 2009) at the end of the growth season. WSP and SSP were measured in following spring (10 September to 10 October 2009).

<sup>Y</sup> abbreviations for variables as described in Table 4.1

'ns', '\*' and '\*\*' indicate no significance at  $P \leq 0.05$ , significance at  $P \leq 0.05$  and  $P \leq 0.01$  respectively.

**Table 4.5 Multiple stepwise regressions between the concentration of TNC<sup>Z</sup>, sucrose or gentianose, within each underground organ from plants of 03/04-114 in autumn, and variables<sup>Y</sup> describing development of crown buds, plant survival or re-growth, following differing periods of defoliation in the preceding growing season.**

Concentrations of NSCs (mg g <sup>-1</sup> FW)			
	Equation	r <sup>2</sup>	P
TCBN	= - 2.63 + 0.393 TNC <sub>crown buds</sub>	0.8946	0.004
WSP	= -73.06 + 7.5 Sucrose <sub>crown buds</sub>	0.8686	0.007
SSP	= - 20.7 + 1.20 Gentianose <sub>storage roots</sub>	0.9237	0.001
LCB	= 1.93 + 0.0811 Gentianose <sub>storage roots</sub>	0.8925	0.004
CBNC	= 1.87 + 0.0275 Sucrose <sub>storage roots</sub>	0.8668	0.007

<sup>Z</sup>NSCs, TCBN, LCB, CN, CBNC and DCB were measured in autumn (20 May 2009) at the end of the growth season. WSP and SSP were measured in following spring (10 September to 10 October 2009).

<sup>Y</sup> abbreviations for variables as described in Table 4.1

#### 4.4 Discussion

In the current study, by utilising different durations of defoliation during the growth season a significant negative effect of defoliation on: carbohydrate reserves, the development of crown buds, winter survival and spring re-growth in the gentian 03/04-114 was evident; and this negative effect increased with the increase of the severity of defoliation (Figure 4.3 to 4.9 and Table 4.2). This is the first time this result has been reported for gentians, albeit similar results have been reported in other perennial plants (Frankow-Lindberg et al., 1997; Dhont et al., 2002; Uleberg et al., 2009). Crown buds are a determinant influencing the yield and quality of floral shoots in gentian cultivation, i.e. plants with more TCBN and larger crown buds develop greater number and longer flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon et al., 2012b). The result in the current experiment, showing positive correlations between the concentration of NSCs and crown bud number, size, overwintering and re-growth (Table 4.4 and 4.5), supports the hypothesis that the availability of NSCs reserves in crowns influences the development of crown buds, such as crown bud number, size, overwintering and re-growth and, in turn, likely influences the yield and quality of floral shoots in the following growth season.

In the current experiment, in addition to the common carbohydrates, i.e. fructose, glucose and sucrose, unique carbohydrates, i.e. gentiobiose and gentianose, and a carbohydrate derivative, i.e. L-bornesitol, were present in the crowns of 03/04-114 in autumn, but no starch was detected. This result of absence of starch was consistent with previous reports in *G. lutea* (Badenhuizen et al., 1964; Keller & Wiemken, 1982). Perennial geophytes rely on the underground crowns for vegetative reproduction in the perennial life-cycle. It has been recognized that the sufficient accumulation of NSCs reserves within underground organs in autumn is a prerequisite for overwintering and initial re-growth in spring (Woolley et al., 1999; Uleberg et al., 2009). NSCs are usually stored as polysaccharides or oligosaccharides, and starch is the most common storage NSC in plants (Lewis, 1984b). In the current experiments, however, in the absence of starch in the crowns of 03/04-114, gentianose was the predominant NSC in storage roots in autumn (accounting for 58.1% of TNC, Figure 4.4A). In addition, the highest concentration of TNC presented in storage roots (approximately 1.8 and 3.8-fold that found in rhizomes and crown buds, respectively (Figure 4.4A to C). Storage roots also accounted for approximately 80% of the biomass of crowns. In contrast, rhizomes and crown buds only accounted for approximately 16% and 4%, respectively (refer Chapter 3). These results indicated that storage roots were the main storage organs in crowns, and gentianose was the predominant NSC stored in crowns. This result implies that gentianose may play a similar role in gentians as starch does in other plant species, and maintaining more leaves may increase the amount of gentianose in crowns of gentians. Given that in the current experiment NSCs were only measured at one point in time during the growth cycle, to further confirm this hypothesis, more detailed information in terms of the distribution of NSCs in various organs, seasonal changes, and the conversion between individual NSCs were investigated in the following chapters.

As evident within the current experiment, the significant reduction in concentration of NSCs in crowns resulting from defoliation during the growing season, was largely due to a decrease in gentianose and sucrose (Figure 4.4). This implies that gentianose and sucrose may play key roles influencing the development of crown buds, winter survival, and re-growth in spring. The details of the possible roles of these NSCs were discussed further, below.

Carbohydrates are the essential carbon and energy source for bud development and maintenance, e.g. the more carbohydrates available, the more or larger buds are able to be

formed (Davidson & Remphrey, 1994; Vesk & Westoby, 2004). Carbohydrates are also directly and/or indirectly involved in plant organogenesis as osmoticum or signalling substances (Brown et al., 1979; Paiva Neto & Otoni, 2003). While exogenously supplied sucrose *in vitro* has been reported to increase the number of crown buds both *in vitro* and in the field after de-flasking in *G. triflora* (NB in a non-peer reviewed report (Sato, 1988a)), a direct relationship between endogenous carbohydrate content and the development of crown buds in gentians has not previously been reported. In the present study the number and size of crown buds were correlated with the concentration of TNC, gentianose, and sucrose (Table 4.4); and further regression analysis indicated that TNC in crown buds and gentianose in storage roots were the largest contributors to the number and size of crown buds, respectively (Table 4.5). These relationships imply specific carbohydrates may play different roles in the development of crown buds. Although the correlation and regression analysis as such does not prove a causal relationship, if solely attributable to carbohydrate content, the reduction of crown bud number and size might directly and/or indirectly be due to the lack of carbohydrate availability in defoliated plants. In the practice of harvesting of flowering shoots in gentians, retaining sufficient leaves for photosynthesis is therefore, essential to optimise carbohydrate accumulation, the number and size of crown buds, and, in turn, to increase the yield and quality of floral shoots.

The formation of crown buds extended over a long period from summer through to winter while, in contrast, the NSCs concentration were only measured at one point in time during the growth cycle in the current experiment. Therefore the comparison between the seasonal changes of individual NSCs in various organs and the development of crown buds throughout the annual growth cycle, would provide a better understanding of the relationships between the development of crown buds and specific individual NSCs. This is addressed in later experiments in Chapter 6. Another limitation of the current experiment, however, was that the removal of foliage was likely to do more than simply reduce carbohydrate acquisition, as defoliation may also affect plant hormonal levels and activity (Musselwhite et al., 2004). Having established the possibility that carbohydrate availability may have some involvement in determining variables such as TCBN and LCB, so as to overcome limitations imposed by defoliation and to further explore this hypothesis, it is considered necessary to establish an alternative experimental system for studying the development of crown buds. As explored further in Chapter 5, an *in vitro* system

independent of seasons, in which the level of individual factors can be controlled, would enable the influence of factors such as carbohydrate supply on the development of crown buds to be studied in gentians.

Winter survival of perennial plants is most commonly reliant upon the storage of NSCs for respiration and to enhance their ability to tolerate cold temperatures (Levitt, 1980; Svenning et al., 1997). It has been recognised that such cold tolerance is associated with the concentration of NSCs stored before winter (Koster & Lynch, 1992; Uleberg et al., 2009). In a previous study with *G. lutea*, Keller et al (1982) suggested the high concentration of gentianose and sucrose in storage roots may serve as cryo-protectants. In the current experiments the concentration of NSCs and biomass of underground organs were reduced by intensive defoliation commencing in January (an accumulation of 1766 GDD), and all the plants in this treatment died during winter. Plants subjected to less intensive defoliation (e.g. commencing in March; an accumulation of 2884 GDD) had a higher level of NSCs stored in storage roots, rhizome and crown buds, and had a higher proportion of plants surviving (87.5%). As no carbohydrate measurements were available when the various levels of defoliation commenced (i.e. only one measurement at the end of autumn in the current experiment), GDD was used for estimating the accumulation of NSCs (Dhont et al., 2002), although GDD may not be able to precisely reflect the reality of carbohydrate accumulation. This result indicated that at least 2884 GDD of growth was required in order for plants of 03/04-114 to survive through winter. Similar to previous reports in other plant species (Frankow-Lindberg et al., 1997; Dhont et al., 2002; Uleberg et al., 2009), this is interpreted as indicating that the more intensive defoliation resulted in the plants being unable to store sufficient carbohydrate reserves to defend against cold temperatures and/or maintain respiration. The positive correlation between winter survival and NSC concentration strongly supported this hypothesis (Table 4.4).

Although the analysis of stepwise regression indicated that sucrose concentration in crown buds contributed the most to winter survival (Table 4.5), the data measured at one point in autumn could not be used to clarify the defence mechanism of carbohydrates against a cold winter. It has been reported that starch degradation during cold conditions increased the concentration of soluble carbohydrates that enhanced the degree of freezing tolerance (Yano et al., 2005). As discussed above, if gentianose (as the main storage NSC in crowns) plays a similar role as starch, does a similar conversion between gentianose and other

soluble NSCs also occur in the crowns of gentians during cold winters? To clarify this hypothesis, the seasonal dynamics of individual NSCs in crowns was further examined in Chapter 6.

For their initial re-growth in spring, perennial plants need to mobilize carbohydrate reserves until they have achieved sufficient rates of current photosynthetic carbohydrate production to more than compensate for that being consumed (Gordon et al., 1986; Danckwerts & Gordon, 1989; Baur-Hoch et al., 1990; Woolley et al., 1999). In alfalfa (*Medicago sativa* L) and white clover, defoliation in autumn reduced re-growth in spring, and the reduction in growth was correlated with the amount and/or concentration of carbohydrate reserves measured in autumn (Dhont et al., 2002). Similarly in the present study, the proportion of crown buds sprouting in Spring (Table 4.2) was significantly reduced, and positively correlated with the concentration of TNC in storage roots ( $r = 0.931$ ,  $P < 0.01$ ) and crown buds ( $r = 0.922$ ,  $P < 0.01$ ) as well as DW of crowns ( $r = 0.857$ ,  $P < 0.05$ ) in the previous autumn (Table 4.3 and 4.4). It is hypothesized that the re-sprouting of 03/04-114 in spring utilized the carbohydrate reserves stored from the previous growth season, and/or the more carbohydrate reserves that could be mobilized for sprouting of crown buds, then the higher the proportion of buds sprouting could be achieved. While stepwise regression analysis indicated that gentianose concentration in storage roots in autumn contributed the most to the proportion of crown buds sprouting in spring, based on the data in the current experiment, whether gentianose is remobilized and how it is used, is still not clear. In any future experiments, the measurement of the dynamics of individual NSCs in crowns during spring re-growth, and using labelled carbon for tracking the carbon flux, would help to determine if gentianose in the roots and/or rhizomes are remobilized for the re-growth of crown buds.

L-bornesitol was reported as only detected in five families (Plouvier, 1963) including the Gentianaceae (Schilling, 1976), but concentration changes have not been reported in gentians. While the physiological roles of bornesitol remains unclear, some reports suggested that bornesitol may serve as an osmoregulator (Ichimura et al., 1999; Ichimura & Suto, 1999). In the current experiments, the concentration of L-bornesitol did not change markedly in crowns with the increase of the severity of defoliation. The specific physiological function of L-bornesitol in gentians therefore, still needs further investigation.

## **4.5 Conclusion**

Defoliation during the growth season significantly influenced the development of crown buds, winter survival, spring re-growth and NSC reserves in crowns (mainly due to the reduction of gentianose and sucrose) in the gentian 03/04-114. The significant positive correlations between carbohydrate reserves and key factors known to influence the yield and quality of flowering shoots, i.e. TCBN, LCB WSP and SSP, provide indirect evidence that carbohydrate reserves stored in crowns are likely to be a limiting factor influencing the development of crown buds, winter survival and re-growth. Different from many other plants, in addition to glucose, fructose, and sucrose, the unique carbohydrates, i.e. gentianose and gentiobiose, were present in the crowns of gentians, and no starch was detected. Gentianose as the predominant NSC reserves in crowns, may play a similar role as starch does in other plant species. Based on the results of the current study, it is important to retain sufficient leaves on gentian plants for accumulating NSC reserves in crowns, with likely implications on maintaining a sustainable yield and quality of flowering shoots in the following year. Within a commercial context, however, further experiments are required to determine the optimum harvest intensity, i.e. how much/many shoots, should be left on the plants when harvesting floral shoots in gentians.

## **Chapter 5 Effect of carbohydrate supply, temperature, photoperiod and plant growth regulators on morphogenesis of crown buds *in vitro***

### **5.1 Introduction**

As introduced in Chapters 1 through 4, the formation of crown buds, including their quality and quantity, is considered to be key in the production of flowering shoots in gentian. An understanding of the factors influencing the morphogenesis of crown buds will therefore be of value, for improving the yield and quality of cut flowers.

The hypothesis that carbohydrates may be an important limiting factor in the morphogenesis of crown buds was explored in Chapter 4. It was inferred from the results that carbohydrate acquisition and/or storage in the current season's growth cycle may be a potential factor directly or indirectly influencing the morphogenesis of the crown buds that develop into the floral shoots in the subsequent growth cycle. In that experiment the severity of defoliation was the treatment used to test this hypothesis, however a limitation noted was that the removal of foliage was likely to do more than simply reduce carbohydrate acquisition, i.e. defoliation not only changes acquisition of photo-assimilate, but also will change other factors such as endogenous plant hormones, reaction to light, etc. Hence the results of the defoliation experiment (Chapter 4) could not, by itself, clarify the physiological effect of carbohydrates on the morphogenesis of crown buds. In order to better understand the roles of different factors on the morphogenesis of crown buds, an *in vitro* system was viewed as providing an ideal alternative option, which, in addition to being less resource-consuming, would ideally provide an experimental system independent of seasons, so as to study factors like carbohydrate supply on the morphogenesis of crown buds.

For gentian a morphological definition for a "crown bud" *in vitro* has not previously been described in any reports. Samarakoon (2012) reported that crown-bud clusters can initiate adventitiously on storage roots and rhizomes in gentian plants. However, from the reports of Sato *et al.* (1988) and Ho and Liu (2001), and the observation in the experiments described in Chapter 3, crown buds were most commonly encountered as dormant axillary buds at the basal nodes of shoots. For the experiments conducted *in vitro*, and reported in this chapter, this latter definition of crown buds was used.

Plant tissue culture has been widely employed in research and commercial production of gentians, for purposes such as propagation, cryopreservation, breeding, genetics and physiological investigations (Hosokawa et al., 1996; Morgan et al., 1997; Hosokawa et al., 1998; Morgan, 2004; Suzuki et al., 2008). While a few technical reports written in Japanese exist (Sato, 1988b; Kawakami & Shimonaka, 1996), no peer-reviewed paper was available demonstrating the characteristics and factors influencing the morphogenesis of crown buds with gentian *in vitro*. As presented within this chapter therefore, the effects of factors such as carbohydrates, photoperiod, temperature, and plant growth regulators on this process were investigated.

Carbohydrates influence the organogenesis of many plant species *in vitro* (Premkumar *et al.*, 2003; Gemas & Bessa, 2006; Jana & Shekhawat, 2011). Sucrose, as a major primary product of photosynthesis (ap Rees, 1984), is most commonly used as a carbon source *in vitro* as it is cheap and readily available (Bhojwani & Razdan, 1996). Although the technical reports by Sato (1988b) and Kawakami & Shimonaka (1996) indicated that sucrose concentration is an important factor influencing the number of crown buds per plantlet formed *in vitro* with gentian, their inconsistent results suggested a lack of clarity for the role of sucrose in the morphogenesis of crown buds. Many studies have been reported that some carbohydrates also play an important role in bud dormancy, through cross-talk with signalling pathways such as light and plant hormones (Anderson et al., 2001; Chao et al., 2006; Chao & Serpe, 2010; Richardson et al., 2010). While dormancy is an important morphogenetic character of crown buds in gentians (refer Section 2.2.2), prior to the commencement of the research reported in this thesis, there was no information available about the effect of sucrose concentration on the dormancy of crown buds. In addition, the endogenous carbohydrate concentration is an important factor governing dormancy, organogenesis and growth of some plant species *in vitro* (Kromer & Gamian, 2000; Shin *et al.*, 2002; Premkumar *et al.*, 2003; Sarkar *et al.*, 2010), and providing an exogenous supply of carbohydrates (e.g. *in vitro*) can influence endogenous carbohydrate concentration (Premkumar *et al.*, 2003). Consideration also needs to be given to the fact that there are unique carbohydrates, i.e. gentianose and gentiobiose, existing in gentians (Bridel, 1911; Keller & Wiemken, 1982). While the metabolism of gentianose and gentiobiose have not been elucidated in plants, sucrose as the primary product of photosynthesis may directly or indirectly be involved in the synthesis of these unique carbohydrates (Section 2.5.5.2). The effect of sucrose supply within the medium

on metabolism of these unique and other endogenous carbohydrates, as well as their possible role in the formation of crown buds, was therefore investigated.

Plants have evolved multiple physiological mechanisms to modify their growth and development for adapting to their environment, including seasonal changes (Franklin & Whitelam, 2004). Light and temperature are important environmental signals regulating plant development, and in gentians technical reports have detailed that at 20 °C and 18 h photoperiod, all plantlets formed crown buds *in vitro* but, at 25 °C, no crown buds formed (Sato, 1988b). Compared to those grown at 20 °C, Kawakami & Shimonaka (1996) reported that gentian plantlets cultivated *in vitro* at 15 °C had more crown buds, as well as a higher proportion of plants forming them, four months after acclimation *in vivo*. Hence, these results were interpreted to suggest that a relatively lower temperature promoted the morphogenesis of crown buds. In the current experiments *in vitro*, the influence of temperatures between 15 and 25 °C were evaluated.

Sato's (1988) technical report showed that an 18 h photoperiod enhanced the morphogenesis of crown buds compared to a 12 h photoperiod *in vitro*. *In vivo*, however, Samarakoon (2012) reported an apparent reversal of this response, wherein a shorter photoperiod increased the number of crown buds to approximately twice that achieved under the longer photoperiod. Therefore the effects of temperature and/or photoperiod on the morphogenesis of crown buds *in vitro*, still need to be investigated. Given the latitude of the regions the parents of 'Showtime Diva' originated from (Section 2.2.2), the shortest and longest day length are between 7 and 17 h (Lammi, 2005), hence in the current experiments 7 h and 18 h photoperiods were used as short and long photoperiodic treatments, respectively.

The regulation of propagation *in vitro* by exogenous plant growth regulators has been investigated for some gentian species (Jomori et al., 1995; Hosokawa et al., 1996; Morgan et al., 1997). Thidiazuron (TDZ) was found to be the most effective cytokinin for *in vitro* propagation of some commercial cultivars of gentian, and 0.01 ppm TDZ was the most efficient concentration for the morphogenesis of adventitious buds/shoots (Hosokawa *et al.*, 1998). Given that adventitious buds were not the focus of the current study, an evaluation of cytokinins within the base medium (BM) was not undertaken within the current experiments.

1-Naphthaleneacetic acid (Vanaartrijk *et al.*) was more effective than indole-3-acetic acid (IAA) or 2, 4-dichlorophenoxyacetic acid (2, 4-D), for rooting (Hosokawa *et al.*, 1996), and gibberellic acid (GA<sub>3</sub>; 0.01 to 0.2 ppm) promoted shoot growth and development *in vitro* (Zhang & Leung, 2002). In contrast however, the effects of plant growth regulators on initiation and development of crown buds (as defined above) *in vitro* have not been reported. Although NAA (0.01 to 0.02 ppm) was used in the BM for studying the effect of sucrose on the morphogenesis of crown buds in the technical reports of Sato (1988b) and Kawakami & Shimonaka (1996), the effect of NAA could not specifically be analysed due to the lack of a comparison with a control treatment in these reports. Hence whether NAA was influencing the morphogenesis of crown buds remained to be examined.

Ethylene is a unique gaseous plant hormone that affects various physiological processes at different stages of plant growth and development (Reid, 1995; Dolan, 1997; Bhat *et al.*, 2010). Although ethylene is generally considered as a growth inhibitor, there is increasing evidence that ethylene can also stimulate plant growth and development (Pierik *et al.*, 2006). Similarly Samarakoon (2012) reported that ethephon (2-chloroethylphosphonic acid; a synthetic compound decomposing into ethylene), alone or combined with either paclobutrazol or TDZ, promoted crown bud formation *in vivo*. Given the preceding results, ethylene, as a decomposition product of ethephon, was viewed as playing a potential role in the morphogenesis of crown buds and, therefore, its influence *in vitro* was examined in the experiments presented within this chapter.

Given the apparent difference in results between previous reports, and the limited information that has been published, the physiological mechanism(s) associated with the morphogenesis of crown buds in gentians remained unclear. The objectives of the experiments presented in this chapter therefore, were to determine the effects of the following factors and/or their interaction on the morphogenesis of crown buds *in vitro*:

- 1) sucrose concentration (including its effect on dormancy status and concentration of endogenous carbohydrates),
- 2) temperature (15 to 25 °C) and photoperiod (18 h and 7 h),
- 3) ethephon concentration (0 to 20 ppm) and NAA (0 and 0.01 ppm).

## 5.2 Materials and methods

### 5.2.1 General materials

Plantlets of the gentian cultivar ‘Showtime Diva’, cultured *in vitro* (Plant & Food Research), were used as the source material for each of the experiments described in the current study. A sufficient number of plantlets for experimental purposes were obtained by first sub-culturing every six weeks for six months. The medium used for proliferation comprised BM as described below, supplemented with 30 g/L sucrose (Morgan, 2004).

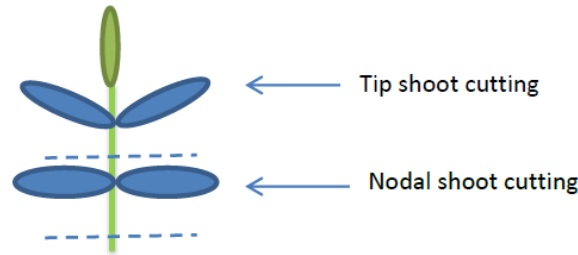
The BM for all experiments comprised macro- and micro-nutrient salts (MS) (Murashige & Skoog, 1962) with macro-salts at half strength, LS vitamins (Linsmaier & Skoog, 1965), and solidified by addition of 7.5 g/L agar (Davis agar, Sigma). The pH of the media was adjusted to 5.7 with either 0.1 N NaOH or 0.1N HCl, prior to autoclaving at 121°C for 15 min.

Culture vessels were disposable 290 ml (80 mm base diameter x 60 mm deep) tubs with snap on lids, manufactured by blow moulding of a general purpose styrene and K-resin plastic (Alto Packaging, Hamilton, New Zealand). Each culture vessel contained 50 ml of medium. Unless stated otherwise, plantlets were cultured at  $25 \pm 1$  °C, at a photosynthetic photon flux density (PPFD) of  $30 \pm 5$   $\mu\text{mol m}^2 \text{s}^{-1}$  at the top of the culture vessels, and 16 h photoperiod, provided by cool-white fluorescent tubes.

### 5.2.2 Experiment One – sucrose concentration

#### 5.2.2.1 Treatments

The treatments comprised seven concentrations of sucrose: 0 % (0 mM), 3 % (87.6 mM), 6 % (175.3 mM), 9 % (262.9 mM), 12 % (350.6 mM), 15 % (438.2 mM), or 20 % (584.0 mM), added to the BM (refer Section 5.2). Tip cuttings (with one pair of expanded leaves) and nodal shoot cuttings (single node; Figure 5.1) were evaluated separately, utilising eight cuttings (i.e. sub-samples) per culture vessel. Each treatment comprised five culture vessels as replicates.



**Figure 5.1 Diagrammatic illustration of positions within the shoot of the gentian cultivar ‘Showtime Diva’ harvested for experimental purposes: shoot tip cuttings and nodal cuttings.**

#### **5.2.2.2 Crown buds**

The proportion of plantlets (i.e. rooted cutting) developing crown buds, and the number of crown buds per plantlet, were determined each week for 12 weeks, after which the proportion of plantlets exhibiting crown buds was considered to be stable.

#### **5.2.2.3 Dormancy assessment**

The clusters of crown buds developed on plantlets cultured on the medium (i.e. cultured for four months) containing 9% sucrose, were transferred to culture vessels containing a BM amended with either 9% or 3% sucrose. Four clusters of crown buds were treated as subsamples within each culture vessel, with three culture vessels as replicates. The crown buds were observed every week for four weeks, noting any visible changes in morphological development.

#### **5.2.2.4 Growth assessment and analysis of endogenous carbohydrates**

After 16 weeks on treatment media the plantlets were washed with tap water, then shoots and roots were separated. Samples (from nodal cuttings) in each culture vessel were pooled, resulting in six replicates of eight plantlets. Three replicates were frozen in liquid nitrogen for subsequent carbohydrate analysis (refer Section 4.2.7), while three replicates of plantlets were used to record fresh and dry weight. Dry weight was recorded after shoots and roots were dried at 60 °C for one week. Because the growth of the plantlets was very limited in the treatments containing 0%, 15% and 20% sucrose within the medium, there was not enough plant material obtained from these treatments for carbohydrate analysis.

### 5.2.3 Experiment Two – temperature and photoperiod

To test the response of plantlets to photoperiod and temperature during culture *in vitro*, two levels of photoperiod and three levels of temperature were utilized in this experiment. Nodal shoot cuttings produced *in vitro* were grown on BM amended with 9 % sucrose (262.9 mM), with eight cuttings (i.e. sub-samples) per culture vessel. Each treatment comprised five culture vessels (i.e. replicates) held in growth cabinets providing one of each of the following treatment environments with a PPFD of between 30 and 40  $\mu\text{mol m}^2 \text{s}^{-1}$ :

- 7 hr photoperiod at 15 °C  $\pm$  1 °C
- 7 hr photoperiod at 20 °C  $\pm$  1 °C
- 7 hr photoperiod at 25 °C  $\pm$  1 °C
- 18 hr photoperiod at 15 °C  $\pm$  1 °C
- 18 hr photoperiod at 20 °C  $\pm$  1 °C
- 18 hr photoperiod at 25 °C  $\pm$  1 °C

The proportion of plantlets developing crown buds, and the number of crown buds per plantlet were observed each week for 12 weeks, after which the proportion of plantlets exhibiting crown buds was considered to be stable, as described in Section 5.2.2.2.

### 5.2.4 Experiment Three – NAA and Ethephon

#### 5.2.4.1 NAA

Nodal shoot cuttings were cultured on BM with 9% sucrose, amended with NAA at either 0 ppm or 0.01 ppm, at 18 hr photoperiod and 25 °C  $\pm$  1 °C. Each treatment comprised five culture vessels as replicates, with eight cuttings (i.e. sub-samples) per culture vessel. The proportion of plantlets developing crown buds, and the number of crown buds per plantlet, were observed each week for 12 weeks, after which the proportion of plantlets exhibiting crown buds was considered to be stable.

#### 5.2.4.2 Ethephon

Ethephon (commercial preparation of Ethrel 48™; 480 g L<sup>-1</sup> ethephon; May & Baker Agrochemicals, NZ) was filter sterilized, and then added to the BM amended with sucrose once autoclaved, but before the medium solidified. Treatments comprised BM amended

with one of four concentrations of ethephon (i.e. 0 ppm, 5 ppm, 10 ppm or 20 ppm) and either 9% or 3% sucrose. Each treatment comprised five culture vessels as replicates, with eight cuttings (i.e. sub-samples) per culture vessel. All treatments were cultured at 18 hr photoperiod and  $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ .

The proportion of plantlets developing crown buds, and the number of crown buds and shoot number (total shoots, including first order, second order and other subsequent order shoots) per plantlet, were observed each week for 12 weeks, after which the proportion of plantlets exhibiting crown buds was considered to be stable. After being cultured for 16 weeks, the crown buds that developed on plantlets on the medium containing 9% sucrose and 10 ppm ethephon (medium concentration of the range from 0 to 20 ppm), were transferred to culture vessels containing BM amended with either 9% or 3% sucrose. The crown buds were observed as described in Section 5.2.2.2.

### **5.2.5 Statistical analysis**

For all three experiments a goodness-of-fit test was used to test the distribution of data. In Experiment One, even after transformation, the data were not normally distributed and thus were analysed using the nonparametric Kruskal-Wallis test, followed by Dunn's procedure for nonparametric multiple comparisons (Zar, 1999). Other data were normally distributed, and were analysed using an analysis of variance utilising GLM (Minitab 16). When significant differences between treatments occurred, mean values were separated using the Tukey method ( $P < 0.05$ ).

## **5.3 Results**

### **5.3.1 Experiment One – sucrose concentration**

#### **5.3.1.1 General growth of plantlets**

As an overview of general growth and development, cuttings cultured with 0 % sucrose did not grow well, and most had died within 12 weeks. On media with 3% to 12% sucrose, both tip and nodal shoot cuttings developed into plantlets with new roots and shoots, but on 15% sucrose plantlets developed very short shoots and roots (Table 5.1). On 20% sucrose medium, there was no visible growth of new shoots and roots for most cuttings. With the increase of sucrose concentration from 3% to 15%, both the shoot and root length of the plantlets from tip cuttings decreased approximately three-fold from an average 13.0

cm to 4.0 cm ( $P < 0.001$ ) and 25-fold from 4.9 to 0.2 ( $P < 0.001$ ), respectively (Table 5.1). Shoot dry weight more than doubled when the sucrose concentration was increased from 3% to either 6% or 9%, but then declined approximately 60% to 0.055 g per plantlet at 15% sucrose ( $P < 0.05$ ). Root number increased approximately three-fold from 8.6 to the maximum of 27.0 between 3% and 9% sucrose ( $P < 0.001$ ), but then declined approximately 70% to 5.8 roots per plantlet at 15% sucrose ( $P < 0.001$ ; Table 5.1). The DW of roots showed a similar trend to root numbers, e.g. root DW increased approximately ten-fold from 0.012 g per plantlet to the max of 0.107 g per plantlet with the increase of sucrose concentration from 3% to 9%, and then declined approximately 90% to 0.010 g per plantlet at a sucrose concentration of 15% (Table 5.1). The nodal shoot cuttings showed a similar pattern of development and growth (data not shown).

**Table 5.1 Effects of different sucrose concentrations *in vitro* on the growth of tip cuttings of ‘Showtime Diva’ after 16 weeks in culture Z, Y.**

Sucrose concentration percentage (molarity)	Shoot length	Root length	Root number	Shoot dry weight (g per plantlet)	Root dry weight (g per plantlet)
0 (0.0 mM)	-	-	-	-	-
3 (87.6 mM)	13.0 ± 1.1 a	4.9 ± 0.2 a	8.6 ± 0.5 cd	0.054 ± 0.007 b	0.012 ± 0.001 c
6 (175.3 mM)	11.6 ± 0.9 a	3.0 ± 0.5 b	19.2 ± 1.0 b	0.143 ± 0.012 a	0.027 ± 0.002 bc
9 (262.9 mM)	9.5 ± 1.2 ab	2.4 ± 0.1 b	27.0 ± 1.5 a	0.137 ± 0.016 a	0.107 ± 0.013 a
12 (350.6 mM)	5.8 ± 1.0 bc	0.7 ± 0.3 c	19.8 ± 1.8 b	0.062 ± 0.008 b	0.047 ± 0.006 b
15 (438.2 mM)	4.0 ± 0.4 c	0.2 ± 0.1 d	5.8 ± 1.2 d	0.055 ± 0.006 b	0.010 ± 0.001 c
20 (584.0 mM)	-	-	-	-	-

<sup>Z</sup> Mean values (± SE), n = 10. Treatments within which cuttings did not develop are represented by ‘-’.

<sup>Y</sup> Within the same column, mean values followed by different letters were significantly different (Tukey test:  $P < 0.05$ ).

### 5.3.1.2 Crown bud formation

Over the initial 12 weeks of observation, no crown buds were formed at sucrose concentrations between 0% and 6%. On the media containing 9% to 15% sucrose, crown buds first became visible after 5 weeks. While crown buds were sometimes found within the nodal axils of the second or higher leaf pairs (Figure 5.2E and F), most crown buds arose in the leaf axils of the first node at the base of shoots, i.e., the leaf axils of the original cuttings (Figure 5.2B to D). After the first crown bud became visible on a plantlet, subsequent axillary crown buds continued to develop within the axils of these initial

crown buds, forming a cluster of crown buds (Figure 5.2C and D). In addition, some crown buds *in vitro* developed roots at their base (Figure 5.2F).

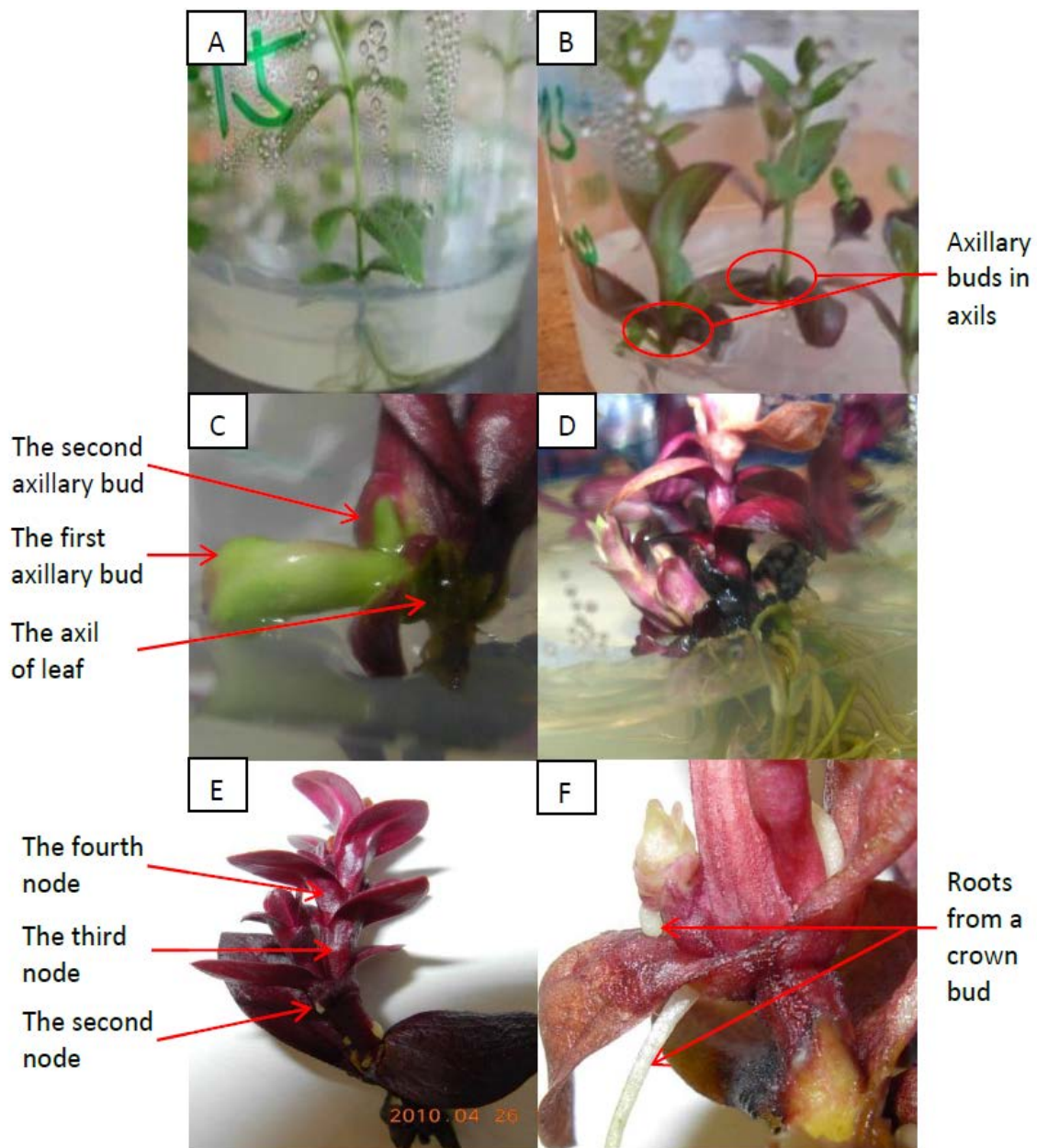
The sucrose concentration significantly affected both the proportion of plantlets developing crown buds (Kruskal-Wallis test:  $P < 0.001$ ) and the number of crown buds per plantlet (Kruskal-Wallis test:  $P < 0.001$ ). However the values were very low, with the highest proportion of 20%, and the highest number of crown buds per plantlet of 0.4, achieved at a concentration of 12% sucrose (Table 5.2). There was no significant difference between tips and nodal cuttings for either the proportion of plantlets forming crown buds (Kruskal-Wallis test:  $P = 0.792$ ) or the number of crown buds per plantlet (Kruskal-Wallis test:  $P = 0.853$ ). For the tip and nodal shoot cuttings, with increasing sucrose concentration the proportion of plantlets developing crown buds increased from 0% and reached a maximum of 20% and 17.5%, respectively, at 12% sucrose (Table 5.2). With the further increase in sucrose concentration to 15%, the proportion of plantlets developing crown buds dropped to 5%. The mean number of crown buds per plantlet, for both tip and nodal cuttings, also followed similar trends with changes in sucrose concentration.

#### 5.3.1.3 Dormancy of crown buds

The crown buds on plantlets transferred to a medium containing 9% sucrose, maintained their dormant status, i.e., did not emerge as shoots (Figure 5.3B). While macroscopically dormant, however, plantlets on the 9% sucrose medium continued to develop additional crown buds following transfer to fresh medium. In contrast, the majority of the crown buds transferred to a medium containing 3% sucrose developed into emerging shoots within one week, and the development of new crown buds stopped (Figure 5.3A).

#### 5.3.1.4 Endogenous carbohydrates

Both in roots and shoots formed *in vitro*, the endogenous carbohydrates comprised gentianose, gentiobiose, sucrose, fructose, and glucose (Figure 5.4), with no evidence of starch. In general, at each concentration of sucrose in the BM, the concentration of endogenous total non-structural carbohydrates (TNC) was higher in roots (ranging from 12.31 to 94.2 mg g<sup>-1</sup> FW) than shoots (11.91 to 37.21 mg g<sup>-1</sup> FW;  $P < 0.05$ ; Figure 5.4).



**Figure 5.2** Morphology of crown buds on plantlets of ‘Showtime Diva’ *in vitro* after different durations on different concentrations of sucrose in the medium, cultured under 18 h photoperiod and 20 °C. (A) no crown buds on 3% sucrose medium after 6 weeks (control); (B) crown buds within the axil of the first node on 9% sucrose medium after 6 weeks; (C) a subsequent crown bud developing within the axil of a previously formed crown bud on 12% sucrose medium after 6 weeks; (D) a cluster of crown buds on 9% sucrose medium after 24 weeks; (E) crown buds within the axils of the second to fourth nodes on 12% sucrose medium after 16 weeks; (F) a crown bud developing roots from its base on 12% sucrose medium after 16 weeks.

**Table 5.2 Effects of sucrose concentration after 12 weeks on the proportion of plantlets developing crown buds and the number of crown buds per plantlet for different shoot cutting types of ‘Showtime Diva’. Plantlets were cultured at  $25 \pm 1$  °C and 18 h photoperiod.**

Sucrose concentration percentage (molarity)	Proportion of plantlets producing crown buds (%)				Mean number of crown buds per plantlet			
	Tips		Nodes		Tips		Nodes	
0 (0.0 mM)	0	a <sup>Y</sup>	0	a	0	a	0	a
3 (87.6 mM)	0	a	0	a	0	a	0	a
6 (175.3 mM)	0	a	0	a	0	a	0	a
9 (262.9 mM)	12.5 ± 3.9 <sup>Z</sup>	b	15.0 ± 4.7	b	0.3 ± 0.10 <sup>Z</sup>	b	0.3 ± 0.12	b
12 (350.6 mM)	20.0 ± 5.1	b	17.5 ± 4.9	b	0.3b ± 0.11	b	0.4 ± 0.18	b
15 (438.2 mM)	5.0 ± 2.5	ab	5.0 ± 2.5	ab	0.1 ± 0.05	ab	0.1 ± 0.02	ab
20 (584.0 mM)	0	a	0	a	0	a	0	a

<sup>Z</sup> Mean values ( $\pm$  SE), n = 40

<sup>Y</sup> Within the same column, mean values followed by different letters were significantly different (Kruskal-Wallis:  $P < 0.05$ ).

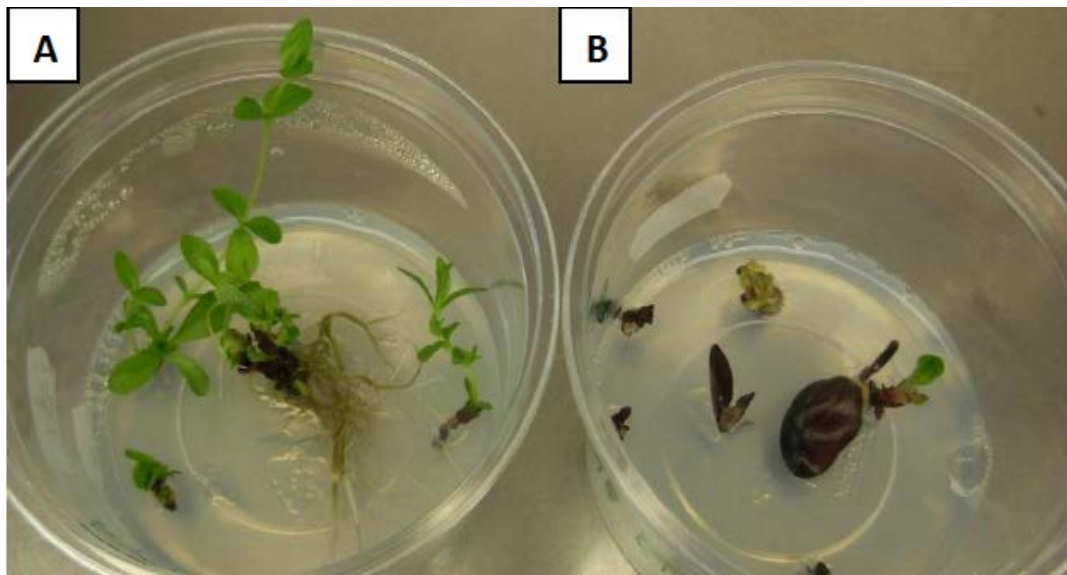
With increasing sucrose concentration in the BM, the concentration of endogenous TNC presented more than seven-fold and three-fold increases in shoots and roots, respectively (Shoots:  $P = 0.005$ ; Roots:  $P = 0.021$ ). This was primarily the result of increases in the concentration of endogenous sucrose and gentianose, e.g. within shoots sucrose increased more than three-fold from 1.58 to 4.82 mg g<sup>-1</sup> FW;  $P = 0.031$ ; and in roots, sucrose increased more than 16-fold from 1.57 to 25.68 mg g<sup>-1</sup> FW;  $P = 0.039$ ). Across this same increase in sucrose concentration within the BM, within shoots the endogenous concentration of gentianose increased almost 20-fold from 2.14 to 42.10 mg g<sup>-1</sup> FW ( $P = 0.005$ ); and in roots, gentianose increased more than three-fold from 1.58 to 4.82 mg g<sup>-1</sup> FW ( $P = 0.048$ ). In contrast, the concentration of fructose, glucose and gentiobiose did not change significantly in either shoots or roots with these same changes in concentration within the BM ( $P > 0.05$ ).

In addition to the acknowledged carbohydrates, the cyclitol known as L-bornesitol (1-O-methyl-myo-inositol) was also identified in shoot and root samples of ‘Showtime Diva’ (refer to Chapter 6). While the concentration of L-bornesitol in shoots, averaging  $3.3 \pm 0.2$  mg g<sup>-1</sup> FW, was approximately three times higher than that in roots, averaging  $1.0 \pm 0.1$

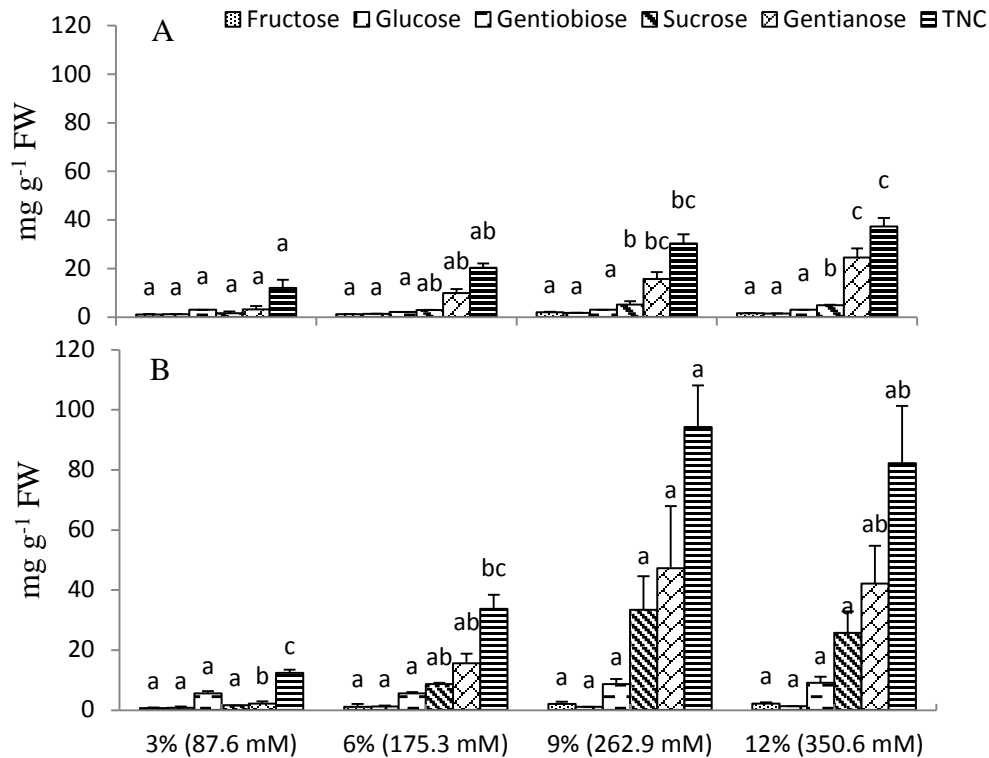
mg g<sup>-1</sup> FW ( $P < 0.001$ ), there was no significant difference resulting from the various sucrose concentrations in the BM (i.e. shoots:  $P = 0.185$ ; roots:  $P = 0.756$ ).

### 5.3.2 Experiment Two – temperature and photoperiod

While neither the effect of temperature nor photoperiod was significant at the 5% confidence interval (i.e.  $P < 0.05$ ), photoperiod significantly affected the proportion of plantlets producing crown buds and the number of crown buds per plant ( $P < 0.10$ ; Table 5.3). Both the proportion of plantlets developing crown buds and the number of crown buds per plant were higher under the 18 h than 7 h photoperiod ( $P < 0.10$ ; Figure 5.5 and Figure 5.6), but the maximum values attained were less than 20% and 0.3 per plantlet, respectively. There was no interaction between photoperiod and temperature.



**Figure 5.3** Extent of shoot emergence from crown buds of ‘Showtime Diva’ after three weeks on BM media amended with; (A) 3% sucrose and, (B) 9% sucrose. The original crown buds were formed after 16 weeks on BM medium with 9% sucrose. Plantlets were cultured at  $25 \pm 1$  °C and 16 h photoperiod.

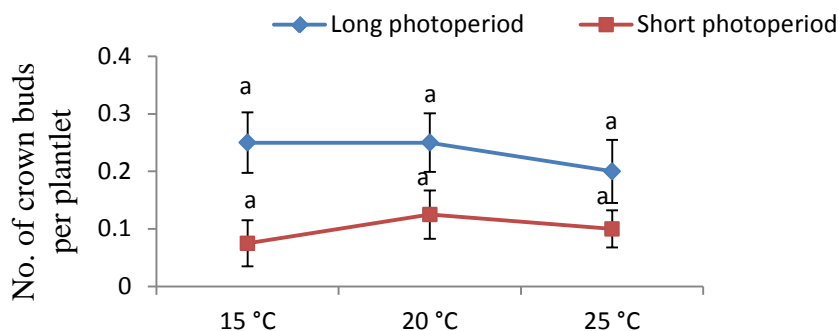


**Figure 5.4** Concentration of individual carbohydrates and total non-structural carbohydrate (TNC) per unit fresh weight (FW) in; (A) shoots and, (B) roots, of ‘Showtime Diva’ when sampled after 16 weeks culture on media with increasing sucrose concentration (%; molarity in brackets). Plantlets were cultured at  $25 \pm 1$  °C and 16 h photoperiod. For each variable, means ( $\pm$  SE) with different letters were significantly different (Tukey;  $P < 0.05$ ).

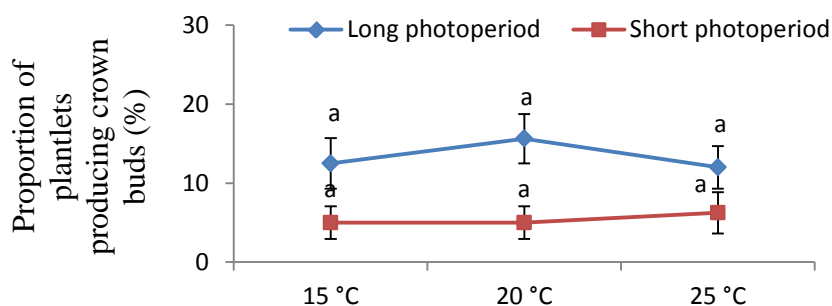
**Table 5.3** Probability values indicating significance of main factors and interactions, determined for influence of temperature and photoperiod on the proportion of plantlets of ‘Showtime Diva’ producing crown buds and the number of crown buds per plantlet after 12 weeks *in vitro*.

	Proportion of plantlets producing crown buds	Number of crown buds per plantlet
	$P^Z$	$P$
Photoperiod	0.084	0.09
Temperature	0.896	0.887
Photoperiod $\times$ temperature	0.773	0.741

<sup>Z</sup> General linear model (GLM) probability value



**Figure 5.5** Effect of photoperiod and temperature on the number of crown buds per plantlet of ‘Showtime Diva’ after 12 weeks *in vitro*. For each variable, means ( $\pm$  SE) within each photoperiod treatment with different letters are significantly different (Tukey:  $P < 0.05$ ).



**Figure 5.6** Effect of photoperiod and temperature on the proportion of plantlets of ‘Showtime Diva’ developing crown buds after 12 weeks *in vitro*. For each variable, means ( $\pm$  SE) within each photoperiod treatment with different letters are significantly different (Tukey:  $P < 0.05$ ).

### 5.3.3 Experiment Three – NAA and Ethephon

#### 5.3.3.1 NAA

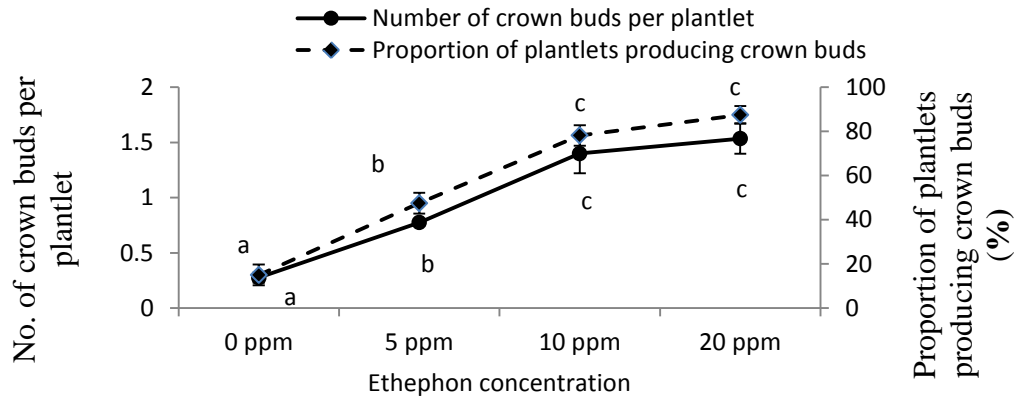
Compared with the control (NAA: 0 ppm), the effect of NAA (0.01 ppm) was not significant on either the proportion of plantlets producing crown buds (DF: 1, 8;  $F = 0.2$ ;  $P = 0.664$ ) or the number of crown buds per plantlet (DF: 1, 8;  $F = 1.1$ ;  $P = 0.327$ ) after 12 weeks *in vitro*. Across all treatments an average  $18 \pm 5.2\%$  plantlets formed crown buds, with an average  $0.3 \pm 0.10$  crown buds per plantlet.

#### 5.3.3.2 Ethephon

##### Crown buds

When cultured on a BM containing 9% sucrose, ethephon increased both the proportion of plantlets producing crown buds ( $P < 0.001$ ) and the number of crown buds per plantlet ( $P < 0.001$ ; Figure 5.7). After 12 weeks in culture, both the proportion of plantlets producing

crown buds and the number of crown buds per plantlet increased by up to approximately five-fold (i.e. from 15% to 78%, and 0.3 to 1.5, respectively) with the increase in ethephon concentration from 0 ppm to 10 ppm (Figure 5.7). This response to increasing concentration of ethephon reached a plateau between 10 ppm and 20 ppm, with no further increase evident at the higher concentration ( $P > 0.05$ ).

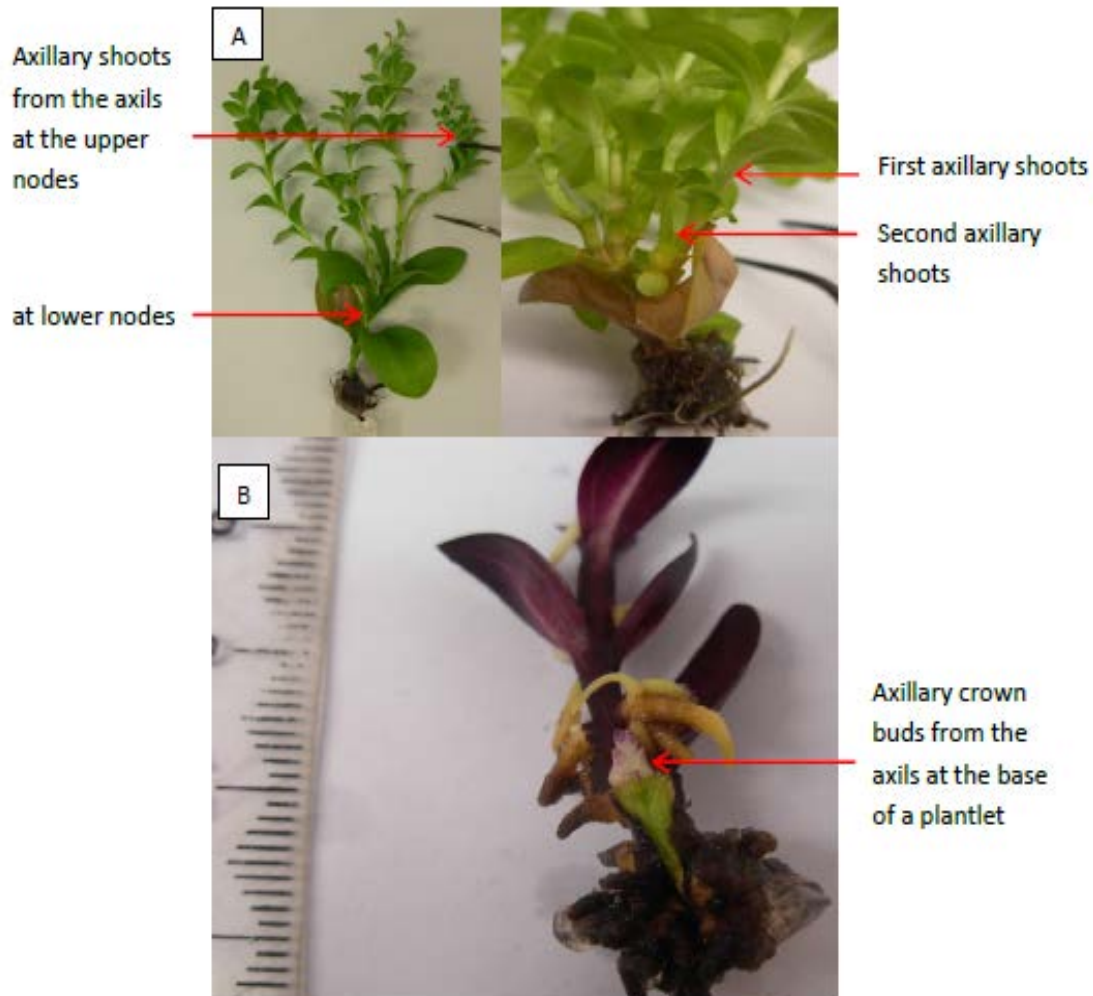


**Figure 5.7** Effect of ethephon concentration on the proportion of plantlets of ‘Showtime Diva’ producing crown buds, and the number of crown buds per plantlet, on basal media containing 9% sucrose after 12 weeks. For values within each variable (means  $\pm$  SE) those with different letters were significantly different (Tukey:  $P < 0.05$ ).

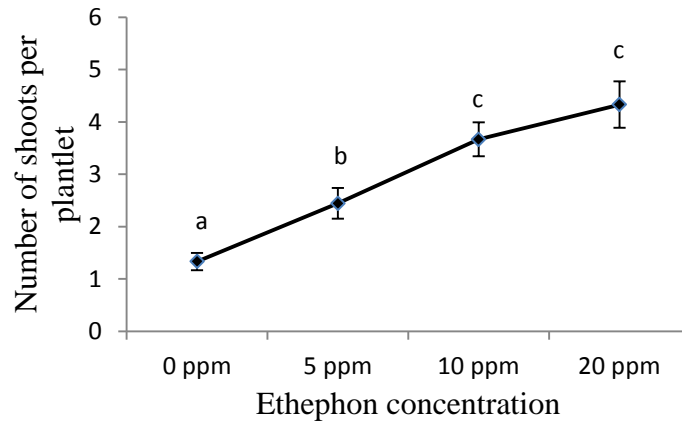
While on a BM containing 3% sucrose no dormant crown buds were evident at any concentration of ethephon, treatment with ethephon resulted in an increased number of shoots per plantlet (DF: 3, 32;  $F = 31.34$ ;  $P < 0.001$ ). Macroscopically, these shoots appeared as axillary shoots (Figure 5.8A). Similar to the crown buds, these axillary shoots predominantly arose from the leaf axils at lower nodes of plantlets; although some of the axillary shoots also arose from the leaf axils at the upper nodes of plantlets. In addition, axillary shoots also arose from the leaf axils of some of the first axillary shoots (i.e. second order axillary shoots; Figure 5.8A). With the increase of ethephon concentration from 0 ppm to 20 ppm, the number of axillary shoots per plantlet increased more than three-fold from 1.3 to 4.3 after 12 weeks (Figure 5.9).

In addition, ethephon also significantly reduced the proportion of plantlets flowering in culture, for plantlets on both 3% sucrose medium (DF: 3, 16;  $F = 31.53$ ;  $P < 0.001$ ) and 9% sucrose medium (DF: 3, 16;  $F = 45.04$ ;  $P < 0.001$ ; Figure 5.10). With the increase in ethephon concentration from 0 ppm to 10 ppm, the proportion of plantlets flowering

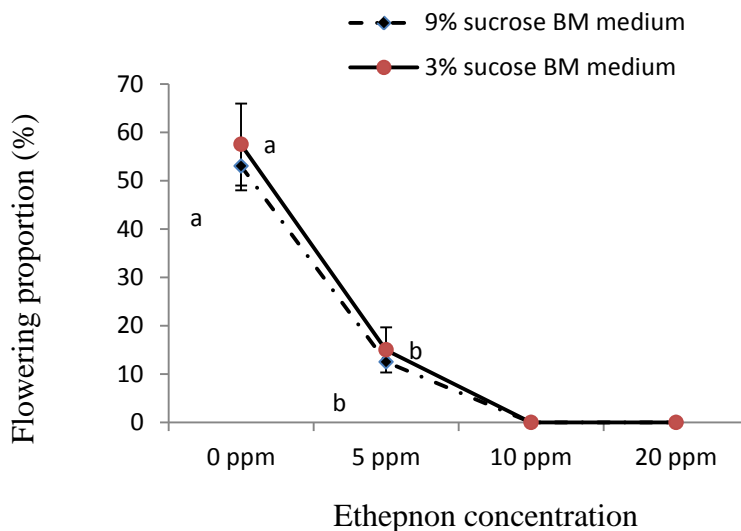
during the 12 weeks of cultivation declined sharply from approximately 60% to 0%, on BM amended with either 3% or 9% sucrose.



**Figure 5.8** Crown buds and shoots on plantlets of ‘Showtime Diva’ *in vitro* following inclusion of ethephon within the BM, cultured under 18 h photoperiod and 20°C. (A) axillary shoots arose from axils at the base of a plantlet on BM amended with 3% sucrose and 20 ppm ethephon after 12 weeks; (B) axillary crown buds within the axils at the base of a plantlet on BM amended with 9% sucrose and 20 ppm ethephon after 12 weeks.

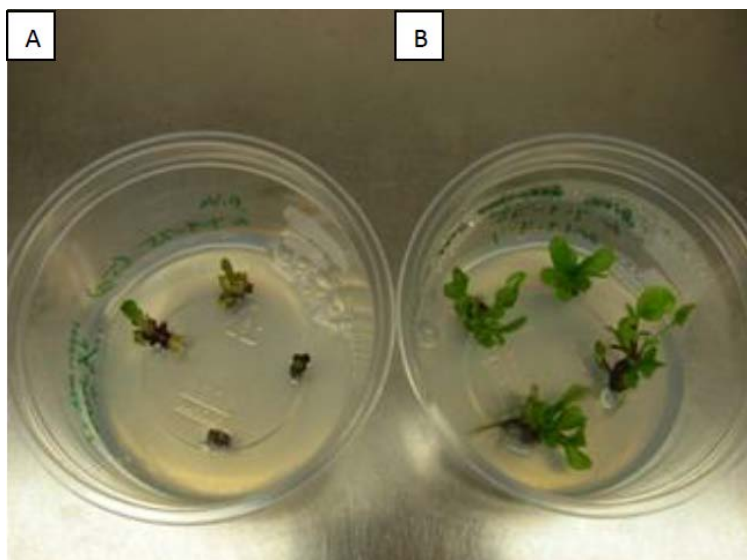


**Figure 5.9** Effect of ethephon concentration on total number(including first and second order axillary shoots) of shoots per plantlet of ‘Showtime Diva’ cultured on basal media amended with 3% sucrose after 12 weeks. Values (means  $\pm$  SE) with different letters were significantly different (Tukey:  $P < 0.05$ ).



**Figure 5.10** Effect of ethephon concentration on proportion of plantlets of ‘Showtime Diva’ cultured on basal media amended with either 3% or 9% sucrose after 12 weeks. Within each sucrose treatment, the values (means  $\pm$  SE) with different letters were significantly different (Tukey:  $P < 0.05$ ).

When the crown buds on plantlets arising from the BM amended with 10 ppm ethephon and 9% sucrose, were transferred to media containing 9% sucrose, they continued to develop additional new crown buds; however, all these crown buds maintained their dormant status (Figure 5.11A). In contrast, those transferred to media containing 3% sucrose developed into shoots within one week, and no further development of additional crown buds or shoots was found over the following 4 weeks of assessment (Figure 5.11B).



**Figure 5.11** Extent of shoot emergence from crown buds of ‘Showtime Diva’ after three weeks on base medium (BM) amended with; (A) 9% sucrose and, (B) 3% sucrose. The original crown buds were formed after 16 weeks on BM amended with 9% sucrose and 10 ppm ethephon.

#### 5.4 Discussion

The current experiments successfully established a protocol for the development of crown buds of ‘Showtime Diva’ within an *in vitro* system. This provides a helpful tool to study the mechanism of morphogenesis of crown buds in gentians and the factors influencing it. Compared to crown buds apparent in the field on plants of gentian 03/04-114 (Section 3.3.3), crown buds of ‘Showtime Diva’ *in vitro* presented similar morphology, i.e. axillary buds that were swollen, in clusters, and covered with purple-coloured scale leaves (Figure 5.2B-F and Figure 5.8B). In addition, some crown buds *in vitro* also developed roots at their base (Figure 5.2F), which was also noted on crown buds in the field (Figure 3.7). As macroscopically evident, the initiation of crown bud clusters of ‘Showtime Diva’ *in vitro* was axillary (Figure 5.2B-F and Figure 5.8B). As discussed in Chapter 3, the fact that initiation of a cluster of crown buds can arise from axillary (Sato et al., 1988; Ho & Liu, 2001) or adventitious buds (Samarakoon et al., 2013), may reflect the plasticity of response in gentian plants.

Sucrose concentration significantly influenced the morphogenesis of crown buds (Figure 5.2 and Table 5.1). Using a historical selection of *G. triflora*, Sato (1988b) reported in his technical report that increasing the sucrose concentration *in vitro* up to 6% (175.3 mM) almost doubled the number of crown buds to approximately 2 and, more than tripled the

proportion of plantlets forming crown buds to 95%. In the current study however, the increase of sucrose up to 12% (350.6 mM) led to an increase in the proportion of plants forming crown buds, but crown buds only formed where the sucrose concentration was higher than 6%, and the maximum percentage of plants forming crown buds was comparatively low, i.e. less than 20% at best (Table 5.1). Together with previously reported findings (Sato et al., 1988; Sato, 1988a; Takahashi et al., 2006), cultivars/genotypes may differ in their response to carbohydrate supply, with the low rates of crown bud formation in the current experiment supporting the hypothesis that sucrose may not be the only factor limiting the morphogenesis of crown buds in ‘Showtime Diva’.

Except for the morphogenesis of crown buds *in vitro*, the effect of sucrose on the growth and development of cuttings, including development of shoots, roots (Table 5.1) and flowering (Appendix IV), demonstrated the important influence of carbohydrates on growth, differentiation, and organogenesis of ‘Showtime Diva’ *in vitro*. Sato’s (1988b) study showed that the enhancement of crown bud formation accompanied an increase of root dry weight. In Experiment One, while the plantlets on both 9% and 12% presented a similar and optimum formation of crown buds and a generally higher root dry weight than other treatments ( $P < 0.05$ ), the dry weight of roots differed significantly ( $P < 0.05$ ) between these two concentrations of sucrose (Table 5.1). This indicated that contrary to Sato’s (1988b) findings, the dry weight of the root may not be directly or closely related to the morphogenesis of crown buds.

It is recognized that carbohydrates may be directly or indirectly involved in regulating the dormancy status of vegetative buds in other plant species (Anderson *et al.*, 2001; Anderson *et al.*, 2005; Chao *et al.*, 2006; Chao & Serpe, 2010). It has been reported in other plant species that high levels of exogenous sucrose seriously restrained germination, seedling development, and carbohydrate metabolism, by restraining the expression of genes involved in energy metabolism such as hexokinase, pyruvate dehydrogenase, citrate synthase, ADP-glucose pyrophosphorylase and glyoxysomal isocitrate lyase (Xu et al., 2010). In contrast, an *in vitro* study focussed on metabolomics in *G. triflora* indicated that a limitation of energy metabolism under phosphate deficiency can induce the formation of crown buds *in vitro* (Takahashi et al., 2012). In the current experiment continuous exposure to a high concentration of sucrose (9%) maintained the dormancy of crown buds formed *in vitro*, however this dormancy was quickly removed once plantlets were

transferred to a medium at a lower concentration of sucrose (3%; Figure 5.3). This may perhaps be similar to findings with kiwifruit (*Actinidia deliciosa*), wherein the accumulation and decline of endogenous carbohydrates coincided with the maintenance and release of dormancy in the meristems of axillary buds *in vivo* (Richardson *et al.*, 2010). Whether there are similar changes of carbohydrates following the transfer of dormancy status in gentian crown buds *in vitro* or *in vivo*, and the possible physiological mechanism underlying it, need further examination. Such investigations in the future will be helpful to understand the role of carbohydrates on dormancy of crown buds in gentian.

In the current study sucrose may not only have served as a carbon source for the morphogenesis of crown buds, but also will have influenced the osmotic potential in the medium and/or plant tissue by changing the concentration of endogenous carbohydrates (Brown *et al.*, 1979; Paiva Neto & Otoni, 2003). As evident in the current experiment (Figure 5.4), increasing sucrose concentration in the medium definitely increased the total concentration of endogenous carbohydrates. This was consistent with previous *in vitro* studies of other plant species such as *Lilium* (Zhao *et al.*, 2010) and *Hosta* (Gollagunta *et al.*, 2004). Hence whether the influence of sucrose on crown bud formation *in vitro* is solely contributed by its role as a carbon source and/or as an osmoticum, deserves further investigation. A suggested research strategy that could be employed in determining this would be the use of osmo-regulators *in vitro*, such as PEG, mannitol or sorbitol (Attree *et al.*, 1991; Sawwan *et al.*, 2000; Paiva Neto & Otoni, 2003; Hongbo *et al.*, 2005) to change osmotic potential independently of the availability of carbohydrates that can be metabolized by gentian plants. In the current experiment the proportion of plantlets developing crown buds and the number of crown buds per plantlet increased with the rise of sucrose concentration in the medium from 6% to 12%, and yet declined when sucrose concentration increased to between 15% and 20% (Table 5.2). This indicated that if osmotic potential is an important factor, the morphogenesis of crown buds needs an appropriate range of osmotic potential. Support for this hypothesis was that cell differentiation *in vitro* was found to be effected by cellular osmotic potential in *Zinnia elegans* L. (Twumasi *et al.*, 2010). Hence the internal osmotic potential in plant tissue, and it's response to external osmotic potential in the medium, also deserve investigation in future experiments.

Different carbohydrates may have different effects on morphogenesis (Petersen et al., 1999; Paiva Neto & Otoni, 2003; Gemas & Bessa, 2006) with different mechanism (Smeeckens, 2000; Teng et al., 2005). For example, in Guinean cashews (*Anacardium occidentale* L.), maltose increased the number of developing shoots *in vitro*, whereas, fructose enhanced the shoot length (Gemas & Bessa, 2006). In gentians, specific sugars, i.e. gentianose and gentiobiose exist (Keller & Wiemken, 1982), so it will be interesting to learn whether these unique carbohydrates have specific influences on the morphogenesis of crown buds or other aspects such as growth, flowering, or resistance to adverse conditions (Tanaka *et al.*, 2004; Takahashi *et al.*, 2006). In the current experiments, the composition of endogenous carbohydrates in both roots and shoots of ‘Showtime Diva’ *in vitro* (Figure 5.4) was similar to that of 03/04114 *in vivo* (Figure 4.4), wherein sucrose and gentianose were dominant, and mainly contributed to the changes of endogenous TNC either via changes of sucrose in the medium (Figure 5.4) or defoliation (Figure 4.4). As endogenous carbohydrates therefore, sucrose and/or gentianose may be more relevant to the morphogenesis of crown buds, and also the difference of growth in ‘Showtime Diva’ in response to different concentrations of sucrose in the medium. On the other hand, carbohydrate metabolism is highly complex, comprising formation of numerous metabolites, breakdown and inter-conversions (Bryant et al., 1999), so carbohydrate concentration is only the balance of accumulation and consumption, which does not reflect the dynamics of carbohydrate metabolism, i.e. how much has been either synthesized or degraded. Extending upon this logic, it is recognised that carbohydrate metabolism in plants is dependent upon the activity of many enzymes. In other plant systems study associated with the carbohydrate-active enzymes (CAZY) has provided additional insight into the role of carbohydrates in physiological functions (Henrissat et al., 2001). Relevant research as to the activity of enzymes hydrolyzing some carbohydrates in gentian plants was therefore explored further in Chapter 7.

In addition, while L-bornesitol was also found in both shoots and roots of ‘Showtime Diva’ *in vitro* (Figure 5.4), no significant changes in concentration were evident from the various sucrose treatments, potentially indicating that L-bornesitol may not be a dominant factor influencing the morphogenesis of crown buds.

As evident in many studies, carbohydrates as signalling compounds, have a phytohormone-like function involving all stages of the plant’s life cycle (Smeeckens, 2000;

Gemas & Bessa, 2006). It has been suggested that in another perennial plant, *Euphorbia esula* L. (leafy spurge), that carbohydrate-signalling may play an important role in regulating the growth and development in underground adventitious buds by enhancing the gene expression of hexokinase and sucrose synthase (Anderson *et al.*, 2005). The net result of this gene expression was an increase in the influx of sugar into these buds. Carbohydrate-signalling pathways closely interact with other signalling networks, especially phytohormones. For example, analysis of the *Arabidopsis*-gin-1 mutant found that glucose signalling, through hexokinase (HXK) and gypsy retrotransposon integrase 1 (GIN1), was involved in ethylene-signalling pathways (Zhou *et al.*, 1998; Iglesias *et al.*, 2006). So far, hexokinase signalling and sucrose-specific signalling pathways, mainly on glucose and sucrose signals, have been proposed (Smeekens, 2000; Rolland *et al.*, 2002; Rook & Bevan, 2003). As one of the unique carbohydrates existing in gentians, gentiobiose has been suggested as playing a role as signals, e.g. gentiobiose was reported as hastening the initiation of ripening in tomatoes, which could be compatible with a proposed intercellular signalling role (Dumville & Fry, 2003). Also in the bacterium *Acetobacter xylinum*, gentiobiose induced endoglucanase gene (cmcax) expression, which plays a pivotal role in the cellulose production system (Kawano *et al.*, 2008). In gentians, little information has been published about the effects of sugars on development of crown buds. The current experiment has shown that while carbohydrates play an important role in the morphogenesis of crown buds *in vitro*, it is still not clear if the carbohydrates work as signals, nor how these signals are transduced.

Photoperiod and temperature are the environmental signals utilized by many perennial plant systems to adapt growth and development to a suitable growing season and escape adverse environmental conditions associated with winter in temperate zones (Jackson, 2008). Photoperiod and temperature have been reported as affecting the morphogenesis of plants *in vitro* (Ronse *et al.*, 1997; Sengar *et al.*, 2011). Based on Sato's (Sato, 1988b) technical report, a relatively lower temperature (i.e. 15 °C and 20 °C) and longer photoperiod (18 h) promoted the morphogenesis of crown buds than higher temperature (25 °C) and shorter photoperiod (12 h). However, in the current experiment, within the range of temperatures between 15 to 25 °C, there was no significant effect of temperature on the formation of crown buds, and also no interaction between temperature and photoperiod (Table 5.3). While a long photoperiod (18 h) in the current experiment resulted in an increase in the formation of crown buds, that was only significant at the 10%

confidence interval, and only by approximately 0.3 crown buds per plant and 20% of plants forming them (Figures 5.5 and 5.6). Using the same cultivar as in the current experiment, i.e. ‘Showtime Diva’, Samarakoon (2012) has recently reported that *in vivo*, short photoperiods (natural progressive decline in day length between autumn and spring, i.e. 9.18 h to 11 h) increased the number of crown buds per plant by twice that achieved under a long photoperiod (2 h night break lighting). Except for the different experimental system (i.e. *in vitro* cf. *in vivo*), another potential explanation for the apparent conflict of results is that the photoperiod may regulate the partitioning of photo-assimilate, which in turn increased the formation of crown buds in this region. For example, it was reported that a short photoperiod could increase carbohydrate allocation to the underground organs (Shillo & Halevy, 1981; Wallace et al., 1993). If also applicable in gentians, the availability of carbohydrates affected by the photoperiod may be one factor influencing the results in Samarakoon’s (2012) study. The analysis of carbohydrate composition within roots and shoots of plantlets under different photoperiodic regimes might provide some useful information to understand the effect of photoperiod on the partitioning of carbohydrates, and in turn influencing the formation of crown buds. In the current experiment *in vitro*, a sufficient supply of carbohydrates, i.e. high sucrose concentration of 9% (262.9 mM) in the medium, may remove a carbohydrate limitation. If so, the results presented here therefore, may support the hypothesis that the long photoperiod acted as a signal, enhancing the formation of crown buds, possibly by directly and/or indirectly regulating genes involved in the morphogenesis of crown buds (Smeekens, 2000; Beveridge et al., 2003; Rolland et al., 2006).

Given the discussion above, only limited influence was achieved by carbohydrates (sucrose), photoperiod and temperature, on the formation of crown buds *in vitro*. Potentially therefore, other factors may influence the morphogenesis of crown buds. It has been recognized that ethylene can either enhance (Vanaartrijk *et al.*, 1985; Kumar *et al.*, 1998; Dias *et al.*, 2010; Liu & Chang, 2011), or inhibit (Sankhla *et al.*, 1995; Reis *et al.*, 2003; Marino *et al.*, 2008; Dias *et al.*, 2010) morphogenesis of either axillary or adventitious buds in some plant species *in vitro*. Morgan and Debenham (unpublished results) reported that the application of ethephon at a concentration of 100 ppm active ingredient, after plants of ‘Showtime Diva’ from tissue culture were established *in vivo*, resulted in 100% of plants forming crown buds, compared to less than 60% of plants in the control treatment. Similarly in the current study *in vitro*, the application of ethephon with

high sucrose concentration (9%) was approximately 5 times more effective in forming crown buds as axillary buds than that achieved under high sucrose concentration (9%) alone (Figure 5.7 and Table 5.2); however, the mechanism that ethylene (ethephon) prompt crown bud formation needs further study in the future.

Since only simultaneous application of both ethephon and high sucrose concentration (9%; 262.9 mM) applied together, resulted in a significant optimization of the morphogenesis of swollen and dormant crown buds, it is reasonable to suggest that ethephon enhanced the differentiation of axillary buds/shoots, while the main role of high sucrose concentration appears to be involved in establishing and maintaining a dormant status within these axillary buds/shoots, i.e. crown buds. This differential role was illustrated by the fact that without high sucrose concentration, ethephon alone only resulted in an increase in the number of axillary shoots.

While the effects of ethylene on plant morphogenesis *in vitro* have been well documented, the mechanism of ethylene action is not well understood (Dolan, 1997). Pua and Lee (1995), using the antisense-RNA approach to expressing the antisense ACC (1-aminocyclopropane-1-carboxylic acid) oxidase gene, developed transgenic plants of *Brassica juncea* L. (mustard), and demonstrated a genetic basis for the regulation of shoot morphogenesis by the direct correlation between ethylene production and the capacity for shoot regeneration *in vitro*. In addition, isolation of mutants in *Arabidopsis thaliana* with different ethylene responses, has led to the identification of receptors and several downstream components in the ethylene signalling pathway (Bleecker & Kende, 2000; Bisson & Groth, 2010). The use of mutants as an experimental system may powerfully advance the understanding of the mechanism of plant morphogenesis in the future and may, in the future therefore, provide new insights to understanding the morphogenesis of crown buds in gentians.

While Sato (1988b) used media amended with 0.01 ppm NAA when studying the effect of carbohydrate on the morphogenesis of crown buds, in the current study, in order to simplify the assessment of carbohydrate supply on crown bud development, no plant growth regulators were added to the BM in Experiment One. In Experiment Three, while NAA (0.01 ppm) improved the rooting of plantlets, this concentration of NAA did not significantly affect the formation of crown buds *in vitro*, even in the presence of 9% sucrose (Section 5.3.3.1). This suggested that NAA may not be an important factor

influencing the formation of crown buds, but it is acknowledged the range of treatments evaluated was limited.

## **5.5 Conclusion**

The results from the current series of experiments indicate that the morphogenesis of crown buds in ‘Showtime Diva’ *in vitro* is significantly influenced by sucrose supply, ethephon, photoperiod and their interaction. It was apparent that for crown buds to occur *in vitro* that an adequate supply of carbohydrates is imperative for the formation of a swollen structure and, establishment and maintenance of a dormant physiological status. Ethephon (ethylene) played a key role involved in enhancing the organogenesis of axillary buds/shoots. However, to fully understand the morphogenesis of crown buds in gentians, future studies that use molecular technologies, investigating relevant environmental factors, signalling and gene expression, may provide valuable mechanistic insights.

## **Chapter 6 Seasonal changes of non-structural carbohydrates in gentians**

### **6.1 Introduction**

Plant species possess elaborate combinations of morphological, physiological, and behavioural traits for adapting to specific environmental and stress factors (Nilsen & Orcutt, 1996a). In this regard the gentians used for cut flower production are typical of perennial geophytes native to alpine and temperate climatic regions (Ohwi, 1965; Ho & Liu, 2001). Being a herbaceous perennial, the growth cycle of gentian plants is controlled seasonally, i.e., normally shoots emerge in spring, flowering in summer/autumn, natural shoot senescence in late autumn, and then dormancy in winter as crowns (Section 2.3.1). The crown is the perennating organ, with storage reserves and crown buds, performing the functions of withstanding the harsh environment of winter, and supporting growth initiation in the subsequent spring (Ohkawa, 1989). Non-structural carbohydrates (NSCs) are the principal organic products of plant photosynthesis, and are used by plants for growth and respiration (Lewis, 1984b). Adequate NSCs reserves are especially important in perennial plants for winter survival, spring re-growth, and surviving other conditions when photosynthesis is not able to provide sufficient photo-assimilate to meet demand (White, 1973). In addition to their use as energy and metabolites, NSCs are also involved in many physiological processes and functions, such as cold hardiness (Palonen, 1999; Patton et al., 2007), bud dormancy (Anderson et al., 2005), morphogenesis (Wyrzykowska et al., 2002), flowering (Corbesier et al., 1998; El-Lithy et al., 2010) and signalling (Rolland et al., 2006). Hence, in the current study, the seasonal changes of the concentration of NSCs were quantified to explore the physiological function of the storage and utilization of NSCs in the growth cycle of gentians.

In commercial production of gentians, the yield and quality of cut flowers are determined by the performance of the flowering shoots. While many factors such as environmental conditions and plant hormones can influence the development of flowering shoots of gentian (Ohkawa, 1989), as discussed within Chapters 3 through 5, evidence supports the hypothesis that the availability of NSCs in gentian plants significantly influences the morphogenesis and dormancy of crown buds, the development of shoots and flowers, and winter survival and re-growth in spring. Changes in any of these variables have the

potential to affect the yield and quality of flowering shoots in gentian. Knowledge of changes in the amount and composition of NSCs in the various plant organs will lead to a better understanding as to the functions of NSCs, the dynamics of their supply and demand, how limitations of NSCs supply influence the production of flowering shoots, and possibly identify improvements in the management practices of the gentian crop (Gesch et al., 2007; Walton et al., 2007).

Seasonal changes in the level of NSCs have been reported in many perennial plants such as *Solidago canadensis* L. (Bradbury & Hofstra, 1977), *Oxytropis sericea* Nutt. (Wyka, 1999), *Lactuca sativa* L. (Lee & Sugiyama, 2006), *Trifolium repens* L. (Turner & Pollock, 1998), *Rosa x hybrida* L. (Roca et al., 2008), *Asparagus officinalis* L. (Pressman et al., 1993), *Paeonia lactiflora* Pall. (Walton et al., 2007), and *Euphorbia esula* L. (Gesch et al., 2007). In contrast, however, information as to the composition and seasonal changes of NSCs in gentian is extremely limited. Most published knowledge about NSCs in gentian has come from studies on the storage-roots of *Gentiana lutea* L. (Great Yellow Gentian), a perennial alpine plant with storage roots used for medicinal purposes. It is interesting that two unique sugars, i.e. gentianose and gentiobiose, are found in the storage roots of *G. lutea* (Badenhuizen et al., 1964; Keller & Wiemken, 1982; Rossetti et al., 1984), but their physiological function(s) are unclear. While Keller & Wiemken (1982) suggested that the high concentration of gentianose and sucrose in storage roots might serve as a cryoprotectant to tolerate the cold winter, incomplete data exists on the seasonal changes of NSCs in the annual growth cycle in gentian that might go some way towards supporting this hypothesis. Although the changes of gentianose, gentiobiose and sucrose in the storage roots of *G. lutea* were measured (Bridel, 1911), as evident data covering winter and early spring, or for other organs, was not presented. Within the experiments presented within this chapter therefore, this omission in the knowledge base was planned to be addressed.

In gentian, flowering shoots originate from crown buds, and the number and/or size of crown buds is a key factor determining the yield and quality of the flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon, 2012). The previous defoliation experiment (refer to Chapter 4) and *in vitro* experiments (refer to Chapter 5) have provided partial evidence implying that carbohydrates can limit the number, size, winter survival, and subsequent re-growth of crown buds. It was recognised, however, that the

data for carbohydrates from one moment in time, in these previous experiments, limited the attainment of a more complete understanding of the effect of NSCs supply on the development of crown buds during the complete annual growth cycle. This limitation was therefore addressed within the current series of experiments.

To endure the cold of winter, until the re-growth in the following spring, a period of dormancy is regarded as essential for crown buds. While the onset, duration and depth of bud dormancy can be affected by plant growth regulators, water status and environmental factors such as photoperiod and temperature (Horvath et al., 2003), the seasonal changes in the level of carbohydrates has also been reported as possibly involved in the regulation of vegetative bud dormancy in other plant species (Ho et al., 2001; Anderson et al., 2005; Chao et al., 2006). In support of this hypothesis, in the previous *in vitro* experiments (Chapter 5) a high sucrose concentration (9%) in the medium effectively inhibited the shoot emergence from crown buds. Thus investigation of the seasonal changes of NSCs in crown buds, and/or the fleshy rhizomes and storage roots that crown buds are associated with, would provide more information on what, if any, potential relationship exists between NSC content and morphogenesis, dormancy, and re-growth of crown buds.

Flowering shoots are the organ harvested for cut flower in gentian, and include leaves, stem and florets. It has been recognized that in other plant species, stems are capable of storing substantial quantities of NSCs, enabling fast reproductive growth, and as a buffer for demand for NSCs under adverse situations when photosynthetic capacity is reduced (Slewinski, 2012). NSCs are also known to be important for ensuring the longevity of flowering stems by providing a carbon source for respiration, continuous development of immature flowers (Adachi et al., 2000; Monteiro et al., 2002), decreasing the responsiveness to ethylene (Verlinden & Garcia, 2004) and, maintaining water uptake to retard wilting (Kumar et al., 2008a). It has been reported that patterns of carbohydrate distribution within the stem of ryegrass (*Lolium multiflorum* Lam.) varied with position within the stem and with age, which related to the eventual reproductive development (Griffith, 1992). However, there is no similar information published in terms of the distribution of NSCs along shoots, seasonal changes of NSCs in shoots and their potential relationship with flowering of gentian; factors considered worthy of investigation in the current chapter. While leaves are the primary photosynthetic organs of plants, diurnal fluctuations in solar radiation substantially influence endogenous leaf carbohydrate levels,

and NSCs generally temporarily accumulate during the light and remobilize during the dark period (Sicher et al., 1984). Building upon this knowledge, a recent study showed that diurnal changes in carbohydrate status affect postharvest shelf life of some vegetables (Clarkson et al., 2005) and cut flowers (Rapaka et al., 2007). While Eason *et al* (2004) reported that pulsing cut flowering shoots of gentian with sucrose (5%) improved their postharvest quality, no relationship with diurnal fluctuation in the composition and levels of NSCs was reported. The experiments reported in this chapter therefore, aimed to determine the diurnal changes of NSCs in flowering shoots (including both leaves and floral stem tissue) in gentian.

In the commercial production of gentian for cut flowers, harvesting unavoidably accompanies removing considerable leaf area with the shoots (Figure 2.1). Because leaves are the main source of new photo-assimilate, it is logical to hypothesize that the harvest of flowering shoots reduces the acquisition and accumulation of NSCs, which in turn leads to a negative effect on formation of crown buds (Chapter 4) and the production of floral shoots in the subsequent year. Empirically, gentian growers retain as many shoots as possible, or at least five shoots per plant (Ohteki, 1982), so as to produce and store enough carbohydrates for overwintering and re-growth in spring. Since the most suitable time to remove any shoots remaining after commercial harvests has not previously been clarified, and what if any influence shoot removal at different times has on the accumulation of NSCs in crowns has not been reported, both were evaluated in this chapter.

Because they are rarely found in large amounts in other plant species, gentianose and gentiobiose are considered unique NSCs in gentian. The occurrence of gentianose in significant concentrations in the Gentianaceae is confined to *Gentiana* and *Swertia* (Kandler & Hopf, 1980; Lewis, 1984b). Except for ivy (*Hedera*) and birch (*Betula*) (Kandler & Hopf, 1984) gentiobiose has only been found in low concentrations in plants other than the Gentianaceae (Dumville & Fry, 2003). While these two NSCs have been identified as occurring in gentian (Meyer, 1882; Badenhuizen et al., 1964), due to the very limited physiological and biochemical studies to date, details of their function and metabolism have not been elucidated clearly (refer to Sections 2.5.4 and 2.5.5).

Gentiobiose is a structural sub-component of gentianose (Figure 2.3), and can be obtained from gentianose treated with invertase (Bourquelot & Herissey, 1901). *In vivo*, however, it has not been shown that gentiobiose is one of the products of the hydrolysis of gentianose

in gentian. The relationship between seasonal changes in concentration and distribution of gentiobiose and gentianose in various organs of gentian and their possible physiological function were therefore investigated in this chapter.

L-bornesitol (L-1-O-methyl-myo-inositol;  $C_7H_{14}O_6$ ), a cyclitol of polyols, is a *myo*-inositol methyl derivative found in several plant species (Plouvier, 1963; Ichimura et al., 1999; Ruiz-Matute et al., 2007), including the majority of species in the Gentianaceae (Schilling, 1976). Because polyols can be a major carbon sink, and are metabolised in close association with other carbon metabolites, some studies of carbohydrate metabolism in other plant species have included bornesitol within the pool of NSC (Ichimura & Suto, 1999). L-bornesitol was previously identified and quantified in the gentians used within this thesis (refer to Appendix I and Chapters 4 and 5), being present in all organs sampled. Due to their potential physiological function in carbon metabolism and stress acclimation, polyols have received increasing attention (Merchant & Richter, 2011). Thus within the current study seasonal changes of L-bornesitol in different organs were also monitored, so as to explore their possible physiological role in the growth cycle of gentian. Because of the absence of a pure standard of L-bornesitol for HPLC measurement in the current experiments, the measured values of L-bornesitol concentration were calibrated against fructose and, therefore, expressed as fructose equivalents. As this measured value does not reflect the actual L-bornesitol concentration, L-bornesitol was not included within the calculation of total non-structural carbohydrate (TNC).

Phloem is the primary pathway for carbohydrate transport, particularly long distance transport (Thorne & Giaquinta, 1984). Sucrose has been found to be the main carbohydrate transported in phloem of most plants, but other monosaccharides, oligosaccharides and polyalcohol (sugar alcohols) may also be transported (van Bel & Hess, 2008; Reidel et al., 2009; Rennie & Turgeon, 2009). Despite the presence of unusual carbohydrates, i.e. gentianose, gentiobiose, and L-bornesitol, the forms of carbohydrate(s) transported in the phloem of gentians have not been reported. Despite attempts earlier within this thesis, both the stylet technique and EDTA-induced exudation (Section 2.5.2.2), were unable to be developed with gentian under the conditions of the current studies (Appendix III).

Taken together, to enhance the understanding of physiological functions of NSCs during the annual growth cycle, and their importance to the commercial production of flowering

shoots in gentian, the broad aim in this chapter was to identify and quantify the NSCs, and describe their dynamics during the growth and development in gentian plants. The specific objectives were to:

- Quantify the seasonal changes of NSCs in different organs, including crown buds, floral shoots, rhizomes and storage roots, and explore the possible relationship between NSCs fluctuation and plant growth and development;
- Quantify distribution of NSCs along the length of flowering shoots and determine what if any relationship exists with flower development;
- Quantify what, if any, pattern of diurnal changes of carbohydrates occur in flowering shoots during a day;
- Quantify the effect of the timing of shoot removal on the accumulation of NSCs in crowns, and determine the suitable time to remove the remaining shoots after the period of commercial harvesting has finished.

## **6.2 Materials and methods**

### **6.2.1 Plant material and cultivation**

Clonally propagated plants of the hybrid gentian cultivar ‘Showtime Spotlight’ were established as a commercial crop in spring of 2003 in the open field at Plant & Food Research, Palmerston North, New Zealand (NZ; 40.38° S 175.60° E). Samples were collected from plants during two growing seasons, either after having been established for four years (i.e. 2007–2008 growing season) or for seven years (2010–2011).

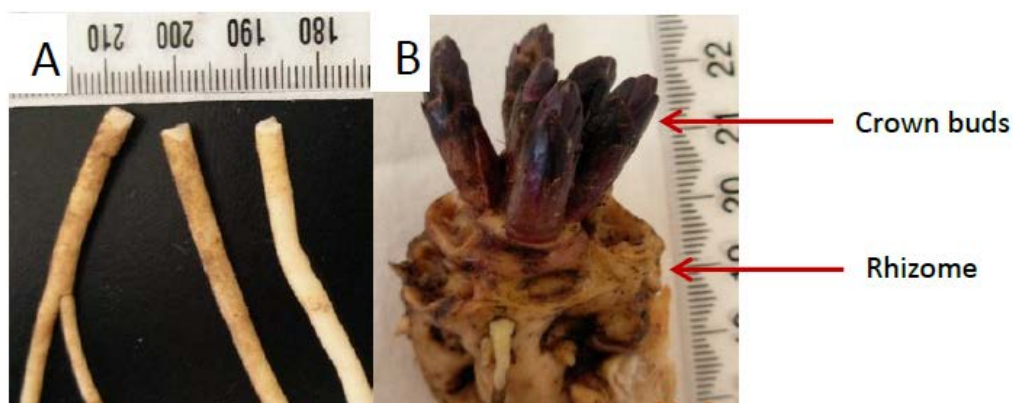
The natural mineral soil within the open field production system was classified as Manawatu fine sandy loam (<http://atlas.massey.ac.nz/soils/profiles/profile.html?P54>). During cultivation fertilisers were applied each season in response to soil analysis, and typically comprised: 15 g/m<sup>2</sup> Slowenne (14N-7P-14K-19S<sub>03</sub>-5Mg-1Fe; Valagro, Atessa, Italy), 2 g/m<sup>2</sup> NPK (12N-5P-15K-S8; Balance Agri-Nutrients; Mount Maunganui, NZ), 2 g/m<sup>2</sup> Sierrablen (21N-1.8P-9K-0.002B-0.005Cu-0.05Fe-0.010Mn-0.002Mo-0.002Zn; Scotts, Heerlen, Netherlands), 1.5 g/m<sup>2</sup> dolomite (21Ca-10Mg; Prebbles Seeds, Christchurch, NZ), 1.5 g/m<sup>2</sup> Gypsum (23Ca-18S; Winstone Gypsum, Auckland, NZ). During the course of the experiment, plants were grown in raised beds with irrigation

supplied by drippers to avoid water stress, and received natural sunlight and photoperiod (9–15 h).

## 6.2.2 Experiments for monitoring NSC

### 6.2.2.1 Seasonal changes

As a preliminary experiment, in the first season of sampling (2007-2008), sample collection started in November (late spring) and continued through until September 2008 (early spring), at two month intervals. On each date samples of shoots, storage-roots and crown buds (Figure 6.1) were taken from three single-plant replicates. During this first experiment rhizome (Figure 6.1B) tissue was not sampled. Shoots were defined as those visibly presenting extended leaves, comprising the apical 100 mm of all shoots, resulting in a mixture of leaf, flower and stem tissue. Crown buds comprised those that had developed in size to between 3 mm and 25 mm in length, and were between 3 mm and 8 mm in diameter. Storage roots comprised those with a diameter between 2 and 5 mm. Immediately following sample collection, samples were washed with tap water, excess moisture removed with paper towels, and the samples were then frozen in liquid nitrogen. All samples were stored at -80 °C until analysis of NSCs.



**Figure 6.1** Plant tissue of ‘Showtime Spotlight’ sampled for analysis of NSC comprised; (A) storage roots with a diameter between 2 to 5 mm and, (B) crown buds arising from the rhizome. In the first experiment (2007-2008 growing season), rhizome tissue was not sampled.

In the subsequent, more detailed, second experiment, sample collection was conducted at one month intervals from October 2010 to July 2011, with three single-plant replicates collected at each date. As data from an interceding experiment (refer to Section 6.2.2.2) in 2009 indicated that the levels of NSCs were significantly different between leaves, floral-

shoot stem tissue and florets, in the second experiment shoots were separated into leaves, florets and floral-shoot stem tissue. In addition, because this interceding experiment (refer to Section 6.2.2.2) also showed significant diurnal changes of NSCs in leaves, sample collection in the second experiment was conducted at midday to avoid potential errors caused by the different sampling time on each sampling day. As noted above, in addition to crown buds and storage roots, samples of rhizome tissue were also collected in the second experiment.

When collecting samples for NSCs measurements, shoot lengths were recorded, and the phenological growth stages of the plants were classified as either; shoot emergence, flowering, or shoot senescence. Additionally, basic air temperature data were collected during both experiments monitoring seasonal changes in NSCs.

#### **6.2.2.2 Diurnal changes**

In late February 2009 (26<sup>th</sup> Feb), sample collection was carried out over a 24 h period on a sunny day when the greatest number of floral shoots was at horticultural harvest maturity. On the sampling day, the light period was defined as the period between sunrise and sunset (from 6:50 am to 7:46 pm, 26<sup>th</sup> Feb, New Zealand summer Daylight saving time); the dark period between sunset and sunrise (from 7:46 pm (26<sup>th</sup> Feb) to 6:50 am (27<sup>th</sup> Feb), New Zealand summer daylight saving time). At each sampling time, leaf and floral-shoot stem tissue were collected from three, single, floral-shoot replicates at optimal harvest maturity, i.e. the apical floret fully pigmented but tightly closed (Eason et al., 2004). Each replicated floral-shoot was randomly selected from the commercial plantings described above (refer to Section 6.2.1). Leaf samples were excised from the third and fourth nodal positions down from the apex, at three-hour intervals between 8:00 am and 8:00 am on the following day. Floral-shoot stem samples were taken from the corresponding third and fourth nodal position from the apex. Immediately upon collection, samples were frozen in liquid nitrogen, and stored at -80°C until NSC were analysed.

#### **6.2.2.3 Changes with position along the flowering shoot**

Samples were collected from three single-plant replicates at optimal harvest maturity (refer to Section 6.2.2.2). Floral shoots were randomly selected from the same commercial plantings noted above (refer to Section 6.2.2.1). In late February 2009, leaf and floral-shoot stem samples were harvested from the upper, middle, and lower positions of

flowering shoots (i.e., between, and including, the second and fourth nodes from the apex, the seventh and eighth nodes, as well as the twelfth and fourteenth nodes, respectively). Collected samples were immediately frozen in liquid nitrogen, and stored at -80°C until NSC were analysed.

#### **6.2.2.4 Changes with the timing of shoot removal**

Commencing after the normal commercial harvest of flowering shoots was completed for the season (2007-2008), the remaining shoots were completely removed at ground level at two-week intervals from March to June 2008. At each date of removal three single-plant replicates were randomly selected for analysis from the same commercial plantings noted above (refer to Section 6.2.2.1). In August 2008, prior to shoot emergence, storage roots (2-5 mm diameter) and crown buds were collected, washed with tap water, surface dried with dry paper towels, and then immediately frozen in liquid nitrogen. All samples were stored at -80 °C until analysis of NSC. The number of calendar days from the end of commercial harvest of flowering shoots was used for timing of the removal of shoots.

#### **6.2.3 NSC analysis**

Individual NSCs were identified and quantified utilising HPLC, following the method described in Section 4.2.7. This data was also used to calculate TNC per unit fresh weight (FW) for each organ, excluding L-bornesitol.

The sample for identifying L-bornesitol was extracted from the leaves of 'Showtime Spotlight' using 62.5% methanol (refer to Section 4.2.7) and purified by HPLC on a Prevail™ carbohydrate column using acetonitrile and water as a mobile phase. Unidentified L-bornesitol was collected during the retention time between 8.0 and 8.7 minutes (refer to Appendix I), and the collected sample was dried using a centrifugal vacuum concentrator (High Capacity Savant SpeedVac® plus SC210A, HiTechTader, USA). The dry sample was sent to Dunedin: Plant Extracts Research Unit, Plant & Food Research NZ, and analysed for compound identification by John van Klink (refer to Appendix I). Briefly, the sample was first dried and flushed with dry nitrogen to remove any residual solvent. For NMR analysis the sample was dissolved in D<sub>2</sub>O and run on a Varian AR shielded 500 MHz instrument. In addition to <sup>1</sup>H and <sup>13</sup>C NMR analyses, a suite of 2D experiments (COSY, NOESY, HSQC and HMBC) was undertaken to determine the structure of the compound (refer to Appendix I).

#### **6.2.4 Statistical analysis**

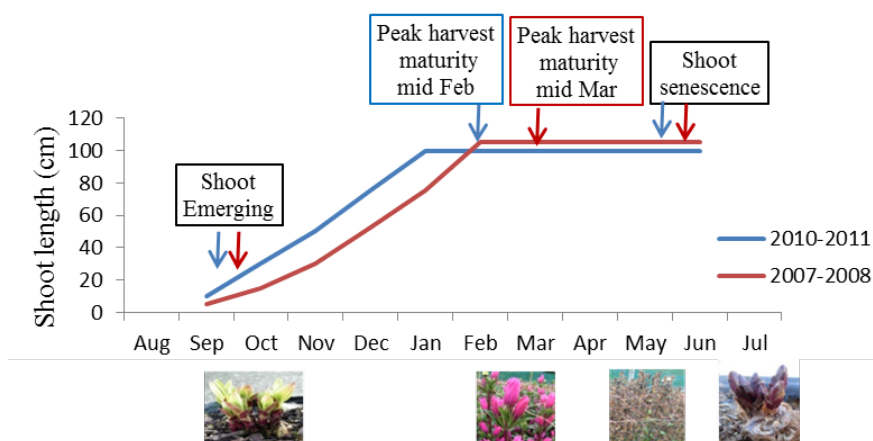
All experiments were conducted as a completely randomised design. The relationships between the concentration of NSCs and the timing of shoot removal were determined using regression analyses; all data were subjected to an analysis of variance utilising GLM (Minitab 16). Each value was expressed as the mean of three replicates  $\pm$  standard error (SE). When significant differences occurred, mean values were separated using the Tukey method ( $P < 0.05$ ).

### **6.3 Results**

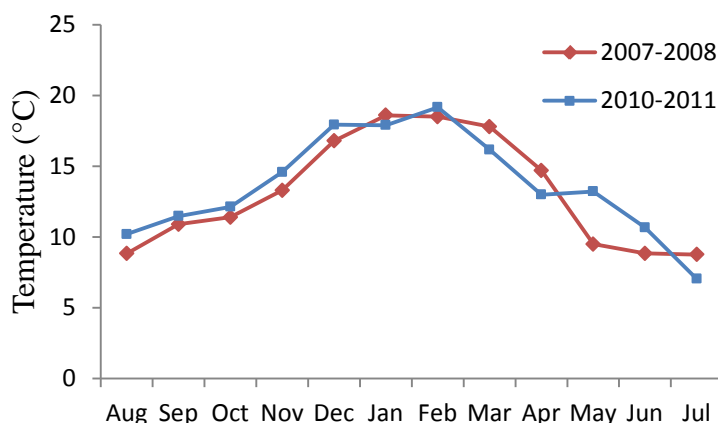
#### **6.3.1 Seasonal phenology**

In both the 2007-2008 and 2010-2011 growing seasons, ‘Showtime Spotlight’ presented a similar pattern of crop phenology, i.e. shoots emerging in spring (September), flowering in late summer/ early autumn (February to March), natural shoot senescence in autumn/ early winter (April to June), and then dormancy in winter (July to August) as crowns (Figure 6.2). Compared to the 2007-2008 growth season, however, floral shoots grew faster in the 2010-2011 growth season and reached harvest maturity approximately one month earlier (Figure 6.2). Comparing the two experiments, daily average air temperature of 2010-2011 were:

- approximately 1.0 °C higher on average than in 2007-2008, during the early growth season from August to November;
- 1.6 °C lower than in 2007-2008, between March and April;
- 2.8 °C higher than in 2007-2008, between May and Jun (Figure 6.3).



**Figure 6.2** Seasonal growth and phenological development of ‘Showtime Spotlight’ during the 2007 – 2008 and 2010 – 2011 growing seasons.



**Figure 6.3** Daily average air temperatures, as averages of each month, during the experimental periods for two growing seasons at Plant & Food Research, NZ. Data are from the weather station located at 40.38° S 175.61° E (NIWA, Palmerston North).

### 6.3.2 Seasonal changes of NSCs

As an overview of the measurement of NSCs in ‘Showtime Spotlight’, over the two growth cycles, gentianose, gentiobiose, sucrose, glucose, fructose and L-bornesitol occurred in all organs and at all sampling dates (if organ present). On average, the concentration of TNCs was highest in storage roots (approximately 100 mg g<sup>-1</sup> FW), followed by rhizomes (45 mg g<sup>-1</sup> FW), dormant crown buds in winter (45 mg g<sup>-1</sup> FW), and floral-shoot stems (35 mg g<sup>-1</sup> FW), while the lowest concentrations were found in leaves (25 mg g<sup>-1</sup> FW). The forms of most abundant NSCs differed between organs, e.g., through the annual growth season, gentianose was dominant in storage roots (Figure 6.4),

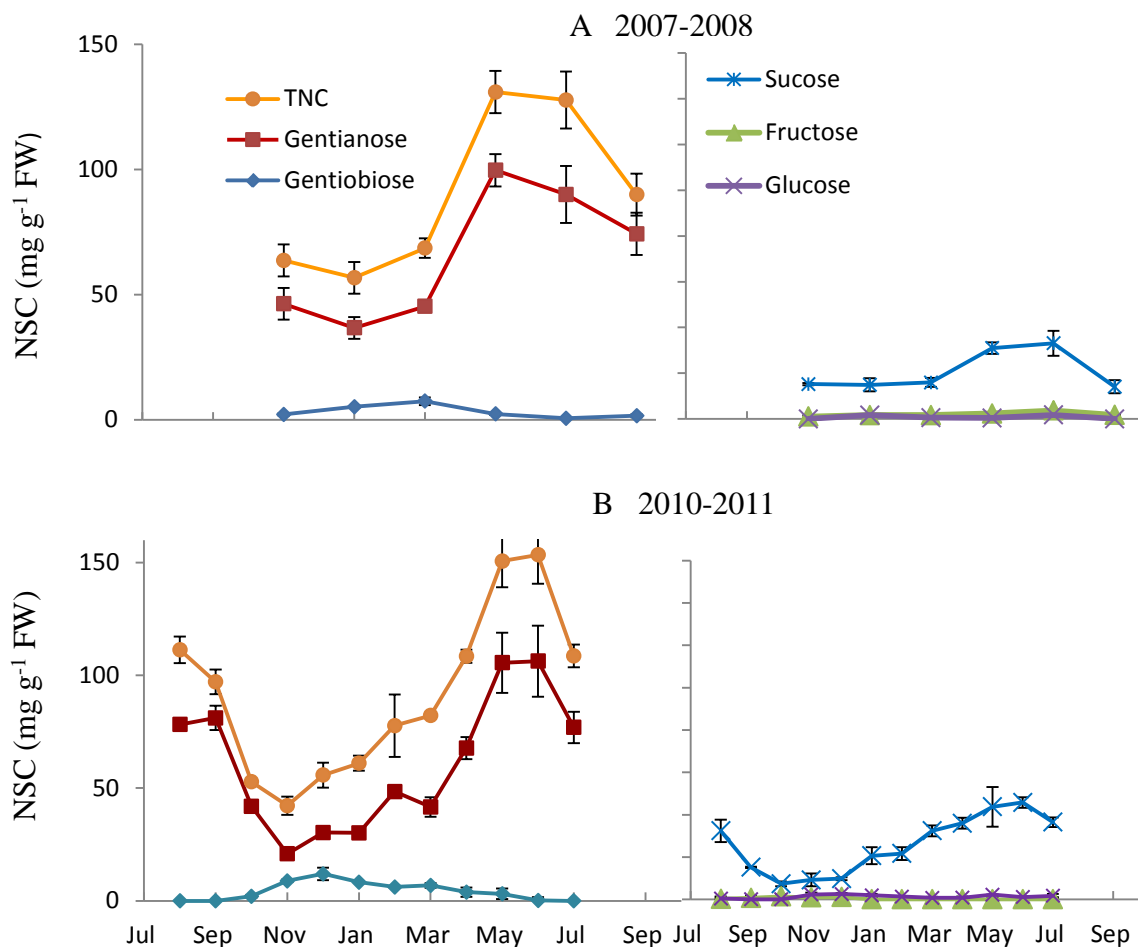
gentiobiose was dominant in the floral-shoot stem (Figure 6.7B), sucrose was dominant in leaves (Figure 6.7C) and crown buds (Figure 6.6) and, gentianose and sucrose were equally dominant in rhizomes (Figure 6.5). As described in the following sections, the concentration of some of these NSCs significantly fluctuated over the growing season.

### **6.3.2.1 Storage roots**

In both years the most abundant NSC in storage roots was gentianose, followed by sucrose. Gentianose alone accounted for between 50% and 75% of TNC in storage roots throughout the annual growth season and, together with sucrose, accounted for more than 90% of TNC in storage roots.

The concentration of TNC exhibited significant seasonal changes ( $P < 0.001$ ), following a similar pattern in both years, i.e., declining from winter through spring, reaching their lowest in early summer (e.g. TNC: 40-60 mg g<sup>-1</sup> FW), then gradually increasing three-fold in concentration to reach their highest levels at the end of autumn (e.g. TNC: approximately 150 mg g<sup>-1</sup> FW; Figure 6.4A and B). Gentianose and sucrose both presented similar patterns of seasonal change to that evident for TNC (Figure 6.4A and B). In contrast, the concentration of gentiobiose, in both experiments, increased during spring to reach a peak in summer, and then dropped during autumn to reach nearly zero in winter. In contrast to the significant fluctuation of gentianose, sucrose and gentiobiose, the concentrations of fructose and glucose showed little change ( $P > 0.05$ ) through the different seasons in both experiments, remaining at a concentration of less than 4.0 mg g<sup>-1</sup> FW throughout (Figure 6.4A and B).

While no samples were taken in December during the 2007-2008 growth season, a difference could be found in the pattern of NSCs changes between the two experiments, i.e., the concentrations of gentianose and sucrose reached their lowest point early during the 2010-2011 season (i.e. November) compared to January within the 2007-2008 growth season (Figure 6.4A and B). Similarly the peak of gentiobiose concentration occurred early in December during the 2010-2011 growth season, compared with the peak occurring in March during the 2007-2008 growth season (Figure 6.4A and B).



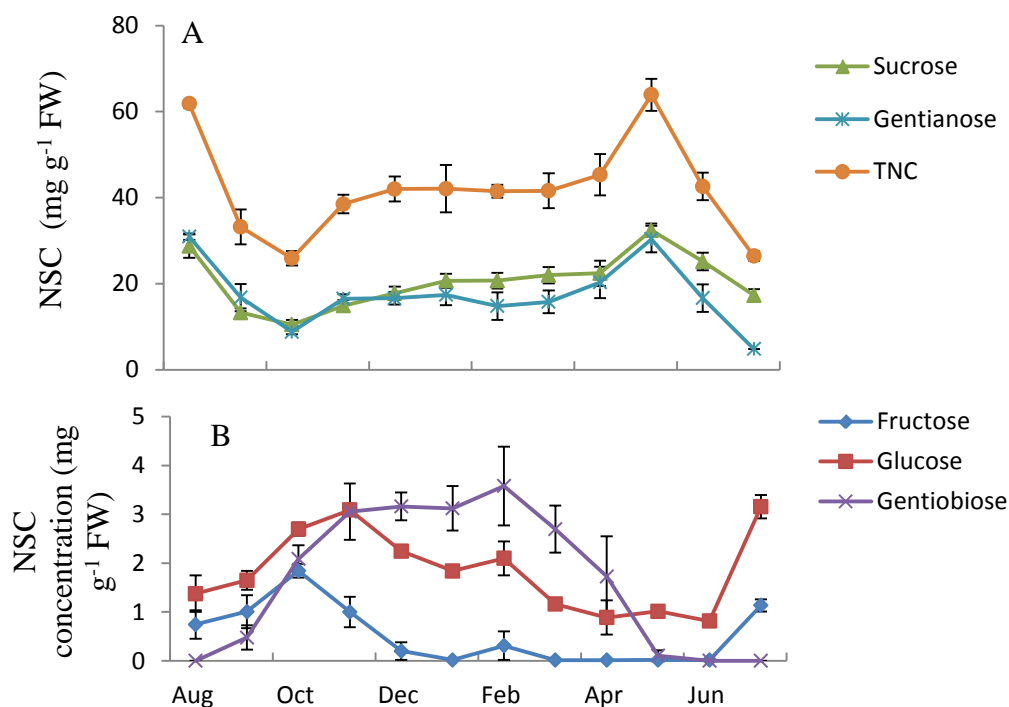
**Figure 6.4** Seasonal changes in the concentration of individual non-structural carbohydrates (NSCs) and total non-structural carbohydrates (TNC) in storage roots of ‘Showtime Spotlight’ during; (A) 2007-2008 and, (B) 2010-2011, growing seasons. Vertical lines =  $\pm$  SE.

### 6.3.2.2 Rhizomes

In the rhizome, gentianose and sucrose were the most abundant NSCs, with similar concentrations of each found at most dates of sampling, ranging between 5.0 and 30.0 mg g<sup>-1</sup> FW (Figure 6.5A). Together, gentianose and sucrose accounted for more than 80% of TNC in the rhizome.

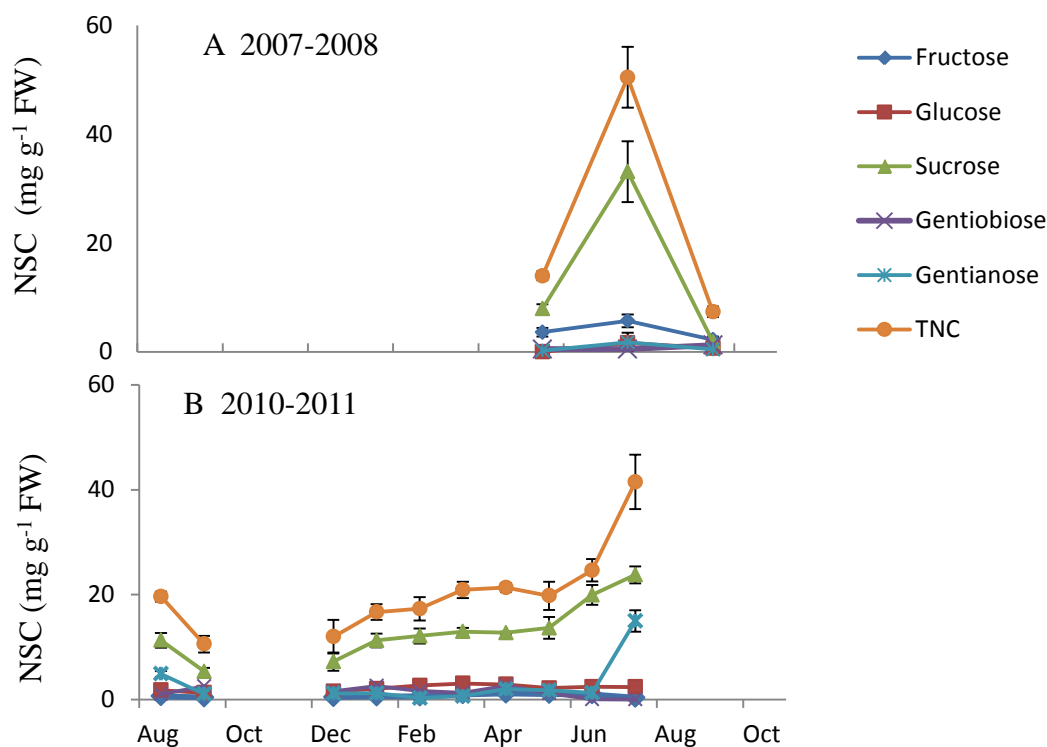
The concentration of TNC, gentianose and sucrose exhibited significant seasonal changes ( $P < 0.001$ ), each following a similar pattern of change. For instance TNC; declined more than two-fold through early spring from August (62.0 mg g<sup>-1</sup> FW) to reach its lowest in October (25.0 mg g<sup>-1</sup> FW), gradually increased through late spring to reach a plateau in summer (approximately 40 mg g<sup>-1</sup> FW), before increasing again in autumn to peak

(approximately  $60 \text{ mg g}^{-1} \text{ FW}$ ) in May. Subsequently the concentration of TNC declined to approximately  $25 \text{ mg g}^{-1} \text{ FW}$  by the middle of winter in July. Of particular note was that in August the concentration of TNC, gentianose, and sucrose, was approximately twice that present in July (Figure 6.5A).



**Figure 6.5** Seasonal changes in the concentration of individual non-structural carbohydrates (NSCs) and total non-structural carbohydrates (TNC) in rhizomes of ‘Showtime Spotlight’ during 2010-2011; (A) TNC, gentianose, and sucrose; (B) gentiobiose, glucose, and fructose. Vertical lines =  $\pm$  SE.

In contrast to the pattern of change evident for the most abundant NSCs (Figure 6.5A), the concentration of the less abundant NSCs (gentiobiose, fructose, and glucose) increased through spring, with glucose and fructose declining gradually through summer and autumn to reach their lowest concentration of  $1.0 \text{ mg g}^{-1} \text{ FW}$  and less than  $0.1 \text{ mg g}^{-1} \text{ FW}$ , respectively, in June (Figure 6.5B). By July, however, the concentration of both glucose and fructose sharply increased more than three and ten times, respectively. In contrast, gentiobiose was maintained at a concentration of  $3.0$  to  $3.5 \text{ mg g}^{-1} \text{ FW}$  through summer, before declining in autumn to undetectable levels by May and through winter (Figure 6.5B).



**Figure 6.6** Seasonal changes in the concentration of individual non-structural carbohydrates (NSCs) and total non-structural carbohydrates (TNC) in crown buds of ‘Showtime Spotlight’ during; (A) 2007-2008 and, (B) 2010-2011. Vertical lines =  $\pm$  SE.

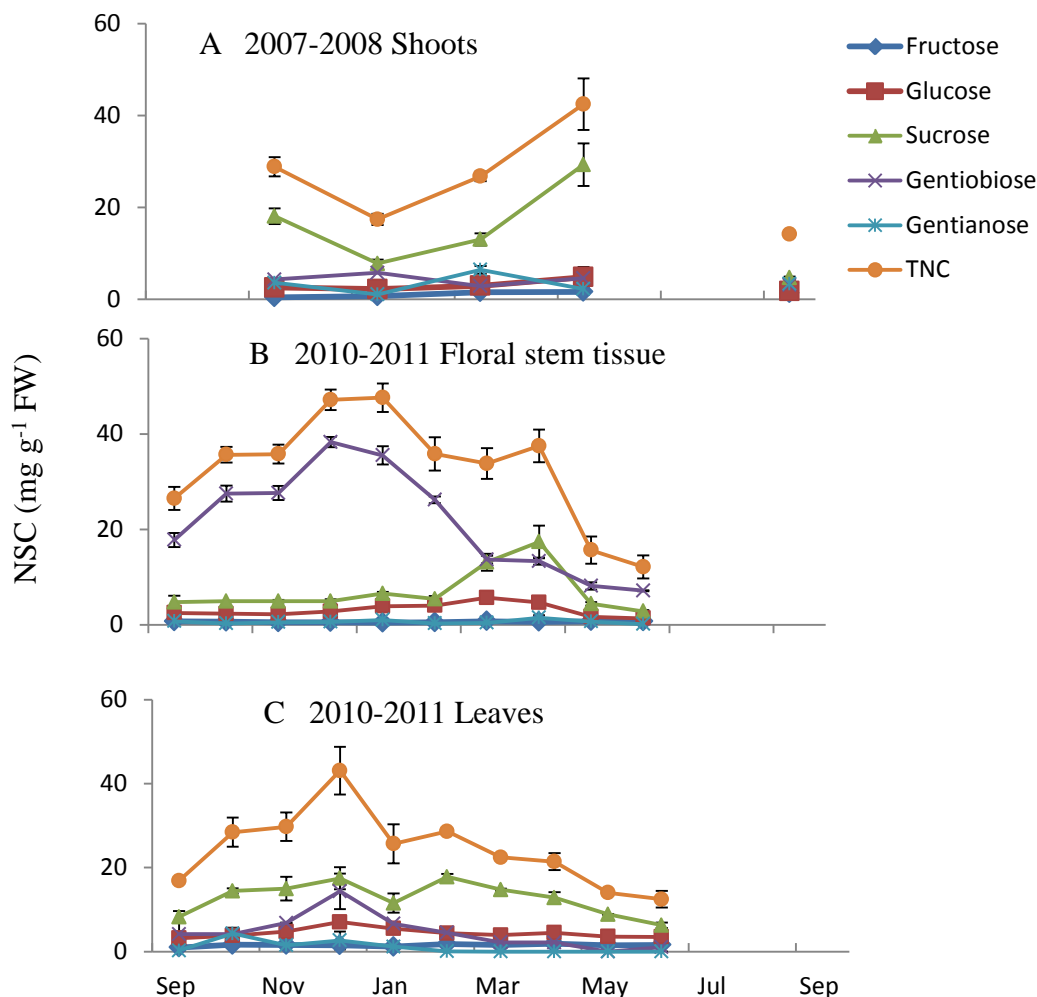
### 6.3.2.3 Crown buds

In crown buds, the most abundant NSC was sucrose, followed by gentianose. Together, both sucrose and gentianose accounted for more than 70% of TNC. In both experiments the concentration of TNC, gentianose and sucrose, exhibited significant changes, each following a similar pattern of a sharp increase during autumn (i.e. May and June) to reach a peak in the middle of winter (i.e. July), before sharply declining in spring ( $P < 0.001$ , Figure 6.6A and B). In contrast, the concentration of the less abundant NSCs of fructose, glucose, and gentiobiose, were consistently low at less than  $3.0 \text{ mg g}^{-1} \text{ FW}$  throughout all seasons in both experiments ( $P > 0.05$  Figure 6.6A and B).

### 6.3.2.4 Floral-shoots

Over the 2007-2008 growing season, sucrose was also the dominant carbohydrate in entire shoots (i.e. mixture of leaves, florets and floral-shoot stem tissue). The concentration of TNC decreased approximately 40% (from  $28.8$  to  $17.7 \text{ mg g}^{-1} \text{ FW}$ ) through spring and summer (November to January), and then increased more than two-fold by May ( $P <$

0.001; Figure 6.7A). In spring (September), when crown buds were just beginning to emerge as shoots, the concentration of TNC in emerging shoots was at its lowest level at  $14.2 \text{ mg g}^{-1} \text{ FW}$  (Figure 6.7A).



**Figure 6.7** Seasonal changes in the concentration of individual non-structural carbohydrates (NSCs) and total non-structural carbohydrates (TNC) in; (A) entire shoots of ‘Showtime Spotlight’ during 2007-2008 growing season and, during the 2010-2011 growing season, (B) floral-shoot stem tissue and, (C) leaves. Vertical lines =  $\pm$  SE.

As evident during the second experiment during 2010-2011, the concentration of TNC in both leaves and stem tissue increased from spring, reached maximum levels in early summer (December) and then gradually decreased through autumn to the lowest level when the stems and leaves died off in the early winter (June). In the floral-shoot stem

tissue gentiobiose was the most abundant NSC, accounting for more than 70% of TNC over spring and summer, and approximately 50% of TNC in autumn. In floral-shoot stem tissue, the concentration of gentiobiose increased two-fold through spring from 18.0 to 38.2 mg g<sup>-1</sup> FW in summer (December), and then gradually decreased over summer and autumn, reaching its lowest level in winter (June) at 7.1 mg g<sup>-1</sup> FW (Figure 6.7B). The concentration of sucrose in floral–shoot stem tissue was maintained at a stable and low level of less than 5.0 mg g<sup>-1</sup> FW, except for a peak in autumn (April) of 17.2 mg g<sup>-1</sup> FW. Fructose and glucose showed little change in this tissue over the different seasons ( $P < 0.05$ ), and both of them were found to be at less than 5 mg g<sup>-1</sup> FW (Figure 6.7B).

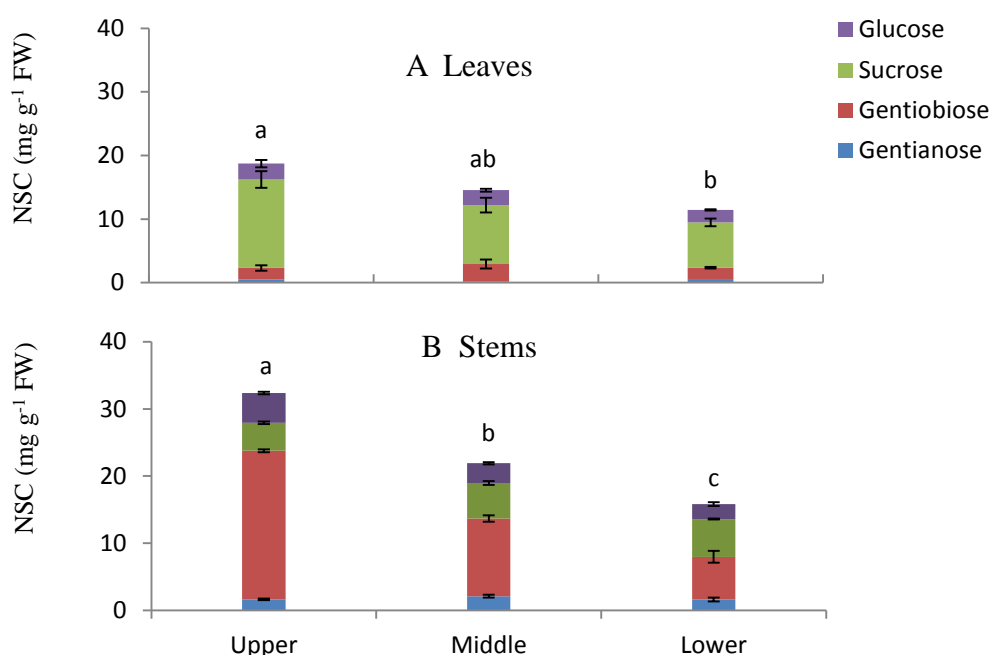
In contrast to the situation evident in the floral-shoot stem tissue (Figure 6.7B), in leaves sucrose was the most abundant NSC, accounting for more than 50% of TNC through spring to autumn. Sucrose concentration increased in early spring, maintained a stable level between 12 to 17 mg g<sup>-1</sup> FW, gradually declining after summer through autumn to reach the lowest level of 6.2 mg g<sup>-1</sup> FW in winter (June; Figure 6.7C). Gentiobiose reached a peak of 14.1 mg g<sup>-1</sup> FW in December in leaves, and was maintained at a low level of less than 5.0 mg g<sup>-1</sup> FW during other periods. Similar to the concentration in floral-shoot stem tissue, the concentration of fructose and glucose in leaf tissue presented little change over the different seasons ( $P < 0.05$ ), with the concentration of fructose never exceeding 2.0 mg g<sup>-1</sup> FW, and glucose always less than 5.0 mg g<sup>-1</sup> FW (Figure 6.7C).

### **6.3.3 Changes in NSCs with position along the floral shoots**

When floral shoots of ‘Showtime Spotlight’ were at optimal harvest maturity, sucrose was the primary carbohydrate in leaves, making up between 58% and 78% of the TNC, depending on the position along the shoot (Figure 6.8A). Gentiobiose was the next most prevalent carbohydrate after sucrose, followed by glucose and gentianose. Fructose was lower than the minimum detectable level.

Moving down the shoots from the upper to lower positions, the concentration of TNC in leaves decreased 18% and 33% at the middle and lower positions, respectively, compared with at the upper position ( $P < 0.05$ ; Figure 6.8A). This was mainly due to the decrease in sucrose concentration, which reduced 36% and 50% at the middle and lower positions, compared to the upper position.

In the stem tissue of floral shoots, gentiobiose was the primary carbohydrate, representing between 45% and 68% of TNC, dependent upon position. Sucrose, glucose and gentianose were the next most abundant carbohydrates, with fructose being undetectable. Along the shoots from top to bottom, TNC concentration declined from 32.5 mg g<sup>-1</sup> FW to half that at 15.6 mg g<sup>-1</sup> FW ( $P < 0.05$ , Figure 6.8B). This was mainly attributed to the reduction in concentration of gentiobiose ( $P < 0.05$ ) which, compared to the upper position, decreased approximately 50% and 73% in the middle and lower positions, respectively. In contrast, other individual NSCs showed little change in concentration ( $P > 0.05$ ) with position.



**Figure 6.8** Concentration of individual non-structural carbohydrates (NSCs) at different positions along the floral shoot of ‘Showtime Spotlight’ at harvest maturity in; (A) leaves and, (B) stems of floral shoots during February 2009. For concentration of total non-structural (TNC) with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines =  $\pm$  SE.

#### 6.3.4 Diurnal changes of NSCs in leaves and stem tissue of floral shoots

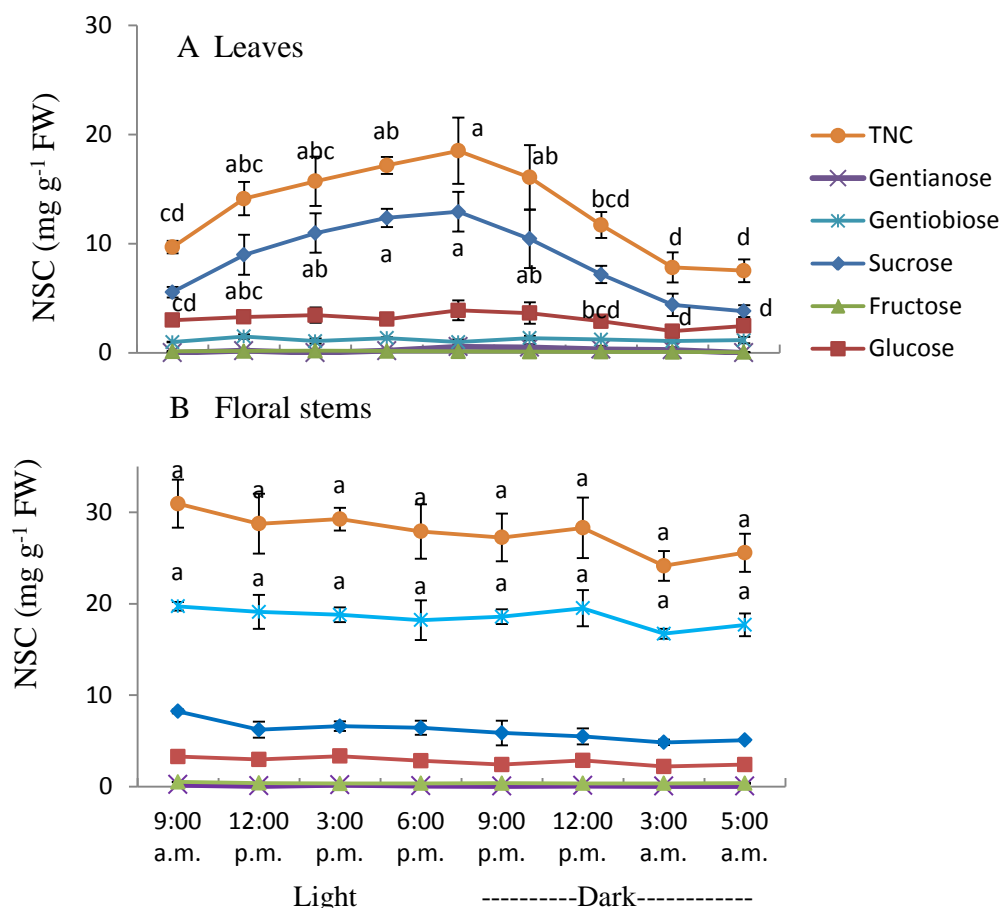
When shoots were at optimal harvest maturity, sucrose was the major NSC in leaves, throughout the 24 hour period (averaging 8.5 mg g<sup>-1</sup> FW), which was more than the total concentration of all other NSCs combined (Figure 6.9A). Glucose was the next most abundant carbohydrate at 3.1 mg g<sup>-1</sup> FW, followed by gentiobiose (1.2 mg g<sup>-1</sup> FW), gentianose (0.3 mg g<sup>-1</sup> FW), and fructose (at only 0.1 mg g<sup>-1</sup> FW).

TNC concentration was significantly affected by the time of sampling during the 24 h period ( $P < 0.001$ ), with differences mainly due to the fluctuation of sucrose concentration (Figure 6.9A). In contrast, the concentration of other NSCs presented little change across the same period ( $P > 0.05$ ). Sucrose concentration increased over the light period (sunrise to sunset: 6:50 am to 7:46 pm, 26<sup>th</sup> Feb 2009, Summer daylight saving time) and reached its highest (13.0 mg g<sup>-1</sup> FW; 70% of TNC) at 8:00 pm (Summer Daylight saving time), being soon after the end of the light period. Subsequently, during the period of darkness (sunset to sunrise: 7:46 pm, 26<sup>th</sup> to 6:50 am, 27<sup>th</sup>), the concentration of sucrose in the leaves declined linearly to its lowest concentration (3.8 mg g<sup>-1</sup> FW; 50% of TNC) in the early morning (8.00 am, summer daylight saving time).

In contrast to the situation in leaves, in stem tissue of floral shoots the TNC and individual NSCs did not show significant changes in concentration over the 24 h cycle ( $P > 0.05$ ; Figure 6.9B). While sucrose was the major NSC in leaves, gentiobiose was the dominant NSC in the floral-shoot stem tissue, averaging 20.0 mg g<sup>-1</sup> FW across the 24 h period, which was approximately twice the concentration of all other NSC in the same tissue (Figure 6.9B). Sucrose was the second most abundant NSC (average 6.5 mg g<sup>-1</sup> FW), followed by glucose (average 2.8 mg g<sup>-1</sup> FW), fructose (average 0.3 mg g<sup>-1</sup> FW), and gentianose (0.1 mg g<sup>-1</sup> FW).

### **6.3.5 Timing of shoot removal**

In both crown buds and storage roots sampled in August, TNC concentration was significantly increased with the delay of shoot removal, i.e. the earlier the shoots were removed, the lower the TNC concentration ( $P < 0.05$ ; Figure 6.10A and B). Compared to March, the TNC concentration for plants with shoots removed in late June, was approximately three-fold greater in crown buds from 7.1 mg g<sup>-1</sup> FW to 21.1 mg g<sup>-1</sup> FW and two-fold greater in storage roots from 58.8 mg g<sup>-1</sup> FW to 118.4 mg g<sup>-1</sup> FW ( $P < 0.05$ ; Figure 6.10A and B). The concentration of both sucrose and gentianose in crown buds followed a similar pattern of change to TNC, and the concentration of gentiobiose, fructose and glucose showed significant changes with the timing of shoot removal.

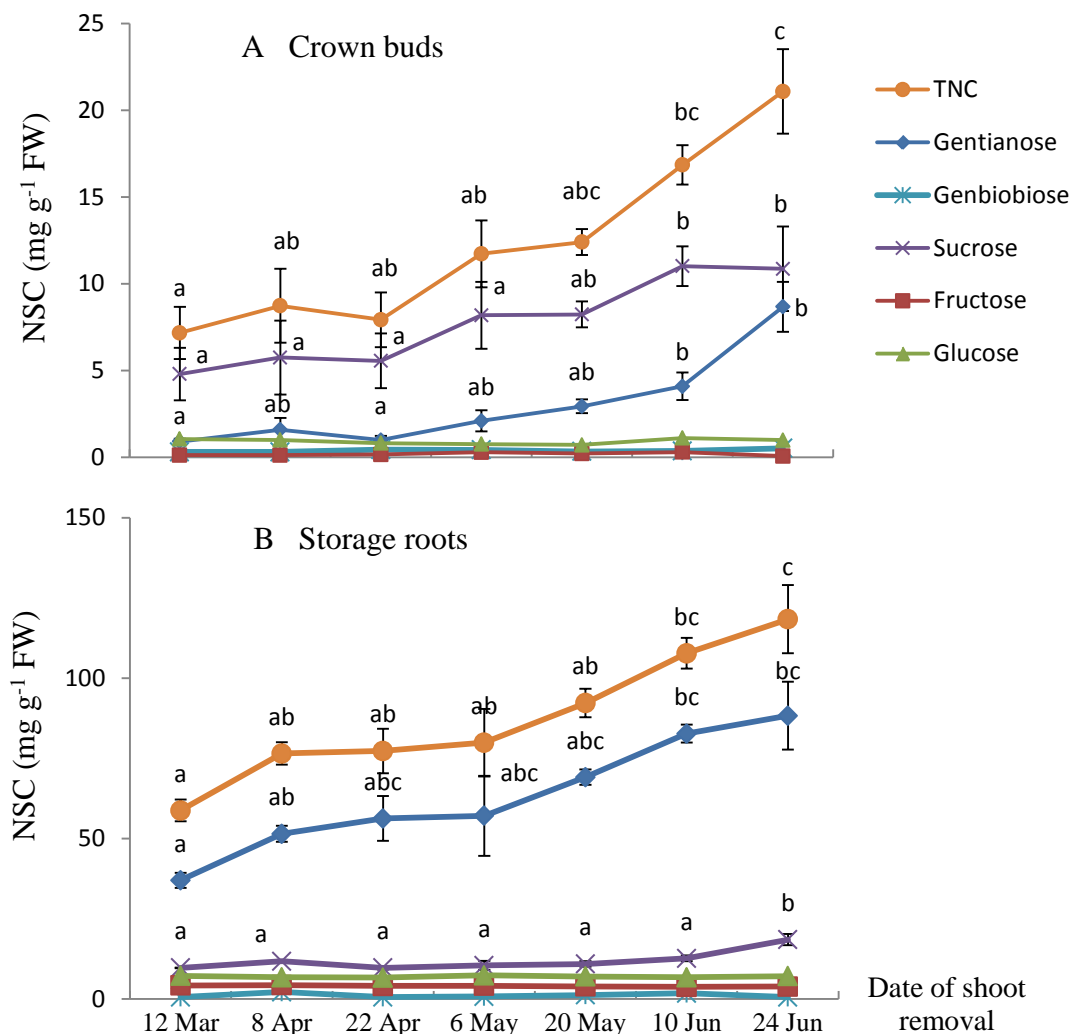


**Figure 6.9** Diurnal changes in the concentration of total non-structural (TNC) and individual non-structural carbohydrates (NSCs) over 24 hours period in; (A) leaves and, (B) floral-stem tissue, at the harvest maturity during February 2009 (summertime). For each variable, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines =  $\pm$  SE. Light period was the period between sunrise and sunset from 6:50 am to 7:46 pm, 26<sup>th</sup> Feb 2009, summer daylight saving time. Dark period was the period between sunset to sunrise from 7:46 pm, 26<sup>th</sup> to 6:50 am, 27<sup>th</sup>, summer daylight saving time.

In storage roots, only gentianose presented a similar increase of concentration, as TNC, with the delay of shoot removal, and sucrose maintained stable at a low concentration of approximately  $10 \text{ mg g}^{-1} \text{ FW}$  before shoots was removed in late of June when sucrose concentration increased to  $15 \text{ mg g}^{-1} \text{ FW}$  ( $P < 0.05$ ; Figure 6.10B). In contrast, other NSCs presented little changes ( $P > 0.05$ ; Figure 6.10B).

Regression analysis illustrated that for crown buds there was no effect of shoot removal on the concentration of any of the individual NSCs up until 22<sup>nd</sup> April. In contrast, from April onwards until 24<sup>th</sup> June, there were positive linear increases between delaying the timing of shoot removal and TNC, gentianose, as well as sucrose ( $P < 0.01$ ; Table 6.1). No

treatment effect of removal date ( $P < 0.05$ ) on the concentration of the less abundant NSCs, i.e., gentiobiose, glucose, and fructose, was evident. For storage roots there were positive linear trends between delaying the timing of shoot removal and TNC ( $P < 0.01$ ), gentianose ( $P < 0.01$ ), and sucrose ( $P < 0.05$ ) through the treatment period from 12<sup>th</sup> March to 24<sup>th</sup> June, while there were no effects ( $P > 0.05$ ) on the less abundant NSC, i.e., gentiobiose, glucose, and fructose (Table 6.2).



**Figure 6.10** For plants of ‘Showtime Spotlight’ concentration of total non-structural carbohydrates (TNC) and individual non-structural carbohydrates (NSCs) in; (A) crown buds and, (B) storage roots, as affected by the timing of shoot removal between March and June 2008. Samples were collected in August 2008. For each variable mean values with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines =  $\pm$  SE.

**Table 6.1 Relationships between the concentration of individual non-structural carbohydrates and total non-structural carbohydrates (TNC)<sup>Z</sup> in storage roots of ‘Showtime Spotlight’ in the middle of the winter (August) and the date<sup>Y</sup> (days from the end of commercial harvest of flowering shoots) when the remaining shoots were removed in the preceding autumn through to early winter (from March to June).**

Concentration (mg g <sup>-1</sup> FW)	Days after harvest the remaining shoots were removed (x)		
	Equation	r <sup>2</sup>	P
TNC	$y = 56.2 + 0.610 x$	0.683	< 0.001 **
Gentianose	$y = 35.2 + 0.590 x$	0.733	< 0.001 **
Sucrose	$y = 8.33 + 0.0777 x$	0.463	0.001 **
Gentiobiose	$y = 1.10 + 0.00018 x$	0.0	0.982 ns
Glucose	$y = 7.05 - 0.00062 x$	0.002	0.870 ns
Fructose	$y = 4.24 - 0.00478 x$	0.154	0.078 ns

<sup>Z</sup> Concentrations for when shoots removed between April and June only. TNC includes gentianose, gentiobiose, sucrose, glucose and fructose.

<sup>Y</sup> The dates (days from the end of commercial harvest of flowering shoots) when the remaining shoots completely removed at ground level at two-week intervals from March to June 2008.

'ns', '\*', and '\*\*' indicate no significance at  $P \leq 0.05$ , significance at  $P \leq 0.05$  and  $P \leq 0.01$ , respectively.

**Table 6.2 Relationships between the concentration of individual non-structural carbohydrates and total non-structural carbohydrates (TNC)<sup>Z</sup> in crown buds of ‘Showtime Spotlight’ in the middle of the winter (August) and the date<sup>Y</sup> (days from the end of commercial harvest of flowering shoots) when the remaining shoots were removed in the preceding autumn through to early winter (from March to June).**

Concentration (mg g <sup>-1</sup> FW)	Days after harvest when the remaining shoots were removed (x)					
	12 Mar to 22 Apr			22 Apr to 24Jun		
	Equation	r <sup>2</sup>	P	Equation	r <sup>2</sup>	P
TNC	$= 9.34 - 0.0037 x$	0.0	0.966 ns	$= 0.77 + 0.220 x$	0.469	0.005 **
Gentianose	$= 0.945 + 0.0105 x$	0.048	0.570 ns	$= -3.44 + 0.119 x$	0.418	0.009 **
Sucrose	$= 6.93 - 0.0117 x$	0.004	0.872 ns	$= 3.00 + 0.096 x$	0.373	0.016 *
Gentiobiose	$= 0.297 + 0.00240 x$	0.194	0.235 ns	$= 0.358 + 0.0009 x$	0.01	0.717 ns
Glucose	$= 1.06 - 0.00542 x$	0.263	0.158 ns	$= 0.576 + 0.0049 x$	0.175	0.120 ns
Fructose	$= 0.102 + 0.00131 x$	0.063	0.516 ns	$= 0.275 - 0.0013 x$	0.054	0.403 ns

<sup>Z</sup> Concentrations for when shoots removed between April and June only. TNC includes gentianose, gentiobiose, sucrose, glucose and fructose.

<sup>Y</sup> The date (days from the end of commercial harvest of flowering shoots) when the remaining shoots completely removed at ground level at two-week intervals from March to June 2008.

'ns', '\*', and '\*\*' indicate no significance at  $P \leq 0.05$ , significance at  $P \leq 0.05$  and  $P \leq 0.01$  respectively.

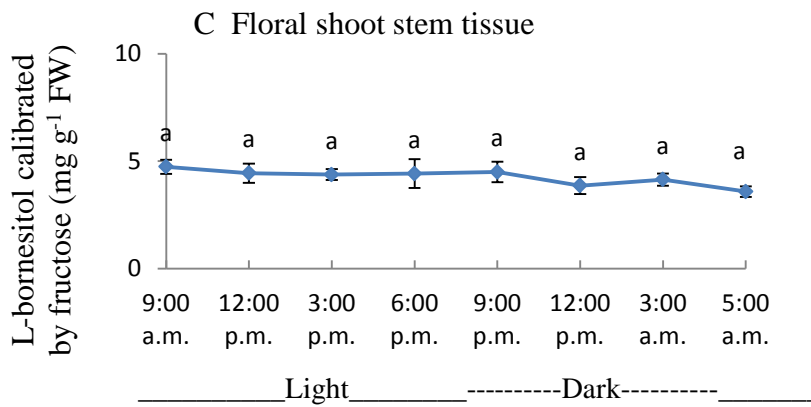
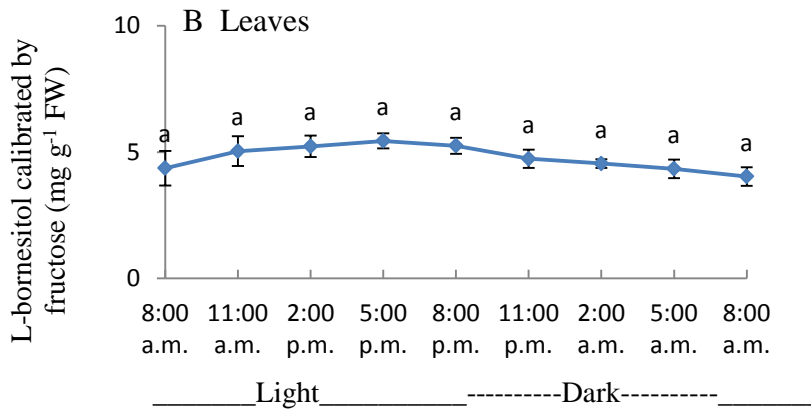
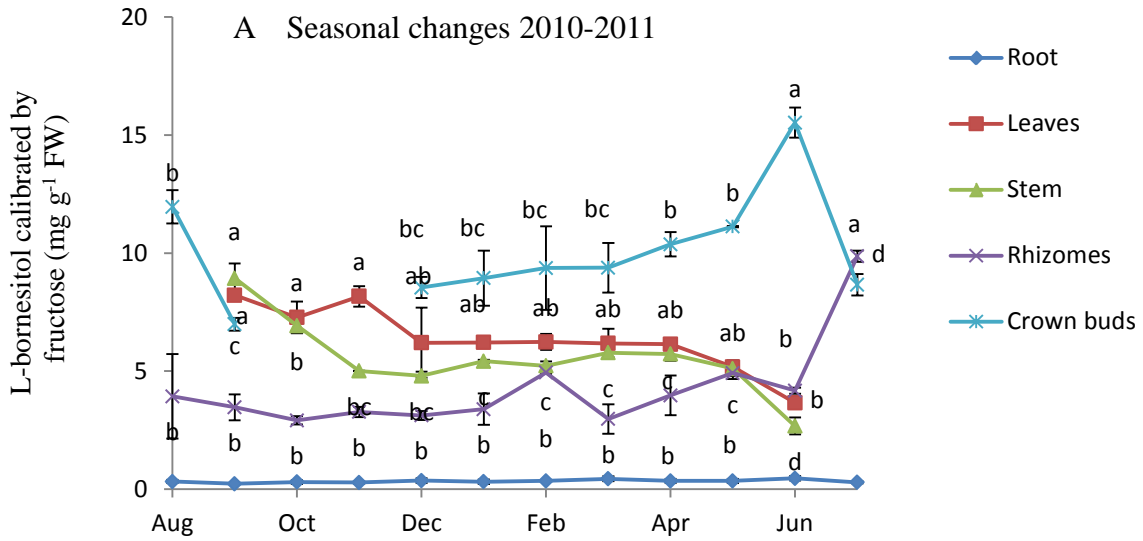
### 6.3.6 L-bornesitol

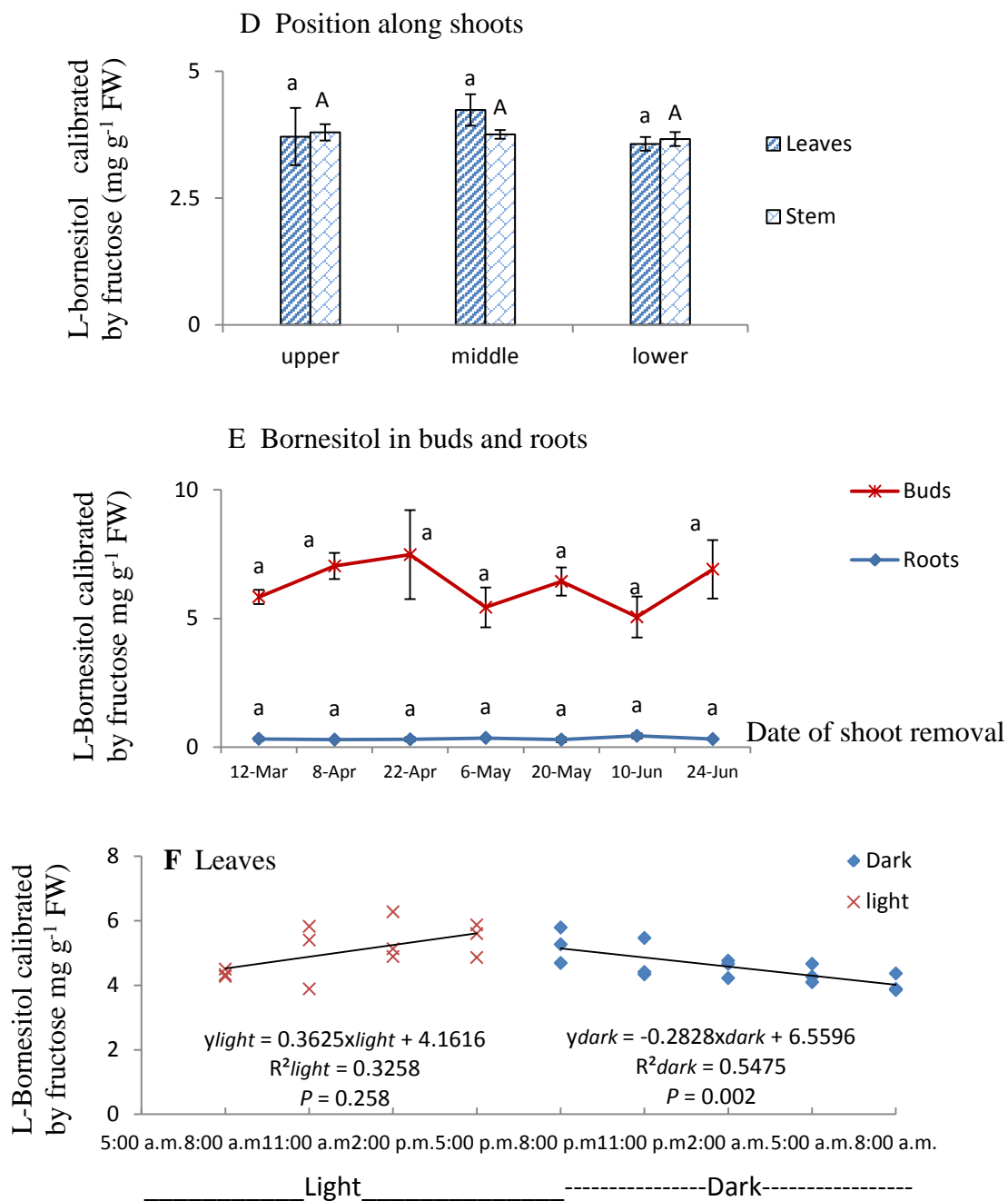
L-bornesitol was distributed through the plant, with concentrations that differed in the different organs. On average the concentration of L-bornesitol was highest in crown buds

(10.2 mg g<sup>-1</sup> FW), followed by leaves and floral-shoot stems (both at 5.0 mg g<sup>-1</sup> FW), rhizomes (4.0 mg g<sup>-1</sup> FW), with the lowest in storage roots at 0.3 mg g<sup>-1</sup> FW ( $P < 0.05$ ; Figure 6.11A).

Over the annual growth cycle in 2010 to 2011, the concentration of L-bornesitol in crown buds decreased more than 40% in spring from 12.0 to 7.0 mg g<sup>-1</sup> FW, before establishing a stable concentration over summer at approximately 9.0 mg g<sup>-1</sup> FW ( $P < 0.05$ ; Figure 6.11A). The concentration subsequently increased 1.6 times over autumn and early winter to reach a peak in June at 15.5 mg g<sup>-1</sup> FW, before decreasing again in winter. The concentration of L-bornesitol in leaves and floral-shoot stems decreased in spring, was stable in summer, and decreased in autumn (Figure 6.11A). In the rhizome, the concentration of L-bornesitol changed little over spring, summer and autumn ( $P > 0.05$ ), but increased more than two-fold (from 4.1 to 9.8 mg g<sup>-1</sup> FW) in winter from June to July (Figure 6.11A).

The concentration of L-bornesitol did not show significant changes during the diurnal cycle in both leaves and stems (Figure 6.11B and C;  $P > 0.05$ ), while linear regression analysis indicated that in leaves there was a significant decrease of L-bornesitol concentration between 8:00 pm to 8:00 am (dark period;  $y = 6.56 - 0.283 x$ ;  $R^2 = 0.548$ ;  $P = 0.002$ ). There was no significant difference with the position along the floral shoot ( $P < 0.05$ ; Figure 6.11D), and even the timing of shoot removal after the completion of flowering, did not influence the concentration of L-bornesitol ( $P < 0.05$ ; Figure 6.11E).





**Figure 6.11** For plants of ‘Showtime Spotlight’, concentration of L-bornesitol (fructose equivalents) in different organs over the 2010 to 2011 growing season; (A) in different organs over the 2010 to 2011 growing season, (B) over a 24 hour period in leaves and, (C) floral-shoot stem tissue, summer daylight saving time in February 2009, (D) at different positions along floral-shoots in February 2009, (E) in crown buds and storage roots in autumn 2008 following different dates of shoot removal, and (F) the regression analysis in the relationships between L-bornesitol and the diurnal cycle over a 24 hour period in leaves (F). For each variable, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines =  $\pm$  SE.

## 6.4 Discussion

NSCs in plants play an important function as a source of energy and metabolites, as well as being involved in essential physiological processes; therefore it is a key area of study for crop production (Farrar, 1999). While numerous studies on NSCs have been conducted in many crops (Lewis, 1984a), the previous literature in terms of NSCs in gentians was very limited. The current series of experiments have for the first time identified and quantified the distribution and seasonal changes of NSCs in gentian plants. Similar to other geophytic plants, the data in this chapter confirm that gentian plants accumulate substantial amounts of NSCs within the crown for overwintering and supporting initial spring re-growth. As discussed above, the concentration of the unique NSCs, i.e., gentianose, gentiobiose, as the most abundant NSCs in various organs of gentian, significantly fluctuated in different organs over the growing season, implying that both gentianose and gentiobiose potentially play important physiological roles related to growth and development, e.g. dormancy, cold tolerance, morphogenesis of crown buds, and the development of floral shoots, and are worth further study.

Geophytes rely on resting buds attached to underground storage organs to survive adverse conditions and initiate new growth in spring. The prerequisite for this growth strategy is sufficient accumulation of reserve substances (primarily carbohydrates) within the underground organs for respiration and demand arising from initial growth in spring. Similar to other geophytes (Bradbury & Hofstra, 1977; Woolley et al., 1999; Orthen & Wehrmeyer, 2004; Walton et al., 2007), including *G. lutea* (Bridel, 1911), the concentration of TNC in rhizomes and storage roots of ‘Showtime Spotlight’ reached their maximum concentration in autumn, declined during winter and spring, followed by gradual replenishment over summer and autumn (Figure 6.4A and B). The point of inflection where the concentration of TNC increased from its lowest, can be interpreted as that point in time when photoassimilate supply started to exceed the demand for growth consumption in January during the 2007-2008 growth cycle, as compared with November in the 2010-2011 growth cycle (Figure 6.4A and B). While the growth cycle evident in both the 2007-2008 and 2010-2011 experiments presented a similar pattern of change in TNC, the lowest concentration of TNC occurred earlier in 2010-2011 than 2007-2008. This difference may have been due to the monthly mean daily temperature in spring during 2010-2011 being 1.0 °C higher than in 2007-2008 (Figure 6.10). As mean

temperature is regarded as the major environmental factor influencing the phenology in geophytes of temperate origin (Rees, 1992; Le Nard & De Hertogh, 1993b), it seems plausible that differences in temperature could account for the differences in timing of changes in NSCs concentration between the two years. This hypothesis is supported by contrasting the phenological calendar, wherein flowering occurred one month early in the 2010-2011 season compared to the 2007-2008 season. In contrast, Samarakoon (Samarakoon et al., 2012b) reported that compared to the outdoor environment in Palmerston North, cultivation of ‘Showtime Spotlight’ in a protected environment (higher mean temperature) resulted in longer shoot length and increased appearance of crown buds, but did not alter the timing of flowering within the current growth season. This contrary result may be because the growth rate of gentian plants only responds to a narrow scale of temperature, i.e., in Samarakoon’s experiments the temperature within the protected environment was higher than the linear temperature range (Arnold, 1959) that gentian plants respond to for changes in growth rate, thus did not affect the timing of flowering. Therefore, how temperature affects the growth and development of gentian plants needs further study, utilising temperature treatments including the linear response range.

In many other perennials the importance of stored NSCs such as starch and fructans etc, for winter respiration, cold tolerance, and early re-growth in spring has been widely recognized (White, 1973; Pressman et al., 1993; Tamura & Moriyama, 2001; Anderson et al., 2005; Walton et al., 2007; Zhang et al., 2011). Keller & Wiemken (1982) suggested that the high concentration of gentianose in the vacuoles of storage roots may have been involved in the cold tolerance for *G. lutea* in alpine areas, although there were no data to clearly support this hypothesis. The results of previous experiments utilising defoliation (refer to Chapter 4) supported the potential role of gentianose (a predominant NSC in storage roots) as a cold protectant and a carbon source, via the highly positive correlations between the concentration of gentianose stored in autumn and both the proportion of plants surviving winter, and re-growth of crown buds in spring. Similarly in the current study, the changing pattern of concentration of gentianose in the rhizome and storage roots of ‘Showtime Spotlight’ (i.e., accumulation during summer and autumn, and decreasing in spring, coordinated with the breakdown of the oligosaccharide, gentianose), logically supports the above hypothesis as to the ecological importance of stored gentianose in underground organs. In order to test these hypotheses, however, plants with differing contents of gentianose in conjunction with cold treatments at various severities would be

required to determine the role of gentianose with cold tolerance. In future experiments, research using labelled carbon for tracking the carbon flux would help to determine if gentianose in the roots and/or rhizomes are remobilized to above ground organs, and could quantify the amount of NSCs used for respiration, or as structural components of re-growth. In addition, cold tolerance would also be affected by other factors. For example, Takahashi et al (2006) identified that some proteins might be related to the cold tolerance and dormancy of crown buds of *G. triflora*. In the current experiments the high level of L-bornesitol concentration found during winter in crown buds and rhizomes (Figure 6.11A), may also be interpreted as relevant to cold tolerance. All these hypotheses, however, require confirmation by further experiments.

In most geophytes studied so far, NSCs stored in underground storage organs are only partially used, i.e., the storage of NSCs is typically greater than the demand for normal plant growth (Wyka, 1999; Kleijn et al., 2005; Walton et al., 2007). The primary hypothesis in this regard is that the unused storage NSC serves as an adaptive buffer, allowing the plant to survive unexpected adverse conditions such as herbivore grazing (Stamp, 2003). In the current two growth cycles of measurement using 'Showtime Spotlight', it was found that at least 30% of the accumulated NSCs (approximately 50 mg g<sup>-1</sup> FW; mainly gentianose) within storage roots, and 40% (approximately 30 mg g<sup>-1</sup> FW; mainly gentianose and sucrose) in rhizomes was not consumed in the normal annual growth cycle and presumably provided this adaptive buffer as long storage NSCs. As evident in the defoliation experiment (refer to Chapter 3), if an adequate leaf canopy was not re-established/maintained, there is a risk of utilizing too much of the reserves within the underground storage structures, potentially resulting in plant mortality. This long-term storage of NSCs, mainly gentianose, can be explained as an evolutionary adaption in the pattern of carbon partitioning in response to adverse conditions, such as grazing (Miller et al., 1999; Huhta et al., 2000).

As discussed within Chapters 3 through to Chapter 5, carbohydrate supply can influence the morphogenesis as well as the dormancy of crown buds; therefore, it is logical to speculate that the concentration of NSC in rhizomes and/or storage roots is able to affect the morphogenesis, dormancy, and re-growth of crown buds. Supporting evidence for this is provided by exogenously supplied sucrose *in vitro* having been reported to increase the number of crown buds both *in vitro* and subsequently in the field after being de-flasked of

*G. triflora* (Sato, 1988a). It was also identified within *in vitro* experiments (refer to Chapter 5) that a high concentration of carbohydrate was a requirement for the formation of crown buds. As the macroscopic formation of crown buds occurred from early summer through autumn (refer to Chapter 3), this period coincides with the increase in concentration of TNC, gentianose and sucrose in storage roots and rhizomes (Figure 6.4A and B). This coordination of timing therefore further supports the hypothesis that the NSCs in storage roots and/or rhizomes may be directly, or indirectly, involved in the morphogenesis of crown buds. The determination of the mechanism of NSCs participating in the morphogenesis of crown buds is particularly complex due to the large number of factors, either physical, chemical or biological, taking part in plant morphogenesis through space and time. Further experiments using molecular technologies, exploring the effect of carbohydrate metabolism on the expression of genes involved in morphogenesis, would greatly contribute to the understanding of the role of NSC in the regulation of the morphogenesis of crown buds (Rolland et al., 2006; Chao & Serpe, 2010; Sairanen et al., 2012).

From an ecological perspective, it is understandable that it is necessary to maintain the dormancy of crown buds during the cold winter, so as to prevent damage to new growth before the arrival of warmer temperatures in spring (Anderson et al., 2005). During summer and autumn, apical dominance may play a key role in maintaining the state of dormancy (paradormancy) of crown buds, while with the senescence of shoots in winter, other internal physiological factor(s) are necessary to regulate dormancy (endodormancy) of crown buds (Lang et al., 1987; Chao et al., 2007). It is recognized that carbohydrates may be directly involved in signal(s) regulating the dormancy status of vegetative buds (Anderson et al., 2001; Anderson et al., 2005; Chao et al., 2006; Chao & Serpe, 2010), or perhaps indirectly via osmotic potential (Green & Jane, 1983; de Fay et al., 2000; Morisset et al., 2012). This hypothesis is supported in part by the concentration of TNC (mainly gentianose) in storage roots during winter, although gradually decreasing, still presented a comparatively high internal concentration (15% FW; 150 mg g<sup>-1</sup> FW; Figure 6.4A and B). In addition, the concentration of TNC (mainly sucrose) in crown buds was also at its highest level (5% FW) in winter, with a sharp decrease in early spring when re-growth started in September (Figure 6.2; Figure 6.6A and B). These patterns of change in TNC in storage roots, rhizomes and crown buds are, therefore, consistent with the changes in dormancy status of crown buds, and support the hypothesis that a high concentration of

NSCs may be involved in maintaining the dormancy of crown buds. In addition, considering the occurrence of the high peak of concentration of sucrose in crown buds in winter when no photosynthesis is producing new assimilate (Figure 6.6A and B), the conversion of carbohydrate forms and remobilisation may be involved in the regulation of dormancy and cold tolerance in crown buds, e.g. sucrose in crown buds may originate from the breakdown of gentianose in storage roots and/or rhizomes, and then move to crown buds. It is also suggested that carbohydrates (mono or oligosaccharides such as glucose, fructose and sucrose) play an important role as signals in regulating vegetative bud dormancy, through regulating gene expression or cross-talking with plant hormones, e.g. inhibiting perception of gibberellic acid and/or, enhancing perception of abscisic acid (Horvath et al., 2003; Anderson et al., 2005; Chao et al., 2006; Chao et al., 2007; Chao & Serpe, 2010). The mechanism of carbohydrates regulation of bud dormancy, however, either as osmotica, signals, and/or through the synthesis of proteins (Takahashi et al., 2006), still needs further study.

The changes in concentration of NSCs in flowering shoots differed between the two experiments (Figure 6.7A to C); primarily this may be due to the differences in the organs sampled (e.g. leaves measured separately or not) and the time of sampling (e.g. sampling in early morning would lead to lower values for the concentration of NSCs). In contrast, sampling later in the day would result in higher concentrations (refer to Section 6.2.2.1). Considering the likely effects arising from changes to sampling procedures, the data reported from 2010-2011 is considered to be more reliable than that from 2007-2008. The results over the 2010 to 2011 growth cycle (Figure 6.8B and C) indicated that the concentration of TNC in both floral-shoot stem tissue and leaves followed similar seasonal changes. The increase in concentration of TNC (mainly gentiobiose) during spring, and its highest concentration achieved in early summer (December), coincided with when initiation of florets were evident in ‘Showtime Spotlight’ under microscopic examination (Samarakoon, 2012), and the continuing rise of temperature (Figure 6.3), which could potentially result in increased photosynthesis (Roca et al., 2005). In contrast, the subsequent decrease in concentration of TNC cannot be interpreted only as being due to the changes in weather conditions, because similar temperatures also occurred during summer through autumn. As an alternative hypothesis for this latter period therefore, it has been reported that temporarily accumulated NSCs in floral-stem tissue and leaves during vegetative growth and/or through to pre-anthesis, can be remobilised for anthesis and

subsequent reproductive growth (Foulkes et al., 2002; Monteiro et al., 2002; Ruuska et al., 2006; Fu et al., 2011; Slewinski, 2012). The development of flowering shoots in gentian, including the extension of shoots and the development of the individual florets are key to determining the pre-harvest quality of the shoot as a cut flower. Hence, it is logical to hypothesize that accumulation of NSCs, particularly gentiobiose, in leaves and floral-shoot stem tissue of ‘Showtime Spotlight’ during spring to early summer, are used for the later development of the flowering shoots. To test this hypothesis, and/or to quantify how much NSCs reserves are remobilized to the florets, further studies using a radioactive carbon tracer could be utilised (Cliquet et al., 1990; Woolley et al., 1999). In addition, the patterns and possible roles of the accumulation and utilization of NSCs during the development of individual florets have been explored in this thesis (refer to Chapter 7).

In terms of postharvest quality, NSCs are widely known to provide a source of carbon for respiration, inhibiting responsiveness to ethylene, and maintaining water uptake (Adachi et al., 2000; Monteiro et al., 2002; Verlinden & Garcia, 2004; Ekman & Worrall, 2005). Similar to other cut flower crops, the function of NSCs in improving the postharvest quality of flowering shoots of gentian has been reported using sucrose pulsing treatments (Zhang & Leung, 2001; Eason et al., 2004; Eason et al., 2007); however, until the current study the internal NSCs in stems and leaves of floral shoots was not reported. In order to maximise the postharvest display life, in commercial production of gentian, flowering shoots are normally harvested when the apical floret is fully pigmented but tightly closed (Eason et al., 2004), i.e. a substantial proportion of the florets are still undeveloped buds. Therefore, abundant gentiobiose accumulated at this pre-anthesis period (Figure 6.7B and C), is likely to become an important carbohydrate source for the continued development of these florets after harvest. To better understand the role of gentiobiose in the postharvest quality of gentian, further investigation is required to examine changes of gentiobiose in leaf and stem tissue of flowering shoots under various postharvest conditions (Bieleski et al., 1992). In support of the hypothesis that such carbohydrates may be involved, it has been found that the vase life of cut flowers in *Helianthus annuus* and *Lilium* was correlated with carbohydrate status under different preharvest conditions, including light and temperature. In particular, if the recognised roles of carbohydrates in improving postharvest quality of cut flowers, as discussed above, are also applicable in gentian, the production practices of plants to increase NSCs reserves, particularly gentiobiose, in the

floral-shoot stem tissue and leaves, should contribute to the improved postharvest quality of flowering shoots in gentian.

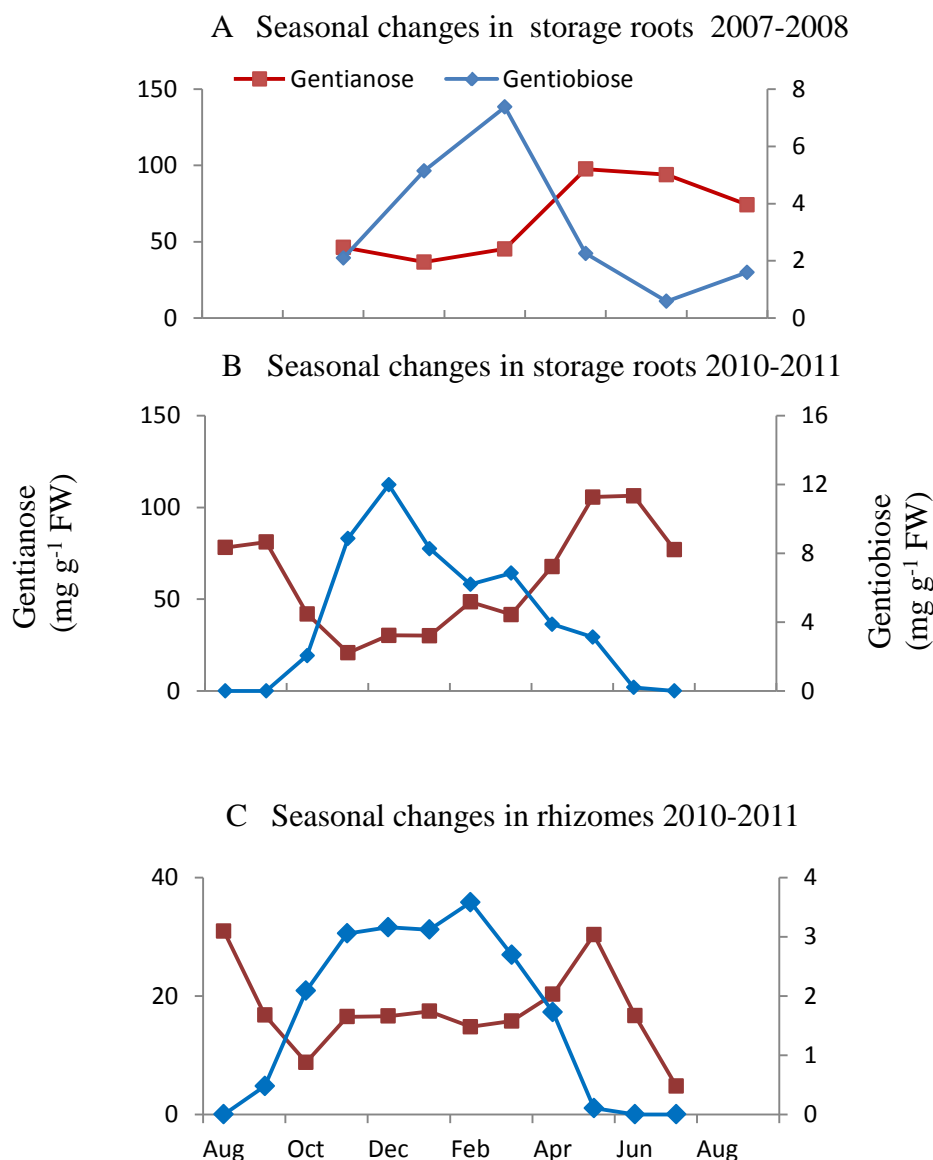
It is well known that NSC accumulates in leaves of some plant species during the light period when photo-assimilate production exceeds utilization (Sicher et al., 1984; Morin et al., 2011). In the present study, the concentration of TNC in leaves demonstrated a clear diurnal pattern when shoots were at harvest maturity, i.e. TNC reached its maximum at sunset (8:00 pm; summer daylight savings time NZ; Figure 6.8A). In ‘Showtime Spotlight’, this increase of TNC was mainly due to an increase in sucrose concentration during daylight hours (Figure 6.8 A), being similar therefore to the result reported in barley (*Hordeum vulgare* L.) (Zeeman et al., 2007). Although the TNC concentration (mainly gentiobiose) was as much as three-fold higher in floral-shoot stem tissue than leaves, there was little diurnal change in the concentration of TNC within the floral-shoot stem tissue, nor the individual NSCs (Figure 6.8 B). In order to maximise the concentration of NSCs within floral shoots at harvest, the best time during the day to harvest is clearly at the end of day, i.e., at sunset. While current commercial production practices emphasize the desire to harvest cut flowers early in the morning when plant water status is high and field heat is low (Halevy & Mayak, 2011 ), the current results lead to the inference that the harvest of flowering shoots of gentians later in the day should be considered. To confirm that this increased content of sucrose does in fact result in an improved postharvest performance for gentian, further experiments are required to examine the vase life and/or the sensitivity to ethylene of flowering shoots when harvested at different times during the day (Rapaka et al., 2007).

Depending on the position along the flowering shoot, the TNC concentration either in leaves or floral-shoot stem tissue decreased 33% and 50% respectively from upper to lower segments (Figure 6.8A and B;  $P < 0.05$ ). This may be due to the distribution and density of the leaf area within the canopy, which determines the light environment influencing photosynthesis, and/or the age of leaves influencing their photosynthetic capacity (Bradbury & Hofstra, 1977; Xie & Luo, 2003). Another possible explanation is that the distribution of NSCs may be related to the competition for carbon between different positions along the shoot, i.e. due to their closer physical proximity to developing florets, the upper segments of the shoots have stronger competency than the lower segments. For example, it has been demonstrated in Italian ryegrass (*Lolium multiflorum*

Lam.) that the quantity of carbon assimilate translocated to the inflorescence within the upper segments of shoots, was considerably greater than that exported to rhizomes underground (Bradbury & Hofstra, 1977). The pattern of NSCs distribution along flowering shoots, i.e., upper segments store more NSCs, may result in the stored NSCs being more easily transported to florets due to their close physical distance, and thereby benefiting the maintenance of postharvest longevity of flowering shoots.

In the current study, where shoot removal was applied, there was a significant reduction in reserves of gentianose within storage roots, and both gentianose and sucrose within crown buds (Figure 6.9A and B). This was consistent with the result reported in the defoliation experiment (refer to Chapter 4). As evident within the current experiment, a reduction in concentration of NSCs resulted from complete shoot removal at any time up until June, i.e. at the end of the annual growth cycle when shoots were naturally senescing (Figure 6.7A and B). In order to achieve maximum NSCs reserves in storage roots and crown buds therefore, allowing the natural senescence of shoots is the optimum strategy. However, within a commercial context, to determine how much/many shoots, should be left on the plants when harvesting, requires further experiments to compare the effect of different severities of harvest on NSCs reserves, development of crown buds, and subsequent commercial yields.

Carbohydrates are typically stored as polysaccharides or oligosaccharides (Lewis, 1984a). Many studies on storage carbohydrates and their seasonal changes in perennial crops have typically focused on starch (Palonen, 1999; Bolding et al., 2000; Kamenetsky et al., 2003; Gesch et al., 2007), fructan (Pressman et al., 1993; Tamura & Moriyama, 2001; Orthen & Wehrmeyer, 2004; Imahori et al., 2010), or raffinose (Bachmann et al., 1994; Peterbauer & Richter, 2001). In the absence of each of these forms of carbohydrate (Section 4.3.2), in the present study with ‘Showtime Spotlight’ gentianose (trisaccharide) was the predominant storage NSC in both rhizomes and storage roots (accounting for 50% to 70% of TNC) through the annual growth cycle. As explored further within the following paragraphs, the predominance of gentianose supports the proposal that gentianose should also be identified as a member of the group of potential storage carbohydrates in plants, as it appears to play a similar role in gentians as starch, fructan or raffinose do in other species.



**Figure 6.12 Reverse relationships of concentration changes between gentianose and gentiobiose during seasonal changes in storage roots of ‘Showtime Spotlight’ during; (A) 2007-2008 season, (B) 2010-2011 season and, (C) in rhizomes during the 2010-2011 season.**

Storage carbohydrates normally occur as poly- or oligo-saccharides, and are synthesised from monosaccharides such as glucose and/or fructose by enzymes, and stored in specific organs (Lewis, 1984b; Thorne & Giaquinta, 1984). When needed, they are cleaved by enzymes into simple carbohydrates or monosaccharides, relocated and further metabolised to produce energy or involved in other metabolic activities. When monosaccharides are not immediately needed, they are often converted to more space-efficient forms, i.e., polysaccharides; these interconversions between various forms of carbohydrates effectively meet the demand of plants during their growth and development (Lewis, 1984b;

Thorne & Giaquinta, 1984). In the various forms of NSCs identified in gentian during the current experiments, fructose and glucose were present at low concentrations, with little changes over seasons (Figures 6.4 – 6.10). This is interpreted as indicating that fructose and glucose are directly involved in metabolic activities rather than existing as storage carbohydrates. In contrast, gentianose, an oligosaccharide, is accumulated and degraded with seasonal changes in gentians, illustrating its role as the main storage carbohydrate, providing the most likely carbon resource for growth in spring by being degraded into monosaccharides such as fructose and/or glucose. In the current experiments it was also interesting to find that there was a reverse trend of changes in concentration between gentianose and gentiobiose within storage roots (Figure 6.12A and B) and rhizomes (Figure 6.12C). If consideration is given to the chemical structure of gentianose (Figure 2.3), i.e. gentiobiose is a structural sub-component of gentianose, this reversal in changes of concentration between gentianose and gentiobiose is hypothesised to imply that gentiobiose may either be the intermediate metabolite in the synthesis of gentianose, or the product of hydrolysis of gentianose. It has been reported that gentianose treated with invertase *in vitro* produced gentiobiose (Bourquelot & Herissey, 1901). *In vivo*, however, it has not been confirmed that gentiobiose is one of the products of the hydrolysis of gentianose in gentians or any other species. To examine this hypothesis, therefore, further experiments to identify the products of hydrolysis of gentianose using enzyme extracts in gentians were conducted and discussed in this thesis (refer to Chapter 8).

Investigations into the function of polyols such as myo-inositol and L-bornesitol, in terms of their contribution to carbon metabolism and stress resistance, appear in recent literature (Merchant & Richter, 2011). L-bornesitol has been detected in several plant families (Plouvier, 1963) including the Gentianaceae (Schilling, 1976; Ichimura et al., 1999), but the distribution as well as both diurnal and seasonal changes have not previously been reported in gentians. In the current experiments, owing to its existence in all organs of ‘Showtime Spotlight’ (Figure 6.11 and Figure 7.6), L-bornesitol may have physiological functions at a whole-plant level in gentians. During the diurnal cycle, the concentration of L-bornesitol presented relatively high levels in leaves during the day and decreased during night, while remaining stable in floral-shoot stem tissue. This may be interpreted as meaning that L-bornesitol is produced in leaves and transported through stems to other organs such as crown buds, where L-bornesitol is accumulated during autumn. Whether

similar pattern of changes in L-bornesitol concentration in crown buds occurs is a topic for future investigation.

The concentration of L-bornesitol being present at higher levels in crown buds and rhizomes in autumn/winter (Figure 6.11A), highlights the potential value of investigating whether such changes might be related to cold tolerance in gentians. While the physiological roles of L-bornesitol remain unclear, some reports suggested that it may serve as an osmoregulator (Ichimura et al., 1999; Ichimura & Suto, 1999) and, therefore, be implicated with cold tolerance and/or organ morphogenesis in gentians. In addition, it has been reported that galactinol, a galactosyl derivative of *myo*-inositol, acts as a galactosyl donor in the synthesis of raffinose (Joersbo et al., 1999). If so, it seems reasonable to speculate that as a derivative of *myo*-inositol, L-bornesitol might be involved in the synthesis/hydrolysis of carbohydrates in gentians via a similar process. Combining the above discussion, L-bornesitol as such may be directly involved in cold tolerance, or indirectly through taking part in the metabolism of other NSCs in gentians. The specific physiological function of L-bornesitol and its role in carbohydrate metabolism in gentians, however, is still worth further experiments to be explored in a future study.

It is important to note that while in the present study the net concentration of extracted NSCs reported in this chapter has provided valuable information in terms of their storage and utilization, this is only part of the knowledge-base required. Details of the dynamics of the synthesis and hydrolysis of NSCs as well as how much is used for respiration, storage or re-growth, even flux, cellular or subcellular distribution, and/or the potential for rapid change, have not yet been reported. Consequently, using other protocols such as full carbon budgets, radioactively labelled carbon to measure the pattern of NSC translocation, and/or determining the respiration rate of component organs during the growth cycle, will result in a more complete picture of the partitioning and the functions of NSCs (White, 1973; Bradbury & Hofstra, 1977). In addition, almost all chemical reactions in a biological cell are catalysed by enzymes. The investigation of carbohydrate-related enzymes therefore, particularly in terms of the unique and less studied gentianose and gentiobiose, is likely to improve our understanding of the process of carbohydrate metabolism and its regulation at a molecular level in gentians. The experiments presented within Chapter 8, therefore, were undertaken with this in mind.

## **6.5 Conclusions**

Gentian ‘Showtime Spotlight’ showed significant fluctuation in the concentrations of various NSCs during annual its growth cycle. This reflected the changes between storage and utilization and also indicated potential physiological functions of NSCs in the growth and development of gentian. Gentianose, as the main storage NSC in storage roots, plays an important role for over-wintering and spring re-growth. The pattern of diurnal changes of NSCs in shoots demonstrated that, harvesting of flowering shoots late in the day should be preferred in commercial production. To achieve maximum NSCs reserves in crowns at the end of growth season, natural die back of shoots is the optimum option for ensuring successful overwintering and re-growth. The reciprocal relationship between concentration of gentianose and gentiobiose implied a potential relationship in their metabolism, and is worthy of further study. The current study, however, is still a long way away from providing a complete carbon budget for gentian plants; this could be achieved by further studies such as those simultaneously monitoring biomass, carbon mobilization by radioactive labelling techniques, and enzyme activities.

## **Chapter 7 Changes of non-structural carbohydrates during floret development in ‘Showtime Spotlight’**

### **7.1 Introduction**

In floriculture, flower development and senescence are critical to the visual appearance, vase-life and, therefore, quality of cut flowers. Flowering is a complex and well-defined procedure, involving responses to environmental factors, hormonal regulation, cell elongation or expansion, a wide spectrum of chemical metabolism and gene expression (van Doorn & van Meeteren, 2003). The actual mechanisms of flower opening also differ between the various genera, species, and even cultivars. Progressing toward a full understanding of the procedure of flower development potentially requires a combination of detailed anatomical, physiological, biochemical, and molecular studies. Extending from the preceding study, this chapter continued to focus on the changes in metabolism of non-structural carbohydrates (NSCs), but by exploring their possible physiological function in the development of florets in gentians.

Carbohydrates are essential for the growth of all parts of the plant as both the main structural components and an energy source. Flowers, as an organ undergoing rapid growth, require large amounts of carbohydrates to meet the demands of growth, while flowers as such conduct no, or very little, photosynthesis (Werk & Ehleringer, 1983). As a result, therefore, the supply of carbohydrates for flower development depends on their importation from carbohydrate sources within the plant, especially leaves. Commercially cut flowers are often harvested before full development and move to postharvest environments where photosynthesis is extremely limited, and a deficiency of carbohydrate supply may lead to a decrease of vase-life and the failure of flower bud opening (van der Meulen-Muisers et al., 2001). Such failures of subsequent opening of flower buds may be particularly evident in those cut flower commodities comprising inflorescence-type structures, where the development of multiple undeveloped buds may be detrimentally affected. In gentians, Eason *et al* (2004) reported that pulsing flowering shoots of gentian with sucrose (5%) improved their postharvest vase-life and pigmentation of petals on developing florets. Zhang & Leung (2001) also reported similar results using sucrose pulsing (3%). As the importance of carbohydrates to floret development and postharvest quality of flowering shoots of gentians has already been established, the focus in the

current chapter on the import, storage and mobilisation of carbohydrates in floral tissue, therefore, provides an opportunity to extend our understanding of how these factors may be related to the physiology of floret development and postharvest performance.

In most species, accumulation and degradation of polysaccharide storage carbohydrates in petals commonly accompanies the procedure of flower development (van Doorn & van Meeteren, 2003), such as starch in *Rosa hybrida* (rose) (Ho & Nichols, 1977) and *Alstroemeria peregrina* (Collier, 1997), fructan in *Campanula rapunculoides* (Vergauwen et al., 2000) and daylily (*Hemerocallis*) (Bielecki, 1993), and both starch as well as fructan in *Chrysanthemum* (Trusty & Miller, 1991). In addition, carbon may also be transferred from senescing flowers to opening ones (van der Meulen-Muisers, 2000). The pattern of change in NSCs within floral tissue during the procedure of floret development and senescence in gentians has not previously been reported, making it unclear which carbohydrates may be used for storage in this tissue. Although Zhang & Leung (2001) detected starch in petals of open florets of *Gentian triflora* after pulsing with carbohydrates, the very low concentration of starch (max value less than 4 mg g<sup>-1</sup> FW) seems insufficient for starch to be the main storage carbohydrate under normal circumstances. Further to this, in previous chapters in this thesis the measurement of NSCs in gentian ‘Showtime Spotlight’ indicated that both starch and fructan were not detectable in other organs, while gentianose, a trisaccharide, was present as a storage carbohydrate at high concentrations (up to 100 mg g<sup>-1</sup> FW). It seemed reasonable therefore to hypothesise that gentianose also plays a role as a main storage carbohydrate in the florets of gentians. The patterns of change of individual NSCs occur during floret development were, therefore, quantified in this chapter to better understand their role in floret development of gentians.

Flower opening is usually accompanied by the increase of both soluble carbohydrate molality and osmotic potential of petal tissue in many species, such as daylily (Bielecki, 1993), *C. rapunculoides* (Vergauwen et al., 2000), rose (Yamada et al., 2007) and *Hibiscus syriacus* (Kwon et al., 2010). It was suggested that carbohydrates may act as a major osmotically active solute in, regulating water potential, promoting water influx for cell expansion, and flower opening (Reid, 2005; Kumar et al., 2008b). Previous studies investigating the pattern of individual carbohydrate metabolism in petals, indicated that the increase of soluble carbohydrate osmolality may be due to the degradation of various

polysaccharides such as starch (Hammond, 1982) and fructan (Bieleski, 1993; Vergauwen et al., 2000), and/or the import of soluble carbohydrates such as glucose and fructose (Yamada et al., 2007). In gentian however, there is no existing data available in terms of the possible involvement of carbohydrates in regulating osmotic potential in petals during floret opening. As presented within this chapter therefore, there was a need to first quantify the dynamics of water, osmotic and pressure potentials (turgor potential) in petal during floret development, and then explore their relationships with the osmolality changes of soluble carbohydrate.

The growth of plant cells is strongly influenced by water potential ( $\Psi$ ) and its components, i.e., osmotic potential ( $\Psi_s$ ), pressure potential ( $\Psi_p$ ) and matric potential ( $\Psi_m$ ) (Reigosa Roger, 2001). Water potential may be expressed as the sum of these components (Eq 1).

$$\Psi = \Psi_p + \Psi_s + \Psi_m \quad \text{Eq 1}$$

In vacuolated cells of high water content, the matric potential is thought to make a relatively minor contribution and, for such cells, the water potential is given simply as expressed in Equation 2 (Eq 2) (Öpik & Rolfe, 2005). Under the assumption that the matric potential made little contribution in the petal tissue of gentian florets, Eq 2 was used within the experiments presented in this chapter.

$$\Psi = \Psi_p + \Psi_s \quad \text{Eq 2}$$

Within plants, water will move from a region of higher water potential to one of lower water potential. When plant cells take up water, the cell wall is stretched and the cell becomes more turgid because of the increased pressure potential. It is important therefore that plants have adequate water to maintain turgidity of petal cells for driving cell expansion, and provide support to the tissues. In contrast, continued water deficit in cells or tissue leads to dehydration and wilting, even death (Öpik & Rolfe, 2005). A certain pressure potential therefore, may be necessary for the rapid cell expansion of petal tissue and supporting flower opening (Reid, 2005). While the changes in, and interrelationships between, carbohydrate osmolality and the sap osmolality in petal tissue have previously been demonstrated (Bieleski, 1993; Yap et al., 2008), the effect of carbohydrate osmolality on water potential and pressure potential have not been clarified or published. Within the experiments presented in this chapter therefore, this omission in the knowledge base was addressed.

Taken together, the aim in this chapter was to reveal the pattern of NSCs dynamics in gentian florets (as a discrete model-organ), and explore the hypothesised mechanism that NSCs regulate osmotic potential during the procedure of flower bud development, with the following specific objectives:

- Quantify the dynamics of NSCs concentration in petal tissue from floret development to senescence, and explore their possible interrelationships and function.
- Quantify the dynamics of NSCs osmolality, osmotic potential, water potential and pressure potential in petal tissue, and explore their interrelationships during floret development and senescence in gentians.

## **7.2 Materials and methods**

### **7.2.1 Plant material and cultivation**

The same plant material and cultivation as described in Section 6.2.2.1 were used in this experiment, i.e. clonally propagated plants of the gentian cultivar ‘Showtime Spotlight’. Samples were collected (Mid Feb 2011) from plants that had been established for seven years.









### **7.2.2 Petal fresh and dry weight measurement**

Individual florets at eight developmental stages from immature through to anthesis and senescence (Table 1) were collected on a sunny day, i.e., at midday 15<sup>th</sup> February 2011, when the greatest number of floral shoots were at horticultural harvest maturity. Five florets at each stage of floret development were randomly sampled. Once sampled, the petals of each floret were immediately dissected and put into pre-weighed plastic vials that were then reweighed to determine petal fresh weight (FW; g), with dry weight (DW; g) determined after drying at 60 °C for one week.

At the same time as sampling to record fresh and dry weight (refer Section 7.2.2), florets at the eight developmental stages (Table 7.1) were also collected, specifically for monitoring NSC content. For each developmental stage, florets were randomly sampled and grouped into three replicates of 10 to 15 florets for each stage (e.g. Figure 7.1). Once sampled the petals, sepals and remaining organs (stamens and pistils) of each floret were

immediately separated, frozen in liquid nitrogen, and then stored at -80 °C until NSCs were analysed. Individual NSCs were identified and quantified utilising HPLC, following the method described in Section 6.2.3.

**Table 7.1 Descriptive characteristics of each of eight stages of development of florets of ‘Showtime Spotlight’ sampled in the current experiment.**

Developmental Stage	Description of florets
1 	small buds with petals 1.5-2.0 cm in length, covered by sepals, without pigmentation.
2 	petals 2.0-3.0 cm in length, floret tightly closed, light pink tips.
3 	petals 3.0-4.0 cm in length, tightly closed, pink tips.
4 	petals 4.0-5.0 cm in length, floret fully pigmented bright pink.
5 	petals 5.0-6.0 cm in length, floret just opening, pink stigma not split, pollen released.
6 	petals 5.0-6.0 cm in length, floret fully open, pink stigma split.
7 	petals 5.0-6.0 cm in length, floret senescing, petals closed, faded from bright pink to dark pink.
8 	petals 5.0-6.0 cm in length, floret senesced, petal tips browned.

### 7.2.3 Monitoring NSCs

Considering the fact that NSCs provide both energy and osmotic function, mass fraction and osmolality were both used in this chapter as concentration units for nutrition and

## Chapter 7 – Changes of NSCs during floret development

osmotic variables, respectively. Mass fraction was defined as mass of a carbohydrate divided by mass of fresh petal tissue and, expressed as  $\text{mg g}^{-1}$  FW. Osmolality of soluble carbohydrates was defined as the amount ( $\mu\text{mol}$  or  $\text{mmol}$ ) of a soluble carbohydrate divided by mass (g) of water ( $\text{H}_2\text{O}$ ) in petal tissue and, expressed as  $\mu\text{mol g}^{-1}$   $\text{H}_2\text{O}$  or  $\text{mmol g}^{-1}$   $\text{H}_2\text{O}$ . This data was used also to calculate both the mass fraction of total non-structural carbohydrate (TNC) and TNC content as a proportion of DW (%), for each organ, but excluded values for L-bornesitol as above.



**Figure 7.1** Typical example of floret samples of ‘Showtime Spotlight’ for three replicates used in the experiment, at stage 4 (refer Table 7.1) for analysis of non-structural carbohydrates. Three replicates, each comprising 10 to 15 florets, were used for analysis.

### **7.2.4 Monitoring Water Potential and its Components**

This experiment was repeated eight times within two weeks commencing mid-February 2011, when the greatest number of floral shoots of ‘Showtime Spotlight’ was at horticultural harvest maturity. On each date of repeating the experiment flowering shoots with florets at each of the eight stages of development (Figure 7.1) were randomly collected at midday on a sunny day. Once sampled, flowering shoots were wrapped in a black plastic bag to prevent desiccation, and quickly transferred to the laboratory ( $23 \pm 2$  °C) within 3 minutes from the field (100 metres away).

Water potential and osmotic potential of petal discs were measured with a dew point micro-voltmeter HR-33T (Wescor Inc., Logan, Utah, USA) in psychrometric chambers (Model C-52; Wescor Inc.) (Teare et al., 1982; Evers et al., 2010). Based on the instruction manual of the HR-33T dew point micro-voltmeter (Wescor, 2001), the method followed comprised:

Fresh petal discs of each stage were sampled using a hole punch (0.65 cm diameter) from the middle of petals (Figure 7.2) and, for each stage of development, each disc was immediately sealed in a psychrometric chamber (eight sample chambers for eight stages) within three seconds after excision. Three hours of equilibration of temperature and vapour pressure was required before measuring water potential using the HR-33T dew point micro-voltmeter. For osmotic potential, the same discs for each developmental stage were wrapped in plastic wrap quickly, and deep-frozen in liquid nitrogen for ten minutes. Samples were subsequently thawed at room temperature before each petal disc was sealed in the same sample chambers for measuring water potential. Half an hour of equilibration of temperature and vapour pressure was required before measuring osmotic potential using the HR-33T dew point micro-voltmeter. Solutions of NaCl (0 to 1.0 M) were used to establish a standard curve for calibrating each C-52 Sample Chamber.

### **7.2.5 Calculation of pressure potential**

Based on Equation 2 above, pressure potential ( $\Psi_p$ ) was calculated using Equation 3 (Eq 3).

$$\Psi_p = \Psi - \Psi_s \quad \text{Eq 3}$$

### **7.2.6 Calculation of osmotic potential derived from soluble TNC**

The osmotic potential measured above was the total osmotic potential of petal tissue that was derived from not only soluble NSCs, but also other components such as soluble proteins, amino acids (e.g. proline), organic and inorganic compounds, etc. (Nilsen & Orcutt, 1996b; Norikoshi et al., 2013). To evaluate the contribution of soluble carbohydrates to the total osmotic potential of petal tissue, according to the molality of soluble TNC measured above, the osmotic potential derived from soluble TNC was calculated using Equation 4 (Eq 4) and expressed as MPa (Reigosa Roger, 2001).

$$\Psi_s = - CiRT \quad \text{Eq 4}$$

Where:

$C$  is the concentration of TNC expressed as osmolality;

$i$  is the van 't Hoff factor which, for soluble carbohydrate  $i = 1$ ;

$R$  is the gas constant =  $8.3144621 * 10^{-3}$  kg MPa K<sup>-1</sup> mol<sup>-1</sup>;

$T$  is thermodynamic (absolute) temperature (K).

## Chapter 7 – Changes of NSCs during floret development

The NSCs measured in gentians within this thesis, i.e. gentianose, gentiobiose, sucrose, fructose, glucose, and L-bornesitol, are all soluble. The osmolality of total non-structural carbohydrate (TNC) was the sum of individual soluble carbohydrates but excluded values for L-bornesitol as the real concentration of L-bornesitol was not available due to lack of standard for L-bornesitol (refer Chapter 6).



**Figure 7.2 Position of sampling discs from the middle of petals of the gentian cultivar ‘Showtime Spotlight’ for water potential and osmotic potential measurement.**

### **7.2.7 Statistical analysis**

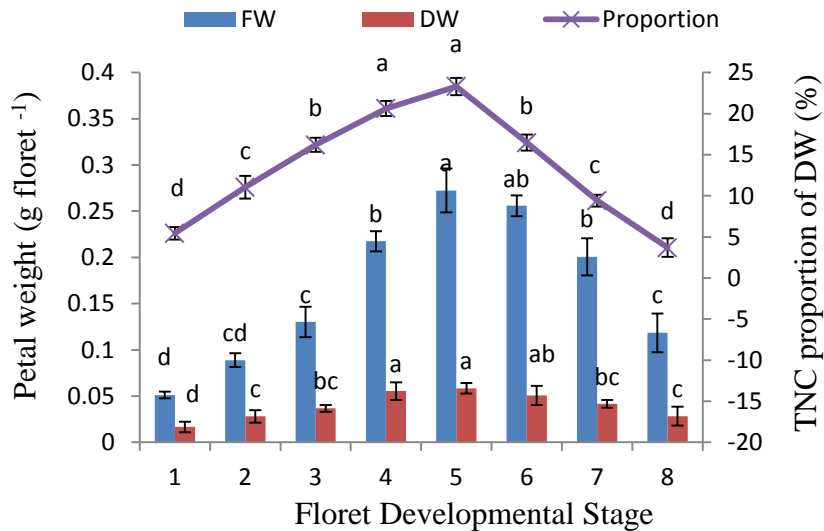
All experiments were conducted as a completely randomised design. The relationships between the osmolality of NSC and water potential, osmotic potential and pressure potential were determined using regression analysis. All other data were subjected to an analysis of variance, utilising GLM (Minitab 16). Each value was expressed as the mean of three replicates  $\pm$  standard error (SE). When significant differences occurred, mean values were separated using the Tukey method ( $P < 0.05$ ).

## **7.3 Results**

### **7.3.1 Changes of petal biomass**

Fresh weight and dry weight of petals increased more than five and three-fold, respectively, from immature small buds (Stage 1) to reach their maximum values when florets were just opening (Stage 5; Figure 7.3,  $P < 0.001$ ). Similarly, during the same period, petal length increased four-fold from 1.5 to 6.0 cm (Table 1). Content of TNC, as a proportion of dry weight, increased linearly more than four-fold from 5% to 23% up to when the floret was just opening (i.e. between Stages 1 and 5) (Figure 7.3,  $P < 0.001$ ). After being fully open (Stage 6) through to senescence (Stage 8), the fresh weight, dry

weight, and TNC content as a proportion of dry weight, all presented a similar decrease through to when florets were senesced at Stage 8 (Figure 7.3;  $P_{DW} < 0.001$ ;  $P_{FW} < 0.001$ ;  $P_{TNC \text{ proportion}} < 0.005$ ).



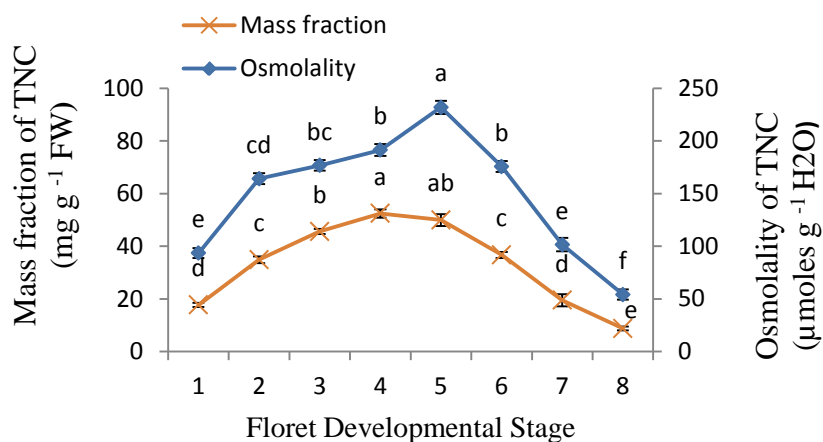
**Figure 7.3** Fresh weight (FW), dry weight (DW) and total non-structural carbohydrate (TNC) as a proportion of DW, in petals of ‘Showtime Spotlight’ during floret development from immature buds through to anthesis, and senescence (i.e. Stage 1 to Stage 8; refer Table 7.1). For each variable, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines =  $\pm$  SE.

### 7.3.2 Changes of NSCs

Concentration of TNC in petals, expressed as the mass fraction, increased approximately three-fold from immature small buds (Stage 1) to reach a maximum value just before florets opened (Stage 4). The mass fraction of TNC then decreased approximately six-fold from Stage 5 when the florets began opening through to Stage 8 when they senesced (Figure 7.4,  $P < 0.001$ ). While the TNC concentration, expressed as osmolality, followed a similar pattern of change with the development of florets, the maximum osmolality was reached when florets were just opening (Stage 5), instead of just before opening (Stage 4; Figure 7.3). When expressed as osmolality there was also a sharp increase just before the opening of florets (Stage 4), followed by more than a four-fold decline when florets senesced (Stage 8, Figure 7.3;  $P < 0.001$ ).

HPLC analysis indicated that gentianose, gentiobiose, sucrose, glucose, fructose and L-bornesitol were present in petals, sepals, and the mixture of stamens and pistils, while no significant peaks of any other compounds, including fructans, were detected in the

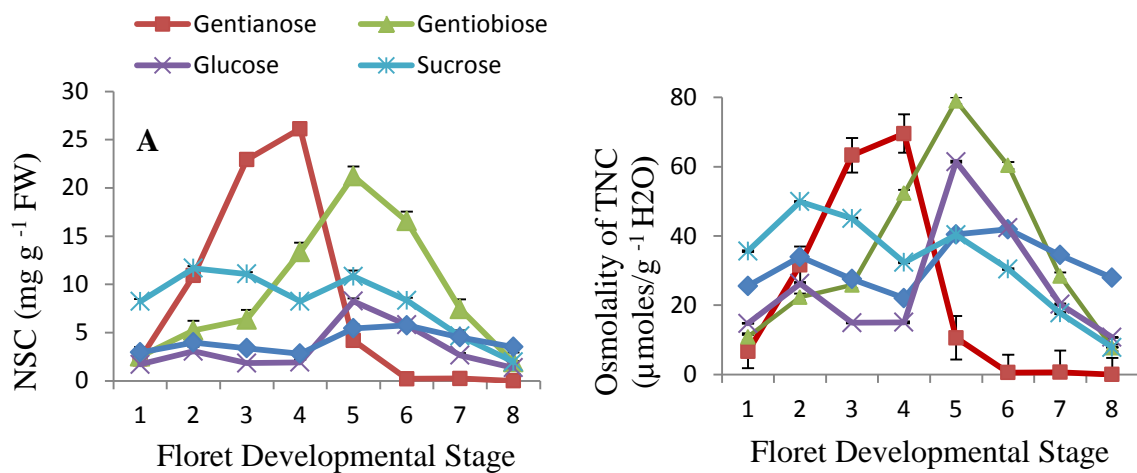
analysis of these organs. Similarly, by using an enzymatic method (Amylase/Amyloglucosidase), starch was also not detected in any floral organ.



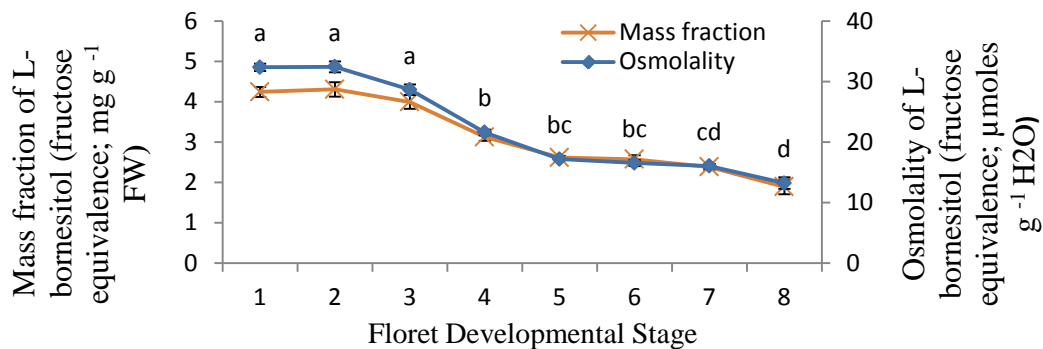
**Figure 7.4 Concentration of total non-structural carbohydrates (TNC), expressed as both mass fraction and osmolality, in petals of ‘Showtime Spotlight’ during floret development from immature buds through to anthesis, and senescence (i.e. Stage 1 to Stage 8; refer Table 7.1). For each variable, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines =  $\pm$  SE.**

Within petal tissue gentianose concentration, as a mass fraction, increased more than eleven-fold with the development of florets to a maximum value of 26.1 mg g<sup>-1</sup> FW just before the opening of florets (Stage 4). The concentration then sharply decreased to approximately 15% as florets began opening (Stage 5), with near zero level from when fully open (Stage 6) through to being senesced (Stage 8, Figure 7.5A). Gentiobiose increased gradually during early development from Stages 1 through 3, but then sharply increased more than three-fold to reach a maximum value of 21.2 mg g<sup>-1</sup> FW as florets began opening (Stage 5), and then decreased 90% through subsequent stages to when senesced (Stage 8, Figure 7.5A). Sucrose concentration remained relatively stable at a level of approximately 10 mg g<sup>-1</sup> FW from early developmental stages through to when florets began opening (Stage 5), and then decreased 80% to approximately 2 mg g<sup>-1</sup> FW in senesced florets (Stage 8, Figure 7.5A). Glucose concentration remained relatively stable at a comparatively low level of 2 mg g<sup>-1</sup> FW between Stages 1 to 4, before it increased four-fold to achieve its maximum value as florets began opening (Stage 5), and then gradually decreased through subsequent stages to senescence (Stage 8, Figure 7.5A). Similar to the pattern of change for glucose, fructose concentration also increased during flower opening. In contrast to the other NSCs monitored, the concentration of L-bornesitol gradually decreased from Stages 1 through to 8 (Figure 7.6;  $P < 0.001$ ).

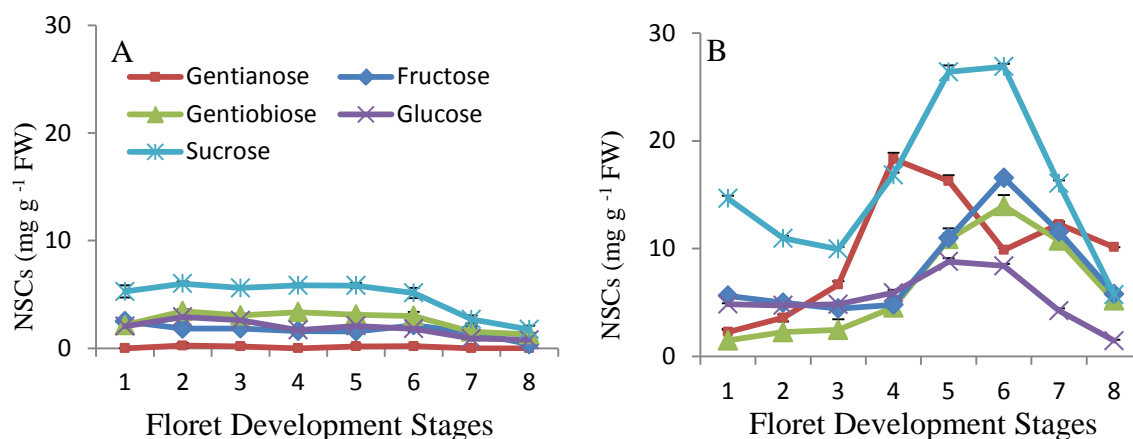
The changes of NSCs concentration with development, expressed as osmolality, presented the same patterns as evident when expressed as mass fraction, while the value of osmolality of low-DP (degree of polymerization) NSCs was relatively amplified. For example, while glucose (DP =1) and gentianose (DP = 3) both presented the same concentration as mass fraction, the value of osmolality of glucose would be three times higher than gentianose.



**Figure 7.5** Concentrations of individual non-structural carbohydrates (NSCs), expressed as; (A) mass fraction and, (B) osmolality, in petals of ‘Showtime Spotlight’ during floret development from immature buds through to anthesis, and senescence (i.e. Stage 1 to Stage 8, refer Table 7.1). Vertical lines = ± SE.



**Figure 7.6** Concentration of L-bornesitol, expressed as mass fraction and osmolality, in petals of ‘Showtime Spotlight’ during floret development from immature through to anthesis, and senescence (i.e. Stage 1 to Stage 8, refer Table 7.1). For each variable, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ). Vertical lines = ± SE.



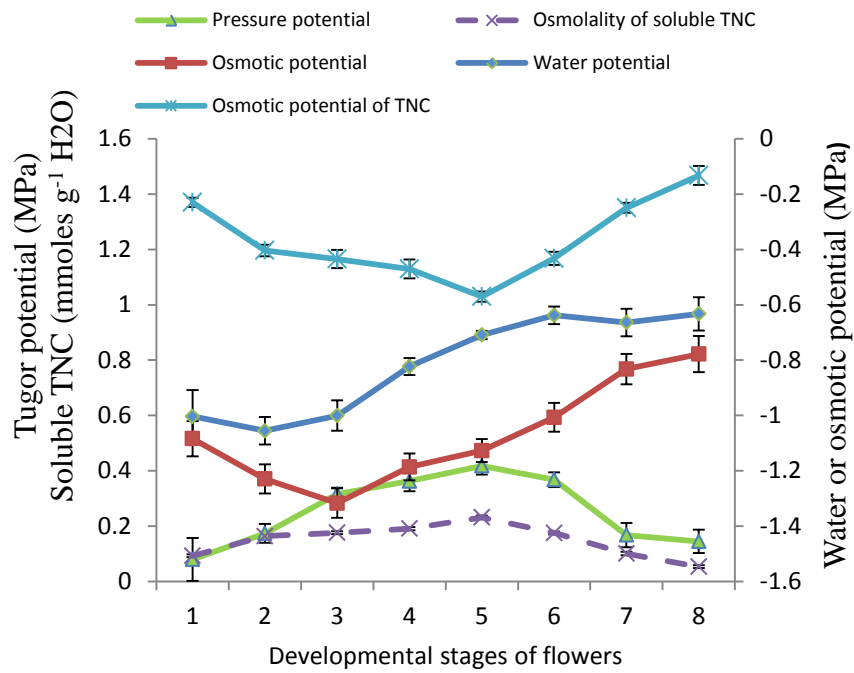
**Figure 7.7** Concentration of individual non-structural carbohydrates (NSCs), expressed as; (A) sepals, (B) the remaining floral organs (i.e. stamens and pistils) in petals of ‘Showtime Spotlight’ during floret development from immature through to anthesis, and senescence (i.e. Stage 1 to Stage 8, refer to Table 7.1). Vertical lines =  $\pm$  SE.

Concentrations of NSCs in sepals were very low, and did not show significant changes with changes in development of the floret (Figure 7.7A). NSCs in the remaining floral organs (i.e. stamens and pistils), presented similar patterns of change as those in petals, with the only difference being that the points of change were delayed to later stages (Figure 7.7B).

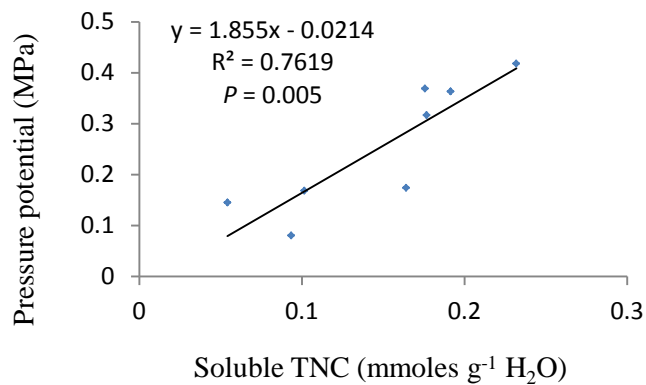
### 7.3.3 Water, osmotic and pressure potential

Osmotic potential of petal tissue decreased during the early developmental stages until florets had coloured tips (Stages 1 to 3) from -1.1 MPa to -1.3 MPa, and then gradually increased to -0.78 MPa when florets were senesced (Stage 8, Figure 7.8;  $P < 0.001$ ). The osmotic potential derived from TNC, in general, only contributed approximately one fourth of the total osmotic potential of petal tissue (Figure 7.8). Water potential was stable at the early developmental stages (Stages 1 to 3), and then gradually increased through to full opening of florets (Stage 6), before remaining stable again as they progressed to senescence (Stage 8). The value of water potential of the petal tissue was always higher than its total osmotic potential (Figure 7.8;  $P < 0.001$ ). In contrast to the changes evident with water potential and osmotic potential, pressure potential increased approximately five-fold from Stage 1 to a maximum value of 0.42 MPa as florets began opening (Stage 5), and then decreased 65% through to when senesced (Stage 8, Figure 7.8;  $P < 0.001$ ). Regression analysis determined a positive correlation between pressure potential and

osmolality of soluble TNC (Figure 7.9;  $y = 1.855x - 0.0214$ ;  $r^2 = 0.7619$ ;  $DF = 1, 6$ ;  $F = 19.2$ ;  $P = 0.005$ ).



**Figure 7.8** Water, osmotic, and pressure potential, and osmolality of soluble total non-structural carbohydrates (TNC), during floret development of ‘Showtime Spotlight’ from immature through to anthesis, and senescence (i.e. Stage 1 to Stage 8, refer Table 7.1). Vertical lines =  $\pm$  SE.



**Figure 7.9** Relationship between the pressure potential and the osmolality of soluble total non-structural carbohydrate (TNC) of petal tissue based on the average value at each stage, during floret development of ‘Showtime Spotlight’ from immature through to anthesis, and senescence (i.e. Stage 1 to Stage 8, refer Table 7.1).

## **7.4 Discussion**

Many different biological mechanisms have been reported for flower development and senescence (van Doorn & van Meeteren, 2003; Kumar et al., 2008b). These mechanisms varied vastly between plant species as well as floral structures, and are sensitive to various environmental influences. In many species significant metabolism of carbohydrates accompanying flower development and senescence has been noted, including the:

- accumulation of starch and/or fructan before the opening of flower buds,
- hydrolysis of these polysaccharides and the subsequent increase of fructose and/or glucose during flower opening and,
- decrease and export of carbohydrates with the senescing of flowers (Bieleski, 1993; Yamane et al., 1995; van der Meulen-Muisers et al., 2001; Waithaka et al., 2001; Le Roy et al., 2007; Yamada et al., 2007).

The results of the current study indicated that the unique carbohydrates, i.e., gentianose and gentiobiose, were the predominant carbohydrates in petals (Figure 7.5), which was consistent with that reported for other organs of ‘Showtime Spotlight’ in previous chapters, but different from the forms of carbohydrates reported in many other plant species. Zhang & Leung (2001) reported the presence of starch in petals, but the concentration reported of less than 4 mg g<sup>-1</sup> FW was much less than the gentianose concentration of more than 25 mg g<sup>-1</sup> FW in the current experiments. Starch was not detected at all in the current experiments, suggesting that ‘Showtime Spotlight’ typically contains little if any starch. The high concentrations and significant fluctuation of both gentianose and gentiobiose with developmental stage (Figure 7.5) was interpreted to imply that both carbohydrates may play important physiological functions in floret development and senescence. In addition to providing energy and structural components, it has been suggested that in other genera the accumulation of soluble carbohydrates, such as sucrose, glucose, and fructose, results from the degradation of starch and/or fructan, or the importation of sucrose, and is a mechanism regulating water potential, promoting water influx driving cell expansion and flower opening (Reid, 2005; Kumar et al., 2008b). The current study indicated a substantial accumulation of gentianose in petals before the opening of florets, and a rapid decrease during their opening (Figure 7.5). This result implies that the accumulated gentianose was likely hydrolysed into carbohydrates with lower degrees of polymerization

(DP) during floret opening. This hydrolysis increased carbohydrate osmolality and, therefore, enhanced pressure potential in petal tissue to drive cell expansion and floret opening. In this study the finding that there was a significant positive correlation between the osmolality of soluble NSCs and pressure potential (Figure 7.9;  $P = 0.001$ ), has provided new evidence supporting the hypothesis, i.e., carbohydrate metabolism in petals is involved in osmotically-driven cell expansion and opening of florets/flowers (Vergauwen et al., 2000; Yamada et al., 2009; Kwon et al., 2010). With the results of the current study in mind, the possible function of carbohydrate metabolism in floret development and senescence in gentians have been discussed in more detail in the following paragraphs.

Similar to many other genera/species of plants (van der Meulen-Muisers et al., 2001; O'Donoghue et al., 2002), the development of florets was accompanied by a substantial and rapid increase in fresh and dry weight of petals, from small buds to when the florets were just opening (Figure 7.3). These changes reflected the cell expansion, water uptake, synthesis of new cell walls, and accumulation of assimilate. In contrast, over the subsequent development through to final senescence, fresh and dry weight of petals significantly decreased. These later phases of development may likely be due to the increase of carbon consumption and export (Waithaka et al., 2001), loss of water, and degradation of cell wall contents (O'Donoghue et al., 2002). These changes in fresh and dry weight can also be considered as indicative of the conversion of petals between sink and source during their development and senescence (Zhou et al., 2005).

During the development of gentian florets, TNC mass concentration in petals showed a significant increase of more than three-fold, and reached the maximum level of  $52.5 \text{ mg g}^{-1} \text{ FW}$ , just before the opening of florets (Figure 7.4). At the same time, TNC as a proportion of dry weight also increased and reached a maximum level of 23% of petal dry weight (Figure 7.3). It has been reported that in other genera the failure of floral bud opening after harvest may be due to limited carbohydrate stored in petals (van der Meulen-Muisers et al., 2001). In contrast, in the current experiment the accumulation of substantial TNC (mainly gentianose) in petals prior to florets opening (Stage 4), presumably would provide sufficient carbon reserves for stressful conditions, such as shading or drought, when photosynthesis is restricted or reduced (Stamp, 2003), and/or for fast development of florets (petal expansion and pigment development) under normal conditions. These

potential functions are particularly important for postharvest performance of gentians (Zhang & Leung, 2001; Eason et al., 2004; Eason et al., 2007). As evident in their research, the most suitable harvest stage of floral shoots of gentians was when the apical florets were fully pigmented (i.e. Stage 4), and pulsing treatments with sucrose could improve the otherwise poor pigmentation of petals if shoots were harvested earlier at Stage 2 or 3. The influence of maturity stage on postharvest quality in Eason's experiments might therefore be explained from the results in the current study, i.e., the accumulated NSCs at the earlier developmental stages were insufficient to provide for optimal development following shoots harvest.

It has been suggested that the rapid hydrolysis of starch and/or fructan in petals during the opening of flowers of other species, results in increases of fructose and/or glucose, which contribute to the osmotic driving force involved in water uptake, cell expansion and flower opening (Kumar et al., 2008b; Norikoshi et al., 2013). The results of the current study illustrate substantial accumulation of soluble gentianose in petals during bud development before opening (accounting for approximately 50% of TNC at Stage 4), which was rapidly hydrolysed during floret opening to reach near zero at full opening (Stage 6). This change during opening was accompanied by increases in gentiobiose, glucose and fructose (Figure 7.5B), the likely by-products of such hydrolysis (Bourquelot & Herissey, 1901; Palmer & Anderson, 1972). This pattern of change was similar to that reported with flowers of other genera/species, such as starch in lily (van der Meulen-Muisers et al., 2001) and rose (Hammond, 1982), and fructan in both daylily (Bialeski, 1993) and *C. rapunculoides* (Vergauwen et al., 2000).

While gentiobiose may be the product of gentianose hydrolysis (Bourquelot & Herissey, 1901), it was evident that the hydrolysis of gentianose alone was not sufficient to provide the gentiobiose present because the maximum concentration of gentiobiose ( $62 \mu\text{moles g}^{-1}$  FW) was higher than that of gentianose ( $52 \mu\text{moles g}^{-1}$  FW; Figure 7.5B). It was also noticed that during the increase in concentration of gentianose, the concentration of gentiobiose started to increase (Figure 7.5). This level of synchronisation indicated the increase of gentiobiose, at least during the period of increase of gentianose, was not likely to be derived from the hydrolysis of gentianose alone. Gentiobiose has been found in other plants without gentianose being detected (Kandler & Hopf, 1984; Dumville & Fry, 2003), and gentiobiose could also be synthesized using UDP-D- $^{14}\text{C}$  glucose as a substrate, with a

dialysed enzyme preparation from ripening seeds of ivy (*Hedera*) (Kandler & Hopf, 1984). In gentian therefore, it is possible that gentiobiose is the product of synthesis rather than the product of hydrolysis of gentianose, or the production of synthesis and hydrolysis of gentianose, but further experiments are needed to support these hypothesis.

Many enzymes have been reported as being related to the hydrolysis of sucrose, starch, or fructan in petals and, therefore, contributing to the osmotic driving force involved in flower opening, such as starch amylase in rose (Hammond, 1982), invertase in *Dianthus caryophyllus* L. (carnation) (Yamada et al., 2007) and, fructan 1-exohydrolase in *C. rapunculoides* (Le Roy et al., 2007). Although gentianose as a soluble carbohydrate is able to increase osmolality, the hydrolysis of gentianose would provide double (hydrolysis to a mono plus disaccharides) or triple (hydrolysis to three monosaccharides) the contribution to an increase in osmolality as that from gentianose itself. This hydrolysis of gentianose therefore, was hypothesised to be the primary cause of the increase in osmolality towards reaching the maximum level at full opening of the florets (Figure 7.4 and Figure 7.5B). In gentian, however, little information has been published regarding the enzymes related to the metabolism of either gentianose or gentiobiose. For example, although gentiobiose, sucrose, fructose and glucose all are possible products of gentianose hydrolysis, what the actual products of gentianose hydrolysis in gentian plants are, has not been reported. Due to the existence of unique carbohydrates, i.e., gentianose and gentiobiose, it seems reasonable to assume that there must be a different enzyme system, with stage-specific dynamics of enzyme activity in petals, which enable tight control of carbohydrate metabolism, and then osmotic regulation involving floret development and senescence. To explore the enzymes related to carbohydrate metabolism in gentians therefore, a preliminary experiment was subsequently conducted to characterize these enzymes (refer Chapter 8).

Sucrose concentration remained stable without significant increase during floret development (Figure 7.5). While Yamada and his co-authors (2007) presented similar results regarding the net concentration of sucrose, invertase activity increased markedly during petal growth of roses. Invertases (including insoluble and soluble acid invertase and neutral invertase) play an important role in sucrose hydrolysis and translocation from phloem to the apoplast by converting sucrose into fructose and glucose. The increase of invertase activity indicated that although net concentration of sucrose remained stable, the

importation of sucrose apparently increased and it was rapidly hydrolyzed into hexoses. In gentians, it was therefore considered possible that the increase of glucose and fructose during floret opening (Figure 7.5) was due to the hydrolysis of sucrose, although they could also be the product of gentianose hydrolysis. To investigate this hypothesis, a preliminary experiment measuring invertase activity was subsequently conducted (refer Chapter 8).

With regard to the senescence of gentian florets (Stages 6 through to 8), the concentrations of the various NSCs (i.e. gentiobiose, glucose, fructose and sucrose) all decreased significantly (Figure 7.5), which was consistent with previous reports with flowers of other genera/species (Bialeski, 1993; van der Meulen-Muisers et al., 2001). This reduction of NSCs is not only due to their consumption during respiration, but also their export from senescing petals (Waithaka et al., 2001). This carbon-saving phenomenon has been demonstrated via the export of radioactive sugars from wilting florets to younger buds in spike-type inflorescences of *Gladiolus grandiflorus* L. (gladiolus) (Yamane et al., 1995). With the inflorescence structure and distribution of floret age in gentians not being too dissimilar from that in gladiolus, if there is an export of NSCs or a transfer of NSCs between senescing florets and young buds, the opportunity exists to quantify its significance in experimental investigations in the future.

Within petal tissue the concentration of L-bornesitol gradually decreased, without extensive fluctuation, throughout the development and senescence of florets (Figure 7.6). This implies that L-bornesitol may not play an important function in floret development of gentian. It is however possible that L-bornesitol is indirectly involved in the metabolism of other carbohydrates (Joersbo et al., 1999).

Although it has previously been suggested that carbohydrate metabolism is involved in osmotic regulation in petals, little direct evidence has been demonstrated in terms of the relationship between carbohydrate concentration and water, osmotic, and pressure potential. Bialeski's (1993) study using the ephemeral daylily flower, illustrated that osmotic potential increased during the early stages of opening and significantly increased with actual opening, accompanying the hydrolysis of fructan, and then increased again with the senescence of the flower. In contrast, during rhythmic expansion of rose petals, osmotic potential increased continuously with the rapid expansion of petals (Evans & Reid, 1988). Another example is flower opening in *Tweedia caerulea*, in which osmotic

potential decreased with the increase of soluble carbohydrate concentration (glucose, fructose and sucrose), but starch concentration remained constant (Norikoshi et al., 2013). In contrast to these patterns of change, in the current study a decrease of osmotic potential was evident at early stages (Stages 1 to 3), followed by an increase through to senescence, with the changes in water potential in petals of gentian presenting a similar pattern (Figure 7.8). The different results evident in these different studies may be due to differences in flower characteristics, for example the ephemeral daylily are only open for less than one day before the onset of senescence while gentian florets can be open for more than one week. In petal tissue of gentians, osmotic potential derived from carbohydrates only contributed approximately one fourth of the total sap osmotic potential (Figure 7.8), and the pattern of its change throughout the development and senescence of florets was not always consistent with sap osmotic potential or water potential. It is therefore concluded that there were other factors contributing to the osmotic potential, such as proteins, organic and inorganic compounds, etc. (Nilsen & Orcutt, 1996b; Norikoshi et al., 2013). The interesting finding within the current study is that the changes of osmolality of soluble TNC was extremely significant in its correlation to the changes in pressure potential (Figure 7.9;  $r^2 = 0.8441$ ;  $P = 0.001$ ), and the absolute value of the pressure potential was slightly less than that of the osmotic potential soluble TNC provided. These results, therefore, lend reasonable support to the hypothesis that the regulation of pressure potential in petals of gentian, which is physically the key driving force involved in cell expansion and opening of florets, is mainly contributed by the activity of carbohydrate metabolism.

The function of carbohydrates inhibiting both the production of ethylene and the sensitivity to ethylene also has been recognised in other flowers. For example in the petals of sweet pea (*Lathyrus odoratus* L.), there was a negative correlation between carbohydrate concentration and ethylene production, and the onset of the climacteric increase in ethylene production was delayed by a pulsing treatment with sucrose (Ichimura & Suto, 1999). In carnation, sucrose treatments delayed flower senescence and decreased the ethylene production and responsiveness in petals (Verlinden & Garcia, 2004). Gentian flowers are ethylene-sensitive and, a pulsing treatment with sucrose improved the vase life of floral shoots (Zhang & Leung, 2001; Eason et al., 2004). The study of Eason *et al* (2007) using the gentian cultivar ‘Showtime Starlet’ showed that application of AOA (Aminooxylvinylglycine) and 1-MCP (1-Methylcyclopropene), inhibited ethylene

production and ethylene perception, respectively, and prevented petal senescence. It can be concluded therefore that ethylene production and perception during senescence of petals are both stage-specific and tissue-specific for tight control of flower senescence. Although ethylene was not measured in the current experiment, the pattern of NSCs fluctuation in petals (Figure 7.5A) is consistent with the logic that the increase and high level of NSCs in petals during early stages, up to the opening of florets, may inhibit the synthesis and/or perception of ethylene. This inhibition will be reduced following the decrease of NSCs with the aging of florets and, finally, the production and perception of ethylene in turn accelerates the process of senescence. To confirm this hypothesis, further experiments using the treatment of carbohydrates, ethylene and/or ethylene inhibitors, as well as detection of ethylene synthesis are needed. Because gentianose and gentiobiose are predominant within petals, and showed extensive fluctuation with floret development, the influence of these unique carbohydrates is particularly worthy of investigation in this regard.

## **7.5 Conclusion**

During the development and senescence of gentian florets, there was a dramatic metabolism of NSCs in petals, including accumulation, hydrolysis, and possible export and conversion. This metabolism of NSCs provided a sufficient carbon source as energy and metabolites for flower development. On the other hand, carbohydrate metabolism in petals also played an important role in osmotic regulation, driving petal expansion and floret opening. The stage-specific and intensive fluctuation of the unique carbohydrates gentianose and gentiobiose imply an important role in the opening of florets. It is concluded that collectively all these changes in metabolism and function of NSCs enabled the development and senescence of gentian florets to be tightly controlled. The results of this chapter also indicated that the process of floret development provided a useful experimental model system to explore the interrelationships between different NSCs in gentian. To better understand the metabolism and function of NSCs in floret opening of gentians, further study is necessary in terms of the enzyme activity and, the interaction between NSCs and other factors such as water status, plant hormones, and environmental influence.

## Chapter 8 Activity of carbohydrate-associated hydrolases in 'Showtime Spotlight'

### 8.1 Introduction

Carbohydrate metabolism is a fundamental metabolic process that is essential for plant development and growth. It has been discovered that carbohydrates and their metabolites are not only important carbon and energy sources (Lewis, 1984b), but also involved in osmotic regulation (Evans & Reid, 1988), signal transduction (Smeekens, 2000), and withstanding adverse environmental stress (Patton *et al.*, 2007). The non-structural carbohydrates (NSCs) are classified into three main groups (refer to 2.4.2.1), i.e. monosaccharides such as glucose and fructose, oligosaccharides such as sucrose and raffinose, and polysaccharides such as starch and fructan. In addition to the common carbohydrates, i.e. fructose, glucose and sucrose, gentian plants have unique carbohydrates, i.e. gentiobiose (disaccharide) and gentianose (trisaccharide), and a carbohydrate derivative, i.e. L-bornesitol (refer to Chapter 6), but are not reported to contain longer chain carbohydrates such as starch and fructan as the main NSCs used for storage (Badenhuizen *et al.*, 1964; Keller & Wiemken, 1982).

In previous experiments with gentians reported within Chapters 4 to 7, gentianose was proposed as the long-term storage carbohydrate, acting in the role of starch or fructan in other plant species and, together with gentiobiose and sucrose, was the dominant carbohydrate in different organs. As evident by changes in concentration over time, their storage and utilization were correlated to important physiological functions such as crown bud development, over-wintering, spring re-growth, development of floral shoots, as well as floret development and senescence (refer to Chapters 4 to 7). As with other metabolic reactions in plants, carbohydrate metabolism comprises enzyme-catalysed biochemical reactions (Turner & Turner, 1975). Carbohydrate-related enzyme activity, therefore, can potentially also be directly related to these same physiological functions in gentians.

Carbohydrates can be transported and stored in some organs or tissue when plants produce more carbohydrates than they can immediately use. These unused carbohydrates are generally stored as oligosaccharides or polysaccharides, with sucrose and starch being the most frequent temporary or long-term stored carbohydrates utilized in plants, respectively

(Lewis, 1984b). During plant development and growth, these oligosaccharide and polysaccharides can be broken down into monosaccharides or shorter-chain NSCs when needed, by glycoside hydrolases (GHs) including glycosidase and transglycosidases (Minic, 2008). The monosaccharide units then enter monosaccharide catabolism to produce energy or become the precursors of other metabolic pathways. The current study therefore, sets out several very preliminary experiments to explore the activity of GHs related to gentianose, gentiobiose and sucrose, with a view to determining methods and detecting changes of activity during growth and development in ‘Showtime Spotlight’.

A comparison between the fluctuation in concentration of NSCs and their related GHs activity would help to better understand both the role of enzymes, and associations between each NSC (refer below), in carbohydrate metabolism during storage and utilization. In addition to crown buds and storage roots, the current experiment also studied the activity of GHs in florets; where visually-defined stages of development were highly correlated with the changes in concentration of various NSCs (refer Chapter 7). As such, this was expected to provide a better experimental system than crowns, to explore the interrelationships between the various NSCs and related enzymes.

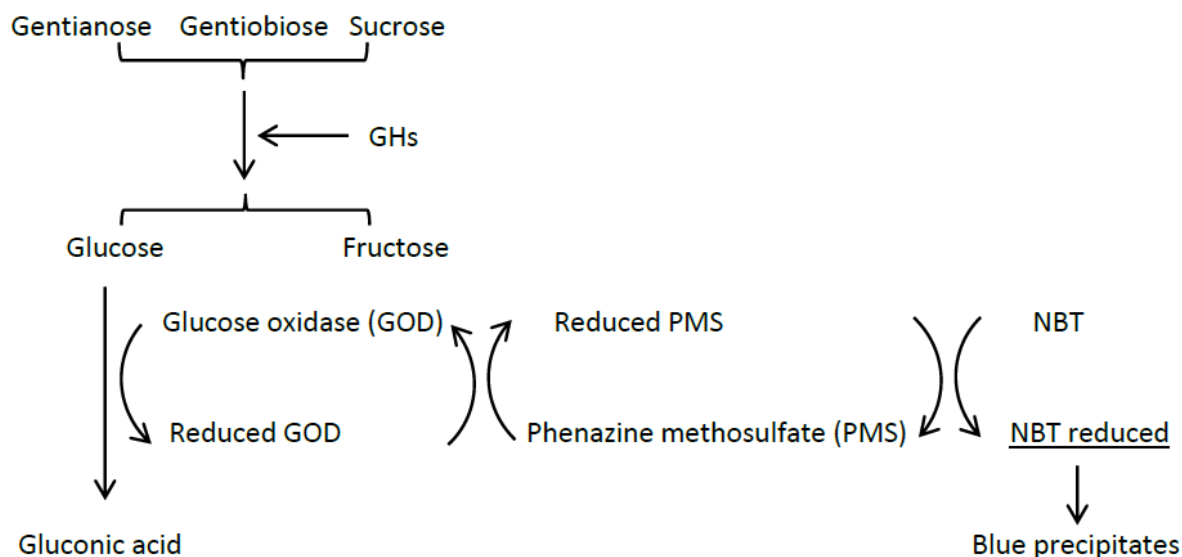
The GHs breaking down sucrose ( $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside), and their physiological roles in plants, have been widely studied (ap Rees, 1984; Sturm, 1999). This process is catalysed by either sucrose synthase (EC 2.4.1.13) or invertase (EC 3.2.1.26) in plants (Sturm, 1999). In contrast, there is very little information detailing the GHs catalysing the hydrolysis of gentianose ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside) and gentiobiose (6-O- $\beta$ -D-glucopyranosyl-D-glucose) in plants in the existing literature. The enzymes responsible for the hydrolysis of sucrose, gentiobiose, and gentianose in gentian plants, have not been reported. It was reported that from *in vitro* experiments that gentianose could be broken down to one unit of fructose and one unit of gentiobiose by invertase (NB. the plant species that the invertase extracted from was not clarified ((Bourquelot & Herissey, 1901), as cited by Badenhcizen et al (1964)); and/or one unit glucose and one of sucrose by emulsin (a preparation containing various enzyme activities that is usually obtained from almonds, and contains glycosidases active on beta stereoisomers of the glycosides such as amygdalin or cellobiose) (Bourquelot & Bridel, 1920). *In vitro*, gentiobiose could be hydrolysed by  $\beta$ -D-glycosidase extracted from *Citrus sinensis* (Cameron *et al.*, 2001), and

the breakdown of gentiobiose was also reported in some micro-organisms (Palmer & Anderson, 1972; Sano et al., 1975). In previous studies (Chapter 6), a synchronised reversed trend of changes in concentration between gentianose and gentiobiose was found within storage roots (Figure 6.12A and B) and rhizomes (Figure 6.12C). This implies that gentiobiose may either be the intermediate metabolite in the synthesis of gentianose, or the product of hydrolysis of gentianose. As a starting point for the current preliminary experiments therefore, crude enzyme extracts from ‘Showtime Spotlight’ were used to examine the enzyme activity hydrolysing these NSCs and the products of these hydrolyses in gentian.

Enzyme activity can be influenced by many factors such as enzyme and substrate concentration, pH, temperature, co-factors and the presence of inhibitors or activators (Halaba & Rudnicki, 1989; Luijendijk et al., 1998; Klotz & Finger, 2001). The activity of most enzymes is sensitive to pH, and extremes in pH usually cause enzyme denaturation. A change in pH can also change the 3-D formation of an enzyme by affecting the state of ionization of acidic or basic amino acids (Shindler & Tipton, 1977). As a consequence, enzymes are only active in a restricted range of pH, and have an optimum pH where activity is maximal, and this has been related to the activity within specific cellular compartments and provides a further level of control within the cell/plant. Similarly, enzyme reactions are strongly influenced by temperature (Peterson et al., 2007). This response of various enzymes to temperature means their activity and function will change with environmental temperature resulting from seasonal changes, even diurnal changes. In addition, different extraction and reaction buffers also affect *in vitro* enzyme activity, for example a  $\beta$ -glucosidase extracted from green vanilla beans (*Vanilla planifolia*) presented higher activity using BisTris propane buffer than phosphate buffer (Dignum et al., 2001). Within the experiments presented in this chapter therefore, the optimum pH, temperature, and influence of buffers were determined prior to any subsequent attempts at quantification of changes in enzyme activity in various organs or stages of the annual growth cycle of ‘Showtime Spotlight’.

While assays of enzyme activity using crude enzyme extracts are convenient and less time-consuming, the characteristics of enzymes are only ever clearly and reliably revealed using purified enzymes, and isolation and purification of enzymes is, therefore, vital to study enzyme characteristics, and further to determine amino acid sequences (Lodish et al.,

2003). Various methods are used in enzyme isolation and purification such as ion exchange chromatography (IEX), polyacrylamide gel electrophoresis (PAGE), and high performance liquid chromatography (HPLC) (Price & Nairn, 2009). In the current experiments, a combination of a Native PAGE and a specific staining procedure was developed, based on previous studies (Dahlqvist & Brun, 1962; Finlayson et al., 1990; Sergeeva & Vreugdenhil, 2002). This staining method relied upon the production of glucose by enzyme action hydrolyzing sucrose, gentiobiose and gentianose, and a set of simultaneous coupling reactions to result in the nitroblue tetrazolium (NBT) being reduced to an insoluble precipitate with a dark blue colour (Figure 8.1). To reduce the diffusion of the coloured precipitate, the effect of a ‘gel sandwich’ system, i.e. using agarose gel to contain the staining system instead of the original liquid staining solution, was also examined (Finlayson et al., 1990).



**Figure 8.1 Schematic representation of the reaction sequence in the staining procedure for detecting the glycoside hydrolases (GHs) of gentianose, gentiobiose, and sucrose, in crude extract samples from ‘Showtime Spotlight’. NBT – nitroblue tetrazolium. Adapted from Dahlqvist and Brun (1962).**

Given the importance of GHs in control of carbohydrate metabolism, the aim of this chapter was to develop a methodology for detecting and characterising the GHs in gentians, so as to determine the possible relationship between enzyme activity and individual carbohydrates during carbohydrate metabolism. Once developed, it was proposed that this could be used to aid understanding of the metabolic mechanism of the unique carbohydrates and physiological roles of related GHs in gentian plants. For this aim, experiments were conducted to achieve the following specific objectives:

- 1) Determine the effects of temperature, pH and buffer solutions on the activity of GHs related to the hydrolysis of sucrose, gentiobiose and gentianose.
- 2) Determine the seasonal changes of activity of these GHs in various organs such as crown buds, storage roots, and floral buds.
- 3) Develop a methodology using PAGE and in-gel activity staining, for the identification and isolation of these GHs.

## **8.2 Materials and methods**

### **8.2.1 Plant material and cultivation**

The same plant material and cultivation as described in Section 6.2.2.1 were used in this experiment, i.e. clonally propagated plants of the gentian cultivar ‘Showtime Spotlight’

### **8.2.2 Sample collection**

#### **8.2.2.1 Effect of temperature, pH, and buffers**

For determining the effect of temperature and pH, the same samples as described in Section 7.2.1 were used, i.e. petals of florets at stage 6 (fully open; 3 replicates per treatment) collected mid-February 2011 from plants established in the field for seven years. Samples were frozen in liquid nitrogen and ground to a powder using a mortar and pestle, which were previously chilled with liquid nitrogen. The tissue in powder form was stored at -80 °C until enzymes were extracted.

#### **8.2.2.2 Seasonal changes**

The same samples of crown buds described in Section 6.2.2.1 collected during the 2007-2008 growing season were used for determining the changes of activity of GHs of sucrose, gentiobiose, and gentianose, i.e. crown buds (3 replicates per treatment) collected in May (autumn), July (winter) and September (spring) 2008, from plants established in the field for five years. Samples were frozen in liquid nitrogen and ground to a powder using a mortar and pestle, which were previously chilled with liquid nitrogen. The tissue in powder form was stored at -80 °C until enzymes were extracted.

The same samples of storage roots described in Section 6.2.2.1 collected during the 2007-2008 growing season were used for determining the changes of the activity of GHs, i.e.

storage roots with three replicates, collected in November 2007, and January, March, May, July, and September 2008, from plants established in the field for five years.

### **8.2.2.3 Changes during floral bud development**

The same samples of petals described in Section 7.2.1 were used for determining the changes of activity of GHs, i.e. petals of florets from Stage 1 to Stage 8 (Table 7.1) with three replicates, collected in mid-February 2011 from plants established in the field for seven years.

All samples were frozen in liquid nitrogen and ground to a powder using a mortar and pestle, which were previously chilled with liquid nitrogen. The tissue in powder form was stored at -80 °C until enzymes were extracted.

### **8.2.3 Glycoside hydrolase enzyme extraction**

Enzyme extractions were based on previously published methods (Cameron *et al.*, 2001; Yamada *et al.*, 2007; Obroucheva & Lityagina, 2009). In summary frozen powdered tissue (0.5 g) was mixed for 1 hour with 25 mg polyvinylpolypyrrolidone (PVPP) and either 1.0 ml Tris-HCL buffer (1M, pH 8.0) or phosphate buffer (0.2M, pH 7.0) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 3 mM dithiothreitol (DTT). The extracts were centrifuged at 4°C for 20 min at 20,000 g. The supernatant was precipitated by slowly adding solid  $(\text{NH}_4)_2\text{SO}_4$  to the supernatant to 80 % saturation (at 4 °C). The precipitate was collected by centrifugation at 20,000 g for 20 min, and then redissolved in a small volume of extraction buffer. The solution was dialyzed using dialysis tubing of 10 kDa MWCO (molecular weight cut off) against 0.01 M phosphate–citrate buffer (pH 6.0), changing dialysis buffer three times totalling 4 L in 24 h. The final volume of extract was recorded and adjusted to a known volume and stored at -80 °C. All steps were performed on ice or at 4 °C.

### **8.2.4 Glycoside hydrolase enzyme assay**

To optimize the conditions for measuring enzyme activity, the frozen enzyme extracts from petals of florets at Stage 6 (florets fully open) were thawed at room temperature. Each 37.5 µl volume of crude enzyme extract was incubated with 75 µl substrate 2-10 mM gentianose (Extrasynthese, France), gentiobiose (Sigma) or sucrose (Sigma) in 0.02 M

phosphate–citrate buffer (pH 3.0, 4.0, 5.0, 6.0, and 7.0) and 0.02 M Tris-HCL buffer (pH 8.0 and pH 9.0) at one of a range of temperatures, i.e. 4 °C, 20 °C, 30 °C, 40 °C, 50 °C, or 60 °C. Because the products of the enzymatic reaction were analysed using HPLC, under the limited conditions for the current experiments (high cost of substrate including gentiobiose and gentianose, and use of crude enzyme extracts), and based on results from preliminary pilot trials (either 0.5, 1.0, 1.5, or 2.0 h; refer 8.3.1) and previous reports (Luijendijk et al., 1998; Klotz & Finger, 2001), a 2.0 h incubation time was used in this study to obtain sufficient hydrolysis products to be measurable by HPLC. In the control reactions, substrates or enzyme extracts were omitted. The reaction was stopped by adding 112.5 µl acetonitrile (Shihabi, 1999). The reacted mixture was centrifuged at 20,000×g for 20 min to remove the precipitate, and the sugars in the supernatant were then analysed by HPLC (as described previously in Section 4.2.7). The enzyme activity was determined by the level of carbohydrates (glucose or fructose) produced from each reaction and expressed as µmol glucose min<sup>-1</sup> g<sup>-1</sup> protein.

Based on the results presented within Sections 8.3.1 to 8.3.3, all subsequent reactions for measuring enzyme activities during seasonal changes in crown buds, storage roots and petals of florets were conducted using the following optimum experimental parameters and expressed as µmol glucose min<sup>-1</sup> g<sup>-1</sup> FW (fresh weight):

GHS of sucrose: 30 °C, pH 5.0, 2.0 hours

GHS of gentiobiose: 30 °C, pH 8.0, 2.0 hours

GHS of gentianose: 30 °C, pH 6.0, 2.0 hours

Total protein content of the crude enzyme extract isolated from tissue samples was measured based on the Bradford method (Bradford, 1976), modified accordingly for use in an absorbance micro-plate reader (SpectraMax Plus384, USA), using Bio-Rad Protein Assay Kit II (Bio-Rad). The protein content was measured against a standard curve of bovine serum albumin (BSA; Sigma).

### **8.2.5 Electrophoresis and staining for glycoside hydrolases:**

The crude enzyme extract was first separated by native-PAGE based on the method of Laemmli (1970) using the BioRad mini vertical slab gel system. The mini-slab gels (length: 7 cm; width: 8 cm; thickness: 1mm), were cast according to the instruction of the

manufacturer of the Mini-PROTEAN II cell (BioRad, California, USA). Native-PAGE was conducted in a discontinuous system consisting of a 3% stacking gel containing 0.625 M Tris-HCL buffer, pH 6.8, and a 10% separation gel containing 0.375 M Tris-HCl buffer, pH 8.8. Crude enzyme extracts (10  $\mu$ l per sample; approximately 20  $\mu$ g total proteins) were mixed with 2 $\times$ sample buffer (62.5 mM Tris-HCl, pH 6.8, 40% glycerol, 0.01% bromophenol blue), and then were loaded into gel wells. The self-cast gels were run in Tris/Glycine Running Buffer (0.025 M Tris, 0.192 M glycine, pH 8.3), at 4 °C, 150 Volt for 2-3 h. The native gels were stained using coomassie blue R-250 (Westermeier et al., 2008) to visualise total proteins in the crude extract.

Glycoside hydrolase enzyme activity was detected by coupling the glucose produced in the enzyme reaction with a set of reactions (Figure 8.1) to form a coloured precipitate of reduced NBT (Doehlert & Felker, 1987; Sergeeva & Vreugdenhil, 2002). To stain for GHs of sucrose and gentianose, the separating gel, after electrophoresis, was placed in a 0.01 M phosphate–citrate buffer solution (pH 5.5) for 10 min at 37 °C, and then washed with the same buffer three times. The gel was incubated in the staining solution (0.01 M phosphate-citrate buffer pH 6.0), containing 4 mg/ml gentianose or sucrose, 0.014% PMS, 0.024% NBT, and 25 U ml<sup>-1</sup> glucose oxidase, at 37 °C until optimum staining was obtained. For staining GHs of gentiobiose, the gel was placed in a staining solution containing 4 mg ml<sup>-1</sup> gentiobiose, 0.014% PMS, 0.024% NBT, and 25 U ml<sup>-1</sup> glucose oxidase, and then incubated at 37 °C until optimum staining was obtained.

Owing to the different optimum pH for glucose oxidase (pH 5.5) and GHs of gentiobiose (pH 8.0), buffers with different pH, i.e. a 0.01 M phosphate-citrate buffer at pH 6.0 and pH 7.0 separately, and a 0.01 M tris-HCL buffer at pH 8 were used to compare the effect of pH on staining the GHs of gentiobiose. To prevent the diffusion of the coloured precipitate, a ‘gel sandwich’ system (Finlayson et al., 1990) was used to improve the effect of using staining solution. Agarose powder (Sigma) was dissolved by boiling in water to make 2% agarose, the solution was maintained at 60 °C and then combined with a solution containing 8 mg ml<sup>-1</sup> gentiobiose, 0.028% PMS, 0.048% NBT, and 50 U ml<sup>-1</sup> glucose oxidase, and immediately cast in the mini slab gels using the same system as the native-PAGE gels (length: 7 cm; width: 8 cm; thickness: 1 mm). The staining agarose gel was overlaid on the separating gel, and then incubated at 37 °C until optimum staining was obtained. All incubations were carried out with continuous gentle agitation.

### 8.2.6 Statistical Analysis

Enzyme activity data were subjected to an analysis of variance, utilising GLM (Minitab 16). Each value was expressed as the mean of three replicates  $\pm$  standard error (SE). When significant differences occurred, mean values were separated using the Tukey method ( $P < 0.05$ ).

## 8.3 Results

### 8.3.1 Optimum incubation time and extraction buffers for glycoside hydrolases

For sucrose hydrolysis, 0.5 h incubation time was sufficient to result in significant peaks during HPLC analysis when the assay conditions were 30 °C, pH 5.0 to 6.0, but hydrolysis of gentiobiose (30 °C, pH 8.0) and gentianose (30 °C, pH 6.0) needed 2.0 h incubation to obtain enough hydrolysis products to be measurable by HPLC (Figure 8.2). There was no significant difference in the activity of GHs of sucrose, gentianose, and gentiobiose for the two extraction buffers, i.e. Tris-HCL buffer (1M, pH 8.0) and phosphate buffer (0.2M, pH 7.0), used within the current experiments (Table 8.1).

**Table 8.1 Effect of two extraction buffers on the glycoside hydrolase (GH) activity of extracts of petals of ‘Showtime Spotlight at Stage 6 of development. GH activity was determined against sucrose, gentiobiose and gentianose substrates.**

Extraction buffers	Activity of GHs ( $\mu\text{mol glucose min}^{-1} \text{g}^{-1} \text{protein}$ ) at optimum pH		
	Sucrose <sup>Z</sup>	Gentiobiose <sup>X</sup>	Gentianose <sup>Z</sup>
Tris-HCL (1M, pH 8.0)	26.0 $\pm$ 2.5 a	46.7 $\pm$ 4.5 a	9.7 $\pm$ 0.9 a
Phosphate (0.2M, pH 7.0)	29.3 $\pm$ 2.8 a	41.2 $\pm$ 3.5 a	8.0 $\pm$ 1.0 a
	n = 3, $P = 0.433$	n = 3, $P = 0.390$	n = 3, $P = 0.275$

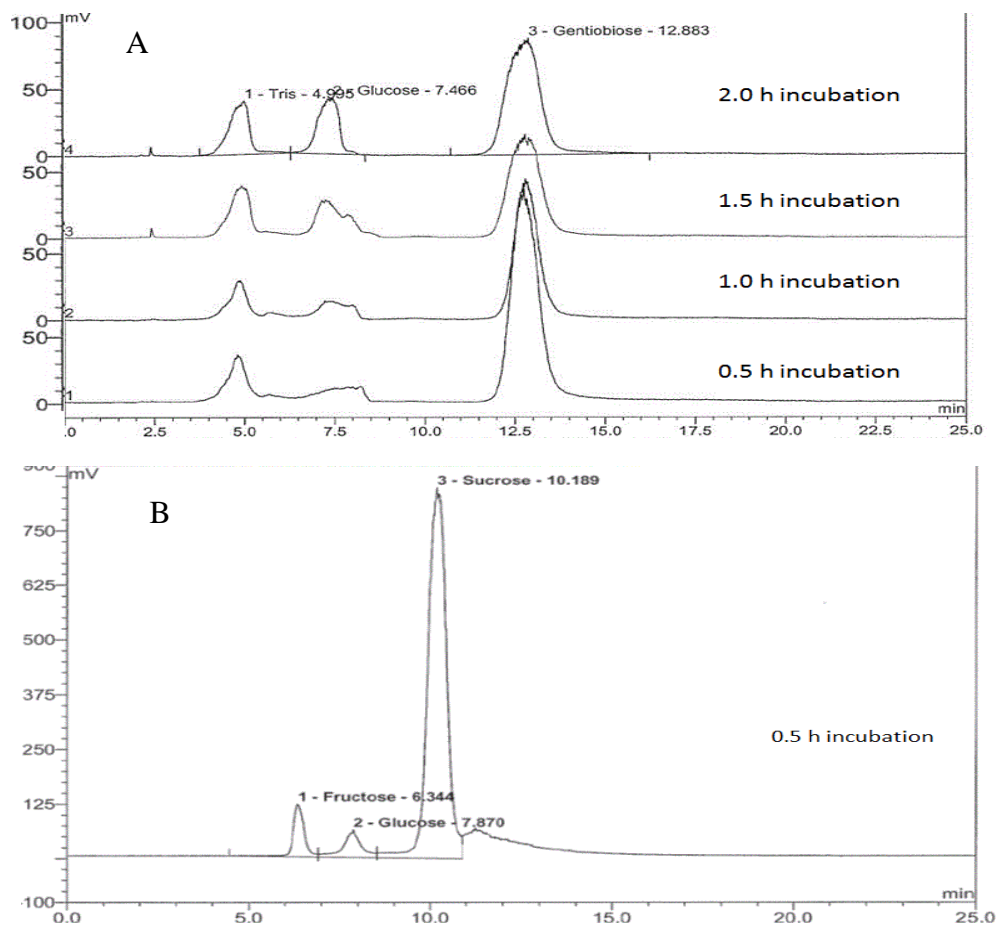
Samples were collected mid-February 2011 from plants established in the field for seven years.

Z Enzyme activity was assayed in 0.02 M phosphate–citrate buffer at optimum pH 6.0

X Enzyme activity was assayed in 1.0 M Tris-HCL buffer at optimum pH 8.0

For each column, mean values with different letters were significantly different (Tukey:  $P < 0.05$ ).

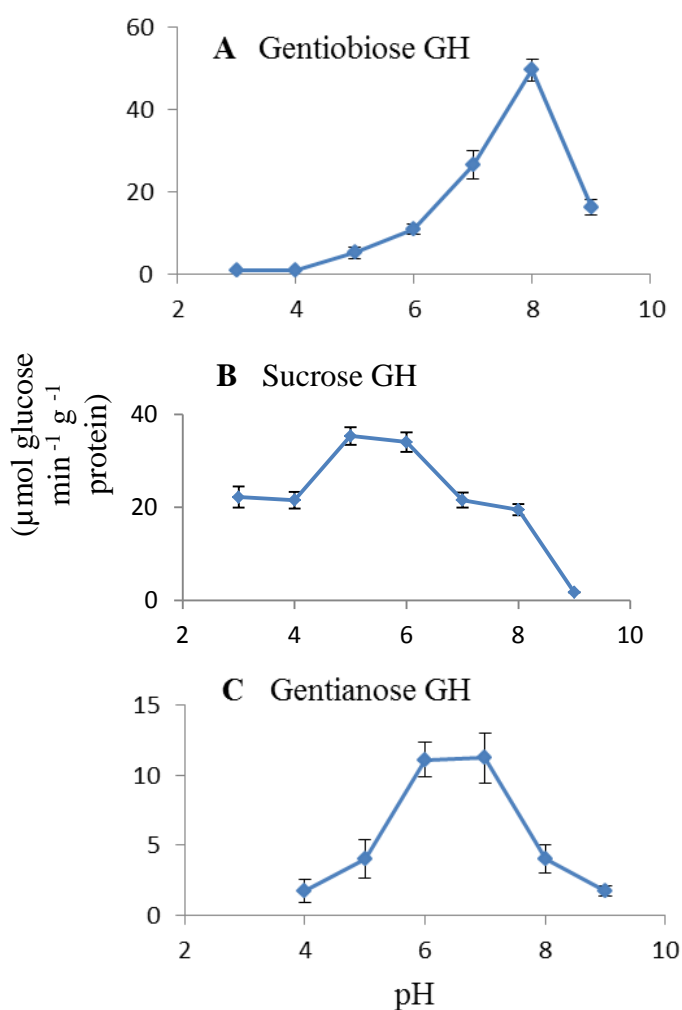
All values are means  $\pm$  SE



**Figure 8.2** Chromatograms derived from HPLC presenting the effect of incubation time on the peaks of products of hydrolysis of gentiobiose (A) and sucrose (B) from crude enzyme extracts of petal tissue of florets of ‘Showtime Spotlight’ at Stage 6.

### 8.3.2 pH optimum of glycoside hydrolases

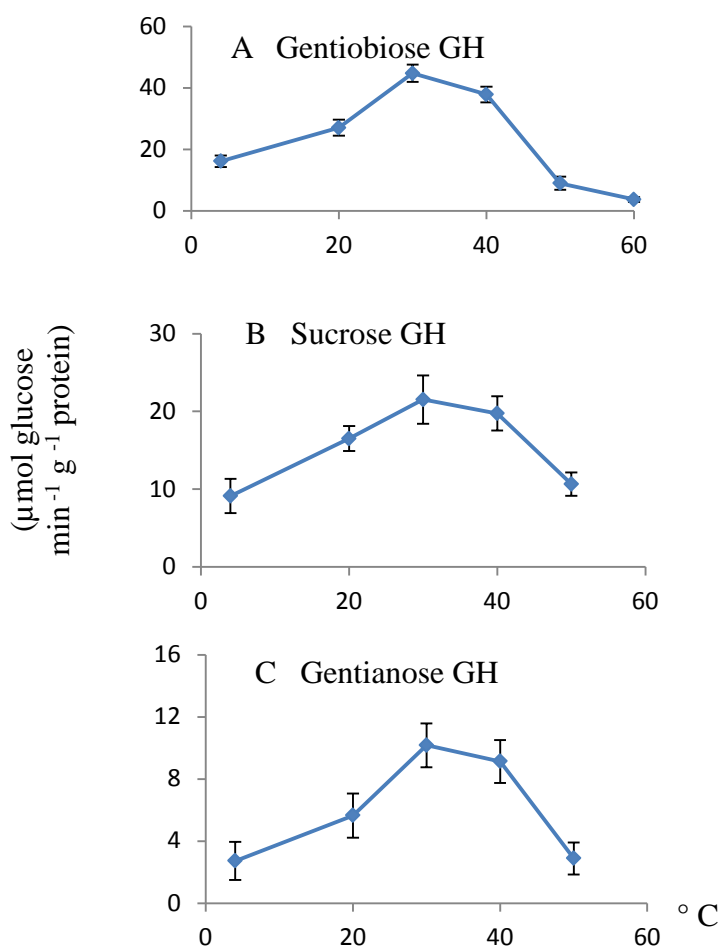
The optimum pH for the GH of gentiobiose in a crude enzyme extract isolated from petals at Stage 6 was pH 8.0 (Figure 8.3A). GH activity decreased sharply toward lower or higher pH values, with one pH unit change, and was completely lost below pH 5. In contrast, sucrose GH had a wide range in activity from pH 3.0 up to pH 8.0, and the highest activity was found at a relatively low pH between pH 5.0 and 6.0 (Figure 8.3B). With pH value increased from 8.8 to 9.0, however, the activity sharply decreased and was completely lost at pH 9.0. The optimum pH for hydrolysing gentianose was between pH 6.0 and 7.0 (Figure 8.3C), which was different from the optimum pH of both sucrose and gentiobiose GHs.



**Figure 8.3** The activity of GHs from crude enzyme extracts of petal tissue of florets of ‘Showtime Spotlight’ against; (A) gentiobiose, (B) sucrose and, (C) gentianose, at a range of pH. All reactions were conducted for 2 h incubation at 30 °C. Vertical bars are  $\pm$  SE,  $n = 3$ . Samples of petals were collected mid-February 2011 from florets at Stage 6 (i.e. fully open).

### 8.3.3 Temperature optimum of glycoside hydrolases

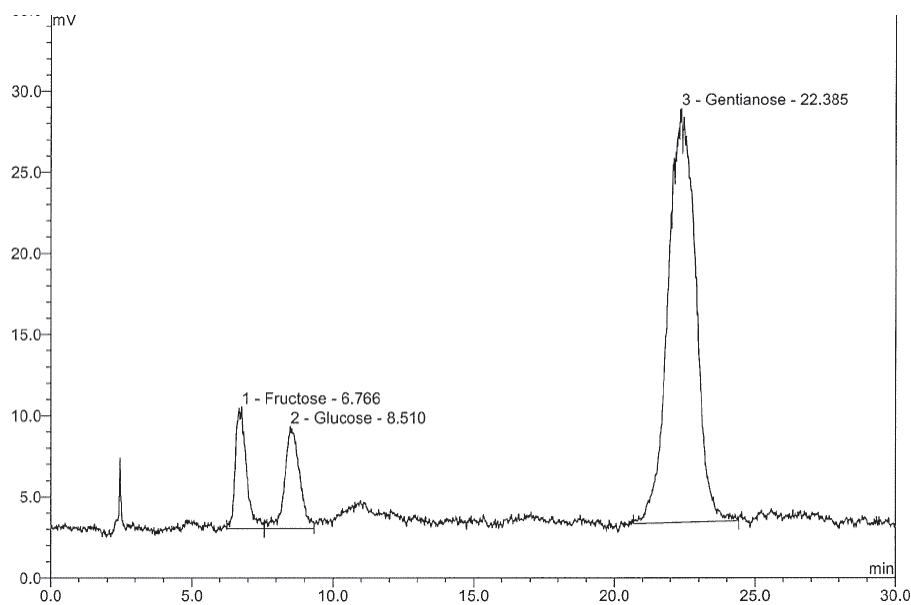
The highest activity of gentiobiose GH was found to occur between 30 °C and 40 °C (Figure 8.4A). At temperatures above 40 °C the enzyme activity dropped sharply and was almost completely lost at 60 °C. Whereas the activity of gentiobiose GH was retained down to 4 °C with approximately 30 - 40% of the activity measured at the optimum temperature of 30 °C. Both sucrose GH and gentianose GH had similar patterns of activity over the 4 °C to 50 °C range, i.e. the optimum temperature for GH activity was between 30 °C and 40 °C, and enzyme activity decreased with higher and lower temperatures (Figure 8.4B and C).



**Figure 8.4** The activity of glycoside hydrolases (GHs) against; (A) gentiobiose, (B) sucrose and, (C) gentianose, at a range of temperatures. Crude extracts were made from petals of floret tissue of ‘Showtime Spotlight’ at Stage 6. The enzyme reactions of gentiobiose were in 0.02M Tris-HCL buffer, pH 8.0, and both sucrose and gentianose were in 0.02 M Citrate-phosphate buffer, pH 6.0. All reactions were conducted for 2 h incubation. Vertical bars are  $\pm$  SE,  $n = 3$ . Samples of petals were collected mid-February 2011 at Stage 6 (i.e. florets fully open).

### 8.3.4 Products of gentianose hydrolysis

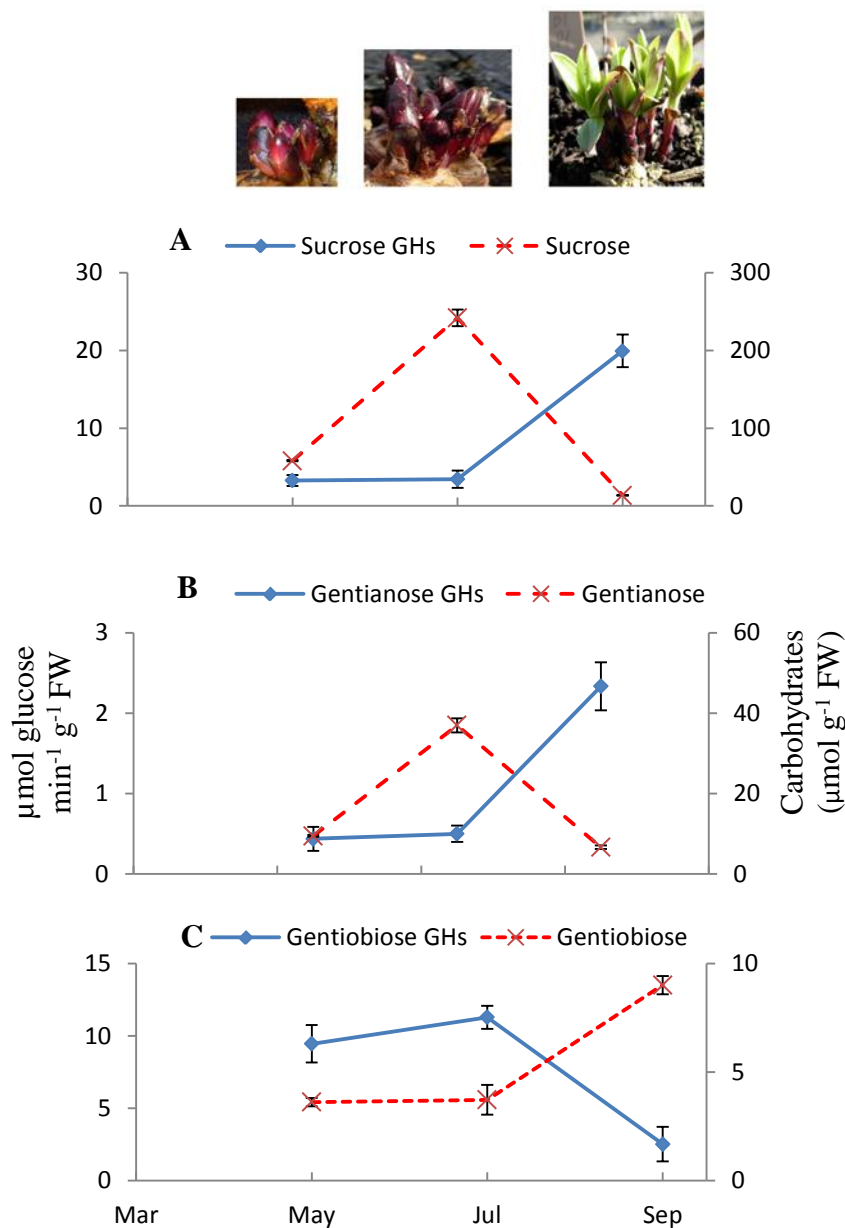
The HPLC chromatograms of the products of gentianose hydrolysis, using the crude enzyme extracts of petal tissue of florets at Stage 6 (i.e. florets fully open), showed that only monosaccharide fructose and glucose were detected, with no sucrose and gentiobiose detected, in the products (Figure 8.5). The ratio of quantities (i.e. moles, calculated using the results in current experiments) of fructose and glucose in the metabolites, was in accordance with the composition of gentianose molecules, i.e. a ratio of fructose : glucose of 1 : 2.



**Figure 8.5 HPLC chromatogram of the products of gentianose hydrolysis. Two non-structural carbohydrates, fructose (Peak 1) and glucose (Peak 2), were detected from the hydrolysis of gentianose (Peak 3), using crude enzyme extracts of petals of ‘Showtime Spotlight’ in 0.02 M Citrate-phosphate buffer for 2 h incubation at 30 °C. Tissue samples were collected mid-February 2011 from florets at Stage 6 (i.e. floret fully open).**

### 8.3.5 Changes in crown buds during the growth cycle

The enzyme activity of sucrose GH in crown buds was low in autumn and winter, and increased more than four-fold in spring, coinciding with when crown buds were re-growing at a fast rate (Figure 8.6A). This change in spring was also correlated with a 90% reduction in the sucrose content of crown buds. A similar trend was obvious between gentianose concentration and the gentianose GH activity of crude extracts from crown buds, i.e. the activity of gentianose GH remained at a low level during autumn and winter, and increased approximately five-fold in spring, with the gentianose content of crown buds dropping almost 80% in spring (Figure 8.6B). Gentiobiose concentration and the activity of gentiobiose GH were also inversely correlated (Figure 8.6C). In contrast to sucrose GH and gentianose GH, the activity of gentiobiose GH was relatively high during autumn and winter and dropped 75% in spring, with gentiobiose concentration increasing in spring almost three-fold.



**Figure 8.6** Seasonal changes in the *in vitro* activity of glycoside hydrolases (GHs) and respective non-structural carbohydrates within crown buds of ‘Showtime Spotlight’; (A) sucrose GH, (B) gentianose GH, (C) gentiobiose GH, with images illustrating typical stages of their development at these time. The reactions of gentiobiose GH were conducted in 0.02M Tris-HCL buffer, pH 8.0, and both sucrose and gentianose GHs were conducted in 0.02 M Citrate-phosphate buffer, pH 6.0. All reactions were conducted for 2 h incubation. Samples of crown buds were collected during the 2007-2008 growth cycle. Vertical bars are  $\pm$  SE, n = 3.

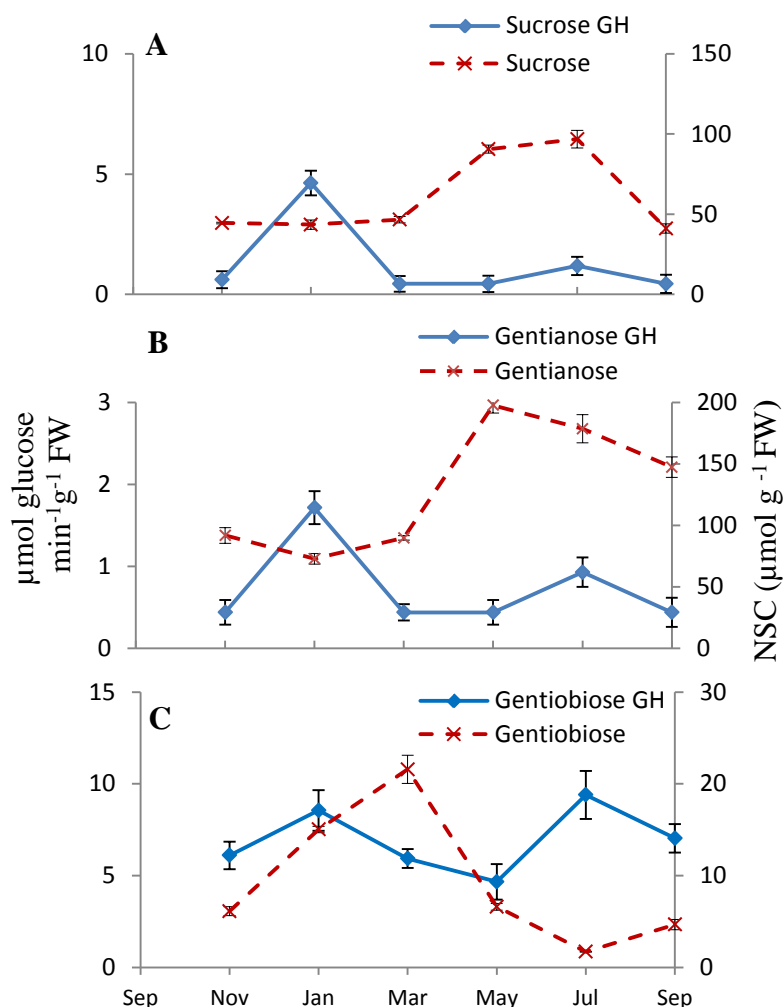
### 8.3.6 Changes in storage roots during the growth cycle

The changes in activity of all GHs for sucrose, gentianose, and gentiobiose in storage roots had one feature in common, i.e. two peaks of activity, with one in summer (January) and one in winter (July; Figure 8.7A to C). The activity of sucrose GH was at a low level in spring (November), and then increased more than seven-fold to reach its maximum in summer (January) and, by March, had dropped back to a similarly low level as in spring. During this period (from spring through to late summer) sucrose content was consistently maintained at a relatively low level. In autumn, from March to May, the activity of sucrose GH dropped to a low level, while the actual sucrose content in storage roots increased one and half fold. By winter (July), the activity of sucrose GH doubled to achieve a second small peak, while sucrose content remained stable at the relatively high level established in May. Thereafter, both sucrose content and sucrose GH decreased until spring.

The fluctuation of the activity of gentianose GH in storage roots showed almost the same pattern as sucrose GH during the annual growth cycle (Figure 8.7B). From spring (November) to summer (January) the activity of gentianose GH increased four-fold and then by March dropped back to the same level as in spring again. From spring (November) to summer (January) gentianose content was at its lowest level. In autumn, the low activity level of gentianose GH established in March was retained until May, while in contrast gentianose content doubled to its maximum by May. Thereafter, the activity of gentianose GH doubled to reach its second smaller peak in winter (July), and then dropped back; during this period gentianose content gradually decreased 30% over winter through to spring (from May to September), which was different from that noted for sucrose content in storage roots, which was retained at a high level in winter and decreased in spring (Figure 8.7A).

The activity of gentiobiose GH and gentiobiose content increased 30% and doubled, respectively, from spring (November) to summer (January). Thereafter the activity of gentiobiose GH decreased approximately 35% to its minimum in May; while the gentiobiose content continued to increase 40% further to reach its maximum in March before it dropped 70% from this maximum in May. From May to July, the activity of gentiobiose GHs doubled to its second peak in winter (July), while the gentiobiose content continuously decreased to its minimum in winter (July). Thereafter, the activity of

gentiobiose GH decreased from winter (July) to spring (September), while in contrast over the same period gentiobiose content increased (Figure 8.7C).

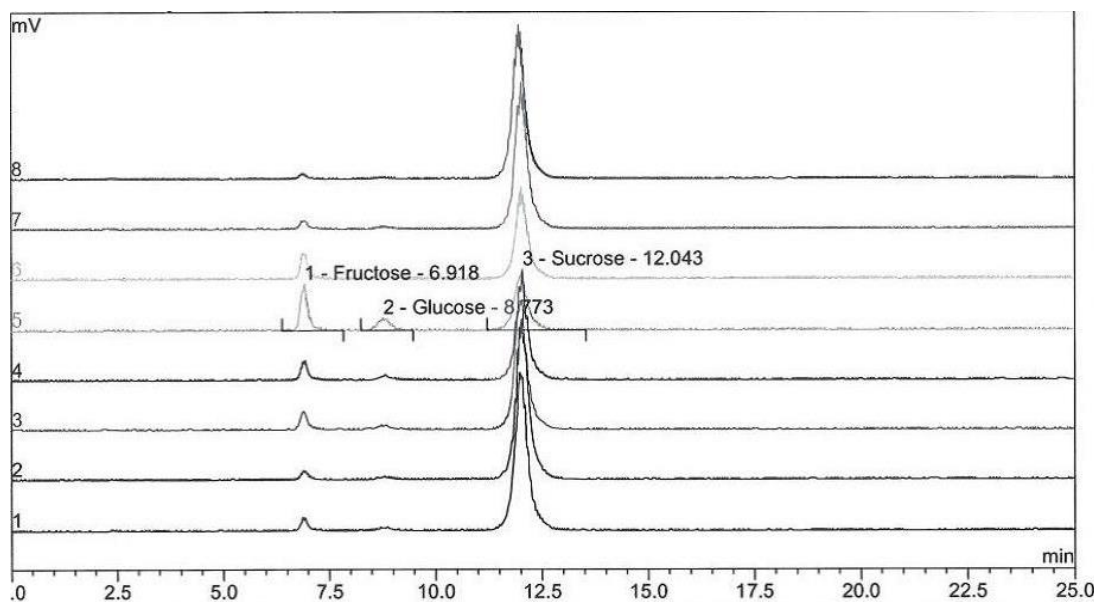


**Figure 8.7** Seasonal changes in the *in vitro* activity of glycoside hydrolases (GHs) and respective non-structural carbohydrates within storage roots of ‘Showtime Spotlight’; (A) sucrose glycoside hydrolases (GHs), (B) gentianose GHs and, (C) gentiobiose GHs, in The reactions of gentiobiose GHs were conducted in 0.02M Tris-HCL buffer, pH 8.0, and both sucrose and gentianose GHs were conducted in 0.02 M Citrate-phosphate buffer, pH 6.0. All reactions were conducted for 2 h incubation. Samples of storage roots were collected during the 2007-2008 growth cycle. Vertical bars are  $\pm$  SE, n = 3.

### 8.3.7 Changes during the development of florets

The activity of sucrose GH in petals remained stable at a relatively low level during the early stages of floret development (i.e. from Stages 1 to 4), as evident by the significant increase in products of this enzyme activity (i.e. fructose and glucose; Figure 8.8). Activity rapidly doubled to reach its maximum at Stage 5 when florets were just opening

(Figure 8.9A). While the sucrose content remained stable from Stage 1 through to Stage 5, it did not decrease, with the rapid increase of sucrose GH at Stage 5 (Figure 8.9A). Thereafter, both sucrose content and the activity of sucrose GH decreased in parallel with each other, coinciding with the progressive senescence of florets until Stage 8 (Figure 8.9A).



**Figure 8.8 HPLC chromatograms illustrating the changes in activity of sucrose glycoside hydrolase from petals of florets of different stages. Crude enzyme was extracted from florets of ‘Showtime Spotlight’ (Stages 1 to 8; stages denoted by second y-axis values). Sucrose (Peak 3) was the substrate and fructose (Peak 1) and glucose (Peak 2) were the products in the enzyme reaction. All reactions were conducted for 2 h incubation in 0.02 M Citrate-phosphate buffer, pH 6.0. Samples of petals were collected mid-February 2011.**

With development, changes in the activity of gentianose GH in petals showed almost the same pattern as with sucrose GH, with the only difference being that in general the activity of sucrose GH ( $5.98 \mu\text{M glucose min}^{-1} \text{g}^{-1} \text{FW}$ ) was approximately three-fold higher than that of gentianose GH ( $2.07 \mu\text{M glucose min}^{-1} \text{g}^{-1} \text{FW}$ , Figure 8.9B). During the early developmental stages of florets (i.e. from Stages 1 to 4), the activity of gentianose GH in petals remained stable at a relatively low level, while gentianose content rapidly increased more than ten-fold during this period, and reached its maximum at Stage 4. Along with the doubling in activity of gentianose GH from Stage 4 to 5 as florets opened, the gentianose content rapidly dropped to only 10% of that at Stage 4, and continuously declined to nearly zero at Stage 6 and beyond. The activity of gentianose GH decreased after its peak at Stage 5, coinciding with the progressive senescence of florets through to Stage 8

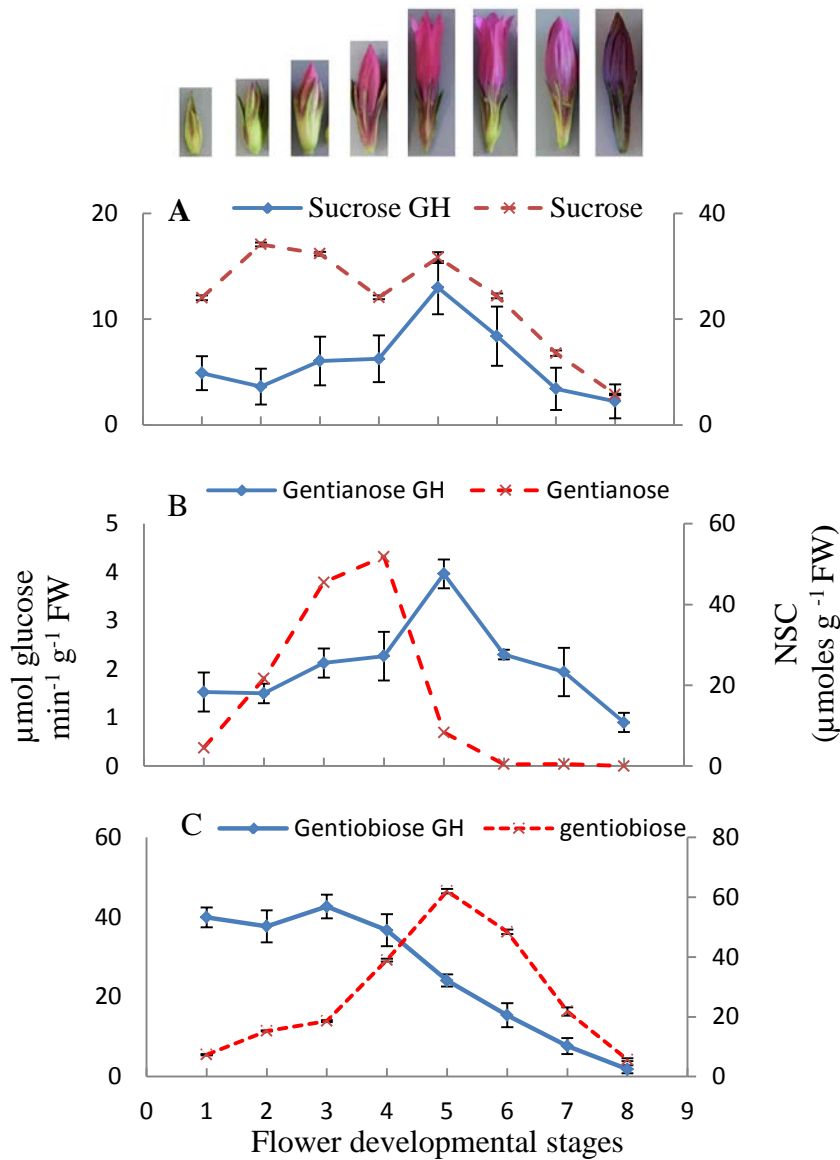
(Figure 8.9B). Worthy of additional note, was that concurrent with the rapid decrease of gentianose content and increase in activity of gentianose GH between Stage 4 and Stage 5, a rapid increase in content of gentiobiose, fructose and glucose occurred (Figure 7.5A and B).

The activity of gentiobiose GH remained at their highest level through Stages 1 to 3, with the gentiobiose content also maintained at relatively low levels during the same period (Figure 8.9C). Thereafter the activity of gentiobiose GH decreased linearly from Stage 3 (i.e. tightly closed with pink tips) to Stage 8 (florets senesced). Concurrent with these changes in enzyme activity, from Stage 3 to 5 the gentiobiose content rapidly increased and reached its maximum at floret opening (Stage 5), and then decreased linearly with the progressive senescence of florets through to Stage 8 (Figure 8.9C).

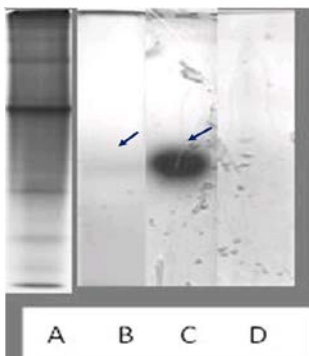
### **8.3.8 Electrophoresis and staining**

In the current preliminary experiments, the proteins in crude enzyme extracts from petals (at developmental Stage 5) were separated using 10% native-PAGE gel, and the GHs of sucrose, gentianose and gentiobiose were detected using the staining reagent. Although the staining of sucrose and gentianose GHs was not achieved, one blue-coloured band of gentiobiose GH was successfully obtained using the coupled enzymatic reactions (Figure 8.10B and C), and no protein was stained in the control, i.e. without adding gentiobiose as the substrate in the enzymatic reactions (Figure 8.10D).

In terms of developing this methodology, staining in a reaction buffer at pH 7.0 was more sensitive (i.e. showing deeper colour) than at either pH 6.0 or pH 8.0. The use of a 'gel sandwich' allowed a more direct supply of the reaction substrate to the partially purified GH enzyme compared with staining in liquid solution, and limited the dilution and dispersal of the final product, thereby significantly improving the staining sensitivity. This resulted in a clear background and a darker blue band (Figure 8.10C and B).



**Figure 8.9** Changes in the *in vitro* activity of glycoside hydrolases (GHs) and respective non-structural carbohydrates within petals of ‘Showtime Spotlight’ at differing stages of floret development; (A) sucrose GH, (B) gentianose GH and, (C) gentiobiose GH. The reactions of gentiobiose GHs were conducted in 0.02M Tris-HCL buffer, pH 8.0, and both sucrose and gentianose GHs were conducted in 0.02 M Citrate-phosphate buffer, pH 6.0. All reactions were conducted for 2 h incubation. Samples of petals were collected mid-February 2011. Vertical bars are  $\pm$  SE, n = 3.



**Figure 8.10** Detection of gentiobiose glycoside hydrolase (GH) in crude enzyme extracts from petals of ‘Showtime Spotlight’ at Stage 5. Approximately 20  $\mu$ g total proteins were electrophoresed on a 10% native-PAGE gel; (A) total proteins stained using Coomassie blue R-250, (B) gentiobiose GH stained in liquid solution (black arrow), (C) gentiobiose GH stained in ‘gel sandwich’ (black arrow), (D) Control, ‘gel sandwich’ without adding substrate gentiobiose.

The staining method used in the current study has also been used for *in situ* localization of enzymes involved in carbohydrate metabolism in plant tissues (Sergeeva & Vreugdenhil, 2002; Sayago et al., 2008), making it possible to visualize the enzyme activity of GHs in tissue sections of various organs of gentians. In contrast to immunological methods that do not distinguish active enzymes from inactive enzymes, this method only detects activated enzymes. Owing to limited time, *in situ* staining of enzymes related to sucrose, gentiobiose, and gentianose, was limited in its application within the current experiments. For completeness, however, the results were included within the appendices (Appendix V).

## **8.4 Discussion**

The identity and physiological function of plant GHs, hydrolysing oligo- or polysaccharides, have been reported for many other plant species (Henrissat et al., 2013). This includes invertase (for sucrose hydrolysis) in *Beta vulgaris* (Sugar beet), amylase (for hydrolysis of starch) in rose (Hammond, 1982), and fructan 1-exohydrolase (for hydrolysis of fructan) in *Campanula rapunculoides* (Le Roy et al., 2007). In contrast, no published information of the GHs in gentian was previously available. As discussed further in the following sections, this chapter reports for the first time:

- 1) the temperature and pH optima for GHs that hydrolyse gentianose, gentiobiose, and sucrose in gentian;
- 2) development of a staining method for detecting in-gel activity of gentiobiose GH, and;
- 3) based on the comparison of the changes in the activity of GHs and content of related carbohydrates during plant development, possible roles of these GHs in carbohydrate metabolism.

The detection of glycoside hydrolase activity in gentians and comparison with fluctuations of carbohydrate content, reveals information about a possible regulation mechanism for carbohydrate storage and utilization in tissues of gentian, as well as their physiological function. During the development of florets, the rapid increase of activity of gentianose GH was detected when the gentianose content declined during floret opening (Figure 8.9B). The activity of sucrose GH rose rapidly during floret opening (i.e. Stages 4 to 5), but sucrose concentration did not decrease (Figure 8.9A). A similar result was reported in rose, where it was hypothesized that the sucrose GHs hydrolyse the sucrose to fructose

and glucose and, at the same time, sucrose was continuously imported to petals from leaves, thereby achieving a balance in sucrose content (Yamada et al., 2007). If applicable to the petals of 'Showtime Spotlight', collectively the increased activity of both gentianose and sucrose GH, combined with the decrease of gentiobiose GH, resulted in the increase of gentiobiose, fructose and glucose concentrations and, in turn, the highest molar concentration of total carbohydrates.

The current results add further information to our understanding of floret opening in gentian, realised previously in Chapter 7 by interpreting carbohydrate changes alone, and also support the hypothesis of the synchrony between a range of enzymes to regulate carbohydrate concentration to meet the requirements of floret development. In addition, this result, showing the very clear interconnection between the various NSC and enzyme activity during the development of florets, highlights the value of using the very tightly defined and rapidly changing stages of floret development as an experimental system in the study of carbohydrate metabolism and their physiological functions in gentians. It is recognised that experiments utilising radioactively labelled carbohydrates would assist confirming the hypotheses associated with metabolism and movement developed from the current data.

In crown buds, sucrose content increased and became the predominant carbohydrate in winter, which was accompanied by a low activity of sucrose GH. During spring, sucrose content rapidly decreased and was accompanied by a rapid increase in sucrose GH activity. These changes in spring also coincided with the period when crown buds started to re-grow and developed new leaves (Figure 8.6B). Similarly the rapid increase of gentianose GHs closely was correlated with a rapid decrease of gentianose content in spring (Figure 8.6A). In contrast, the rapid decrease in activity of gentiobiose GHs in spring coincided with an increase of gentiobiose content (Figure 8.6C). These close relationships between each GH and its related carbohydrate in crown buds, can be used to imply that the changes of carbohydrate content were controlled and catalysed by the GHs (Irving et al., 1997; Minic, 2008) for the purpose of shoot growth and development in spring.

In storage roots, gentianose was the predominant carbohydrate in all seasons. The highest activity of gentianose GH was in summer, which corresponded to the lowest content of gentianose. In contrast, the lower levels of activity of the gentianose GH in autumn were associated with a near doubling in the content of gentianose. In winter, the increased

activity of gentianose GH accompanied a decrease of gentianose content (Figure 8.7B). While the activity of sucrose GH presented the same seasonal pattern of change as gentianose GH, the difference being that the sucrose content in winter did not decrease with the increase of the activity of sucrose GH (Figure 8.7A). This was interpreted as supporting the hypothesis that the hydrolysis of gentianose may produce sucrose, compensating for the hydrolysis of sucrose by sucrose GH. This is supported by previous reports that found the importation of sucrose into rose petals maintained a stable net concentration of sucrose in the tissues despite the activity of invertase being increased (Yamada *et al.*, 2007). The gentiobiose content had a reverse pattern of changes in activity to that for gentiobiose GH, i.e. high activity of gentiobiose GH corresponding to low gentiobiose content (Figure 8.7C).

A clear mechanism of carbohydrate storage and utilization and physiological function in gentian becomes apparent by comparing the changes of GHs with related carbohydrates in both crown buds and storage roots. Plants of ‘Showtime Spotlight’ stored gentianose in storage roots in autumn and, in winter, when no newly synthesised carbohydrate is available from leaves, part of the gentianose stored within the storage roots is hydrolysed by GH for both respiration and mobilization to crown buds. These carbohydrates, once moved to the crown buds, presumably may then be used to; restore the carbon source utilised for growth and respiration, increase the osmotic potential as a protectant against cold weather, and/or maintain crown bud dormancy (Chao *et al.*, 2006). In spring the GHs hydrolyse the stored carbohydrates again, thereby providing the necessary carbon source and energy for re-growth.

### **What is the potential identity of the enzymes?**

Owing to the structural diversity of carbohydrates with respect to diversity of monosaccharides and their linkages (refer to Section 2.5.1), there is a vast spectrum of enzymes with specificities involved in carbohydrate biosynthesis, breakdown and modification (Cantarel *et al.*, 2009). Currently, the Carbohydrate-Active Enzyme (CAZy) database has described up to 132 families of GHs, based on the similarity of amino-acid sequences (Henrissat *et al.*, 2013) and, in *Arabidopsis* for example, GHs are represented by 379 genes/proteins, classed in 29 families (Henrissat *et al.*, 2001). Unlike the widely studied enzymes related to the hydrolysis of carbohydrates in other plant species that

contain sucrose, starch, and fructan, there is very little information available for GHs in gentians that act on the unique carbohydrates, i.e. gentianose and gentiobiose.

Sucrose is a major product of photosynthesis and the principal form of translocated carbon to sites of utilisation or storage in sink organs (ap Rees, 1984). Utilization of sucrose in plants as a carbon and/or energy source requires its breakdown into monosaccharides, i.e., glucose and fructose; this process is catalysed by either sucrose synthase (EC 2.4.1.13) or invertase (EC 3.2.1.26) in plants (Sturm, 1999). Sucrose synthase is a glycosyl transferase that converts sucrose into UDP-glucose and fructose under the presence of uridine diphosphate (UDP), while invertase directly cleaves sucrose into glucose and fructose. In higher plants, invertases are classified into three types (Sturm, 1999; van Doorn & Woltering, 2008; Kim *et al.*, 2010):

- 1) Insoluble acid invertases bound to the cell wall and involved in the ‘loading and unloading’ of sucrose in long-distance transport. Optimum activity ranging from pH 3.5 to 5.6.
- 2) Acid soluble invertases located in vacuoles related to the breakdown of storage sucrose. Optimum activity ranging from pH 3.5 to 5.6.
- 3) Neutral and/or alkaline invertases located within the cytoplasm with optimum activity close to pH 7.0 to 8.0 and possibly involved in regulation of intracellular glucose and fructose levels.

It is acknowledged that pH is one of the most important factors influencing enzyme activity (Luijendijk *et al.*, 1998; Dignum *et al.*, 2001; Klotz & Finger, 2001), and optimum pH is an important characteristic of any enzyme. In the current experiments therefore, the highest activity of sucrose GHs in crude extract from ‘Showtime Spotlight’ was found between pH 5.0 and 6.0 (Figure 8.2 B), which is in accordance with the optimum pH of soluble acid invertases reported in other plants (Klotz & Finger, 2001; Obroucheva & Lityagina, 2009). The crude enzyme extract also had a wide range in activity up to pH 8.0, which implies the possible existence of neutral or alkaline invertase within the crude extract that present activity at high pH. In the current study therefore, using the soluble crude enzyme extract, types 2 and 3 of the above list of invertase isoforms were considered most probably included in these measurements of invertase activity in ‘Showtime Spotlight’. In order to identify if any isozymes of neutral or alkaline invertase

exist in gentian, further experiments involving the isolation and purification of enzymes are needed.

Gentiobiose is a disaccharide composed of two units of D-glucose joined with a  $\beta$  (1 $\rightarrow$  6) linkage. It was reported that gentiobiose can be broken down into two equivalents of glucose by  $\beta$ -D-glycosidase extracted from *C. sinensis* (Cameron *et al.*, 2001). The same enzyme catalysed reaction ( $\beta$ -D-glycosidase) was also reported in a bacterium of *Flavobacterium* (Sano *et al.*, 1975). In contrast, in the bacterium *Aerobacter aerogenes* the cleavage of gentiobiose was first catalysed to form gentiobiose monophosphate [6-O-phosphoryl- f-D-glucopyranosyl- (1 $\rightarrow$  6) -D-glucose] by a  $\beta$ -glycoside kinase, in the presence of adenosine 5'-triphosphate (ATP), and then a phospho- $\beta$ -glucosidase catalysed the hydrolytic cleavage of gentiobiose monophosphate to form equimolar amounts of D-glucose and D-glucose 6-phosphate (Palmer & Anderson, 1972). For 'Showtime Spotlight' tissue samples the GH hydrolysing gentiobiose had a narrow pH range with an optimum of pH 8 (Figure 8.2A). These results differed from those reported for other plant species, including the  $\beta$ -glycosidase extracted from fruit tissue of *C. sinensis* (Cameron *et al.*, 2001), which could hydrolyse gentiobiose, and had optimum activity at pH 5.0. Also at variance to the current results, pH 5.8 was reported as the optimum pH for a  $\beta$ -1,6-glucosidase glycosidase extracted from the microorganism *Flavobacterium* (Sano *et al.*, 1975). The narrow pH range noted in the current experiments was also different from the broad pH optima, ranging from 6.0 to 8.5 noted for *Catharanthus roseus* (Luijendijk *et al.*, 1998). Other non-specific  $\beta$ -glycosidase enzymes in plants usually have their optimum pH between 4.0 and 6.5 (Marquez & Waliszewski, 2008). As a result of the current experiments therefore, it is hypothesised that the enzyme responsible for hydrolysis of gentiobiose in 'Showtime Spotlight', and perhaps *Gentiana* itself, may be very different from the other  $\beta$ -glycosidase enzymes in terms of pH stability. Clearly future research targeted at first purifying and identifying the enzyme responsible for hydrolysis of gentiobiose would assist in preparing to examine this hypothesis.

Gentianose is a trisaccharide. In structure, one molecule of gentianose can be broken down to one molecule of glucose and one of sucrose, or to one molecule gentiobiose and one of fructose (Figure 2.3). Although it was reported that gentianose could be broken down to one unit of fructose and one unit of gentiobiose by invertase ((Bourquelot & Herissey, 1901), as cited by (Badenhuizen *et al.*, 1964)), or one unit glucose and one of sucrose by

emulsin (Alagarsamy, 2012), it is still not clear what the products of the hydrolysis of gentianose are in gentians. In the current experiments the optimum pH for hydrolysing gentianose was between pH 6.0 and 7.0 (Figure 8.3); this result was different from the optimum pH of both sucrose and gentiobiose hydrolysing enzymes, and no previous published reports were found that made any comparison for the optimum pH for gentianose GHs. Only fructose and glucose were detected by HPLC after gentianose hydrolysis in the current experiments. The reason that gentiobiose or sucrose was not detected may have been due to their rapid hydrolysis by invertase or gentiobiose GH in the crude enzyme extract. In the current study, the results derived from measuring enzyme activity using crude enzyme extract, were inevitably influenced by other components such as other enzymes, or co-factors, inhibitors or activators, etc. (Halaba & Rudnicki, 1989). Purifying and further biochemical analysis of these enzymes therefore, is a vital next step in any further study, to provide definitive information in terms of the products and process of gentianose hydrolysis.

Temperature is another important factor that affects enzyme activity (Copeland, 2000; Daniel *et al.*, 2010). Determining the optimum temperature where maximum activity occurs is crucial for characterizing an enzyme, measuring its activity and understanding its physiological role in plants. In the current experiment the optimum temperature for both the sucrose and gentianose GHs were the same, i.e., 30 to 40 °C (Figure 8.5B and C). This is similar to the optimum temperature of 35 °C determined for sugar beet GH (Klotz & Finger, 2001), while being lower than the 45 °C optimum for invertase in rose and 60 °C for invertase in *Saccharomyces cerevisiae* (Tanriseven & Dogan, 2001). Gentiobiose GHs exhibited highest activity at the optimum temperature of 30 °C (Figure 8.5A), which is close to the 38 °C optimum of  $\beta$ -glucosidase (Marquez & Waliszewski, 2008), but lower than both the 40 – 50 °C optimum of  $\beta$ -glucosidase found in *C. sinensis* (Cameron *et al.*, 2001) and 40 °C of  $\beta$ -glucosidase in *Flavobacterium* (Sano *et al.*, 1975). In the current experiment even at 4 °C all these GHs still retained approximately 30 to 40% of the activity measured at the optimum temperature of 30 °C (Figure 8.5A, B and C). This means that *in vivo* these GHs still could be expected to achieve a functional level of activity of carbohydrate storage and utilization during the cold of winter in NZ. It is noted, however, that although samples were collected at different times of the year, the measurement in current experiments was conducted under optimum conditions, i.e., optimum pH and temperature, and the specific data therefore, would not necessarily reflect

the real condition where gentian plants grow in the field. Collectively, the optimum reaction temperatures for GHs of sucrose, gentiobiose and gentianose in ‘Showtime Spotlight’ were relatively low, and may be useful to explain the physiology underlying the growth and development of gentians with respect to climate, i.e. growth is optimal in regions with cooler climates, with an average optimum temperature range from 16 °C – 25 °C, and plant growth is retarded above 25 °C (Ohteki, 1982; Ohkawa, 1989). It was reported that as compared with natural conditions outside, the warmer temperatures of a heated greenhouse did not change the flowering schedule of ‘Showtime Spotlight’ (Samarakoon et al., 2012b). This result, as discussed in Chapter 6, may be due to that the temperature within the protected environment in Samarakoon’s experiments was higher than the range that gentian plants respond to for changes in growth and development; however, the mechanism underlay this phenomenon is not known. If link this phenomenon to the result in the current chapter, i.e., the highest activity of GHs occurred at a range of relatively low temperatures, it will be an interesting topic to determine whether the influence of temperature on enzyme activity is related the response of gentian plants to environmental temperature in their growth and development.

While it has been reported that different extraction and reaction buffers may affect enzyme extraction and activity (Doehlert & Felker, 1987; Dignum et al., 2001), in the current series of experiments no significant difference was noted between the two extraction buffers (i.e. Tris-HCL buffer (1M, pH 8.0) and phosphate buffer (0.2M, pH 7.0)) on the activity of these GHs (Table 8.1). It is acknowledged, however, this may be due to the limitation of evaluating only two buffers at two pH levels, so a wider range of buffers and pH need to be examined in future studies, before reaching a more definitive answer.

As discussed above, while assays of enzyme activity using crude enzyme extracts are convenient and less time-consuming, such assays do not distinguish isozymes and, avoid the effects of other substances in crude extracts (Minton, 2001). It is recognised that the characteristics of enzymes are only ever clearly and reliably revealed using purified enzymes, and isolation and purification of enzymes is, therefore, vital to study enzyme characteristics, and to determine their amino acid sequences (Lodish et al., 2003). These strategies remain to be explored using gentian as an experimental plant.

Electrophoretic separation of proteins is most commonly performed in polyacrylamide gels. In native-PAGE, the proteins remain in their native state, so were able to be

visualised not only by general protein staining reagents, but also by specific enzyme activity-linked staining. Preliminary experiments were able to successfully stain one gentiobiose GH polypeptide using coupled enzyme reactions to produce a blue coloured NBT precipitate. This also indicated that possibly only one polypeptide that has activity, hydrolysing gentiobiose into glucose, existed in the crude protein extract, while the broad band presented in the current experiments implies this hypothesis needs to be further examined using a higher sensitive gel electrophoresis such as two dimensional and three dimensional gel electrophoreses. Utilisation of the ‘Sandwich gel’ method resulted in a clearly stained protein band of gentiobiose GH by limiting the diffusion of blue-stained glucose product in the gel (Figure 8.11). This method now provides a useful mechanism for further isolating and purifying gentiobiose GHs in gentians. The failure of this method to stain sucrose and gentianose GHs, however, may have been due to;

- *Limited mobility of large multimeric proteins.* In native-PAGE, protein-protein interactions are retained during separation, wherein some proteins may form large multi-subunit complexes and cannot move in the separating gel; for example a 500 to 550 kD multimer of invertase was detected in native-PAGE in the seeds of *Aesculus hippocastanum* (Obroucheva & Lityagina, 2009). If this is also true for GHs in gentian, the larger pore sizes of agarose gels may prove useful to overcome such problems during electrophoresis.
- *Denaturation.* The sucrose and gentianose GHs were denatured during running the gel. If so, to retain the activity of these enzymes, alternative gels and buffers need to be evaluated to find the suitable system.
- *Co-factors.* Many enzymes depend on the presence of cofactors for catalysing biochemical reactions. Such cofactors include metal ions, organic molecules and metal organic complexes, and can be classified as co-enzymes (loosely bound) and prosthetic groups (tightly bound). Loss of these essential elements for the enzyme reaction may occur during electrophoresis. Hence, the effect of adding some of cofactors in the staining reaction solution may be worthy of attempting.
- *Presence of inhibitors.* Further steps to remove inhibitors before native-PAGE without damaging the GH activity may be necessary.
- *pH.* The reaction pH may be further optimised to promote the enzymatic reactions.

## **8.5 Conclusion**

The activity of GHs in gentian was closely related to the changes in carbohydrate metabolism, and potentially plays an important seasonal physiological function during plant growth and development. The in-gel staining technique for GHs in native-PAGE gels provides a method for isolation and purification of gentiobiose GHs. Although crude enzyme extract was used, the basic characteristics of GHs such as the effects of temperature and pH provided valuable descriptive information to assist further study into the role of these GHs in gentians.

## Chapter 9 General discussion

### 9.1 Introduction

Gentian is regarded as a potential new crop for export as a cut flower in New Zealand (NZ). With novel cultivars being developed and introduced in NZ, the gentian industry has been well supported. However, fundamental knowledge of plant development and physiology is also required, so as to continue to underpin the technology for improving sustainable production of this crop.

It was hypothesized that the availability of carbohydrate is a limiting factor influencing the development of crown buds, winter survival, spring re-growth, and the development of flower buds; this in turn influences the yield and quality of flowering shoots of gentians in commercial production. Studies within this thesis, therefore, mainly focused on the effect of carbohydrate supply (experimentally induced) on development of gentian plants (Chapters 4 and 5), distribution and seasonal (natural) changes of non-structural carbohydrates (NSCs) in various organs (Chapters 3, 6 and 7), and activity of glycoside hydrolases (GHs) that contribute to the breakdown and utilization of stored NSCs (Chapter 8).

In the current chapter, the main findings from all experimental chapters are summarized and discussed. The aim of the thesis was to understand the physiological function of NSCs, their metabolism, and their relevance to the growth and development of perennial gentians (i.e. over-wintering, spring re-growth, and the development of flowers and crown buds), to provide valuable information for improving the commercial production of flowering shoots. Further possible studies, based on the findings in the current study, are also raised in this chapter.

### 9.2 Unique NSCs

Plants contain a wide range of NSCs that vary in different species; however, the most common NSCs are glucose, fructose, sucrose and starch (Lewis, 1984b; Miller, 1992; Zeeman et al., 2010). The unique soluble carbohydrates, gentianose and gentiobiose, were previously reported as occurring in storage roots of a medicinal gentian, i.e. *Gentiana lutea* L. (Bridel, 1911; Badenhuizen et al., 1964; Keller & Wiemken, 1982). Within the

current study, using HPLC and NMR analysis (Chapters 4, 5, 6, and 7), it was confirmed that, in addition to sucrose, fructose and glucose, both gentianose and gentiobiose and L-bornesitol existed in all organs of the new selections of gentians for cut flower production (i.e. ‘Showtime Spotlight’, ‘Showtime Diva’, and the genotype 03/04-114, with parents including *G. triflora* and *G. scabra*); but no starch was detected. Although Zhang & Leung (2001) reported the presence of starch in petals of open flowers of *G. triflora*, considering the maximum concentration of starch they reported (approximately 4 mg g<sup>-1</sup> FW), which was much less than the maximum concentration of gentianose in petals (25 mg g<sup>-1</sup> FW) and almost negligible when compared with the maximum concentration of gentianose in storage roots (106 mg g<sup>-1</sup> FW) in the current study, it was concluded that these new selections of gentians typically contain little if any starch.

Relatively high concentrations and significant fluctuation of gentianose, gentiobiose and sucrose, occurred in various organs during the annual growth cycle (Figures 6.4 to 6.10) and experimental treatments affecting carbohydrate supply (i.e. defoliation: Figure 4.4 and *in vitro*: Figure 5.4), indicated that they were the main storage NSCs (both long-term and/or temporary) for gentians (Table 9.1). In contrast, fructose and glucose were present at low concentrations (less than 8.2 mg g<sup>-1</sup> FW in all organs over an annual cycle), with little changes due to treatments (Figures 4.4 and 5.4) and over seasons (Figures 6.4 to 6.10). This is interpreted as indicative that fructose and glucose in gentian are most typically directly involved in metabolic activities rather than existing as storage carbohydrates.

The predominant NSCs varied in gentians, dependent upon the organ. For example, gentianose was predominant in storage roots, rhizomes and petals; gentiobiose in stem tissue of floral shoots; and sucrose in leaves, crown buds and rhizomes (Table 9.1). As discussed in Chapters 6 and 8, the changes in content of NSCs and enzyme activity indicated that gentianose in storage roots may be the main carbon source for initial re-growth in spring. Storage roots were the main storage organs, accounting for approximately 80% of biomass (either fresh weight or dry weight) of crowns (comprised of crown buds, rhizomes and storage roots; Table 3.1). Of the individual NSCs reserved within all organs of gentians, gentianose was present at the highest concentration in storage roots at the end of autumn (Table 9.1). The highest concentration of total non-structural carbohydrate (TNC) also occurred in storage roots in autumn; and gentianose

was the predominant NSC over an annual growth cycle (Figure 4.4; Figures 6.4 to 6.10). Taken together, these data indicate that gentianose, as a trisaccharide with the highest degree of polymerization within the major NSCs present within gentians was, therefore, the predominant storage NSC. This result also implies that gentianose should be considered as a new member of the group of storage carbohydrates in plants, playing similar roles in gentians as starch, fructan or raffinose do in other plant species (Peterbauer & Richter, 2001; Orthen & Wehrmeyer, 2004; Walton et al., 2007).

**Table 9.1 Maximum concentration per unit fresh weight (FW) of total non-structural carbohydrates (TNC) and individual non-structural carbohydrates (NSCs) in various organs of 'Showtime Spotlight' over the 2010-2011 growth cycle<sup>Z</sup>.**

Organ	TNC (mg g <sup>-1</sup> FW)	Predominant NSCs	Concentration (mg g <sup>-1</sup> FW)	Timing
Petals	50 ± 1.6 <sup>Y</sup>	Gentianose	25 ± 1.1	Just before opening (Stage 4; Table 7.1)
Leaves	43 ± 5.7	Sucrose	14 ± 2.6	Early summer
Stems of floral shoots	48 ± 1.9	Gentiobiose	38 ± 1.1	Early summer
Crown bud	42 ± 5.2	Sucrose	23 ± 1.6	Winter
Rhizomes	64 ± 3.7	Sucrose	32 ± 2.1	Late autumn
		Gentianose	31 ± 3.1	Late autumn
Storage roots	153 ± 12.8	Gentianose	106 ± 15.8	Late autumn

<sup>Z</sup> Data derived from Chapters 6 and 7.

<sup>Y</sup> Mean values (± SE).

As a trisaccharide, structurally one molecule of gentianose can be broken down to one molecule of glucose and one of sucrose, or to one molecule of gentiobiose and one of fructose (Figure 2.3). Although it was reported that *in vitro* gentianose was broken down to one unit of fructose and one unit of gentiobiose by invertase ((Bourquelot & Herissey, 1901) as cited by (Badenhuizen et al., 1964)), it is still not clear *in vivo* what the products of the hydrolysis of gentianose are in gentians, or if gentiobiose is the product of hydrolysis of gentianose. In the current study (Chapter 6) a synchronized trend change, in reverse direction, between concentrations of gentianose and gentiobiose was found within storage roots (Figure 6.12A and B) and rhizomes (Figure 6.12C) and, therefore, it was speculated that gentiobiose is the product of gentianose hydrolysis in gentians. However, experiments using petal tissue during floret development (Chapter 7) indicated that the hydrolysis of gentianose alone was not sufficient to produce the amount of gentiobiose

present, and also both gentianose and gentiobiose concentration showed a synchronous increase before floret opening (Figure 7.5). Gentiobiose, therefore, may be produced independent of gentianose, or at least is not likely to be derived from gentianose hydrolysis alone. Studies in Chapter 8, detecting the activity of gentianose GH and products of gentianose hydrolysis, further supported this reasoning. Firstly, the optimum pH of the GH for hydrolysing gentianose was between pH 6.0 and 7.0 (Figure 8.3C), which differs from the optimum pH (pH 5.0 to 6.0; Figure 8.3C) of soluble invertase (a mix of acid and alkaline invertases) proposed to hydrolyse gentianose into gentiobiose. Secondly, from the HPLC chromatograms in the current experiments (Figure 8.5), only fructose and glucose, but not gentiobiose, were detected in the products of gentianose hydrolysis, using the crude enzyme extract from petal tissue. It needs to be noted that the results using crude enzyme extract, is inevitably influenced by other components such as other enzymes, or enzyme inhibitors or activators, etc. (Halaba & Rudnicki, 1989). Purifying these enzymes, therefore, is seen as a vital next step towards confirming the products and process of gentianose hydrolysis.

L-bornesitol (1-*L-O*-methyl-*myo*-inositol), as a cyclitol, is another unique chemical compound of polyols found in gentians (Schilling, 1976). As a methyl derivative of *myo*-inositol (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>), L-bornesitol has only been detected in a few plant families (Plouvier, 1963) including the Gentianaceae (Schilling, 1976; Ichimura et al., 1999). Polyols, as a carbon sink, are closely associated with the metabolism of other carbon metabolites, and some studies of carbohydrate metabolism in other plant species have included bornesitol within the pool of NSCs (Ichimura & Suto, 1999). Due to the absence of a pure standard of L-bornesitol for HPLC measurement, the measured value (fructose equivalent) presented within the current study cannot reflect the actual L-bornesitol concentration and, therefore, L-bornesitol was not included in calculation of TNC. To complement this omission of information, further experiments are needed to quantify the concentration of L-bornesitol in gentians. The current study, however, confirmed that L-bornesitol was present in all organs of the cultivars and genotypes of gentians used in this thesis, with fluctuations monitored over the seasons (Chapters 4, 5, 6, and 7). The function of polyols such as *myo*-inositol and L-bornesitol has recently attracted attention in relation to carbon metabolism and resistance to stresses such as heat, freezing, dehydration or high salinity (Merchant & Richter, 2011). In the current study, the significant peaks of L-bornesitol concentration in crown buds and rhizomes in winter are consistent with its potential

function with cold tolerance (Figure 6.11A). It has been reported that a galactosyl derivative of *myo*-inositol, galactinol, acts as a galactosyl donor in the synthesis of raffinose (Joersbo et al., 1999). While, as another derivative of *myo*-inositol, if and how L-bornesitol is involved in carbohydrate metabolism in gentians is worth further experimentation in a future study.

In addition to the summary and discussion above, the existence of unique carbohydrates also opens various channels for future study. For example:

- can these unique carbohydrates be used as effective chemotaxonomic markers to distinguish the differences and/or similarities between species within *Gentiana* and/or between *Gentiana* and other genera (Schilling, 1976; Miller, 1997) ?
- why these chemical compounds evolved to be central to carbohydrate metabolism in gentians, or what if any advantages might result from the metabolism of these carbohydrates to adapt to any specific environment from which gentians originated (Degani & Halmann, 1967; Lazcano & Miller, 1999; Zeeman et al., 2010) ?
- study the enzymes related to the synthesis and hydrolysis of gentianose and gentiobiose, and regulation of their activity (Li et al., 2005; Minic, 2008; Henrissat et al., 2013).

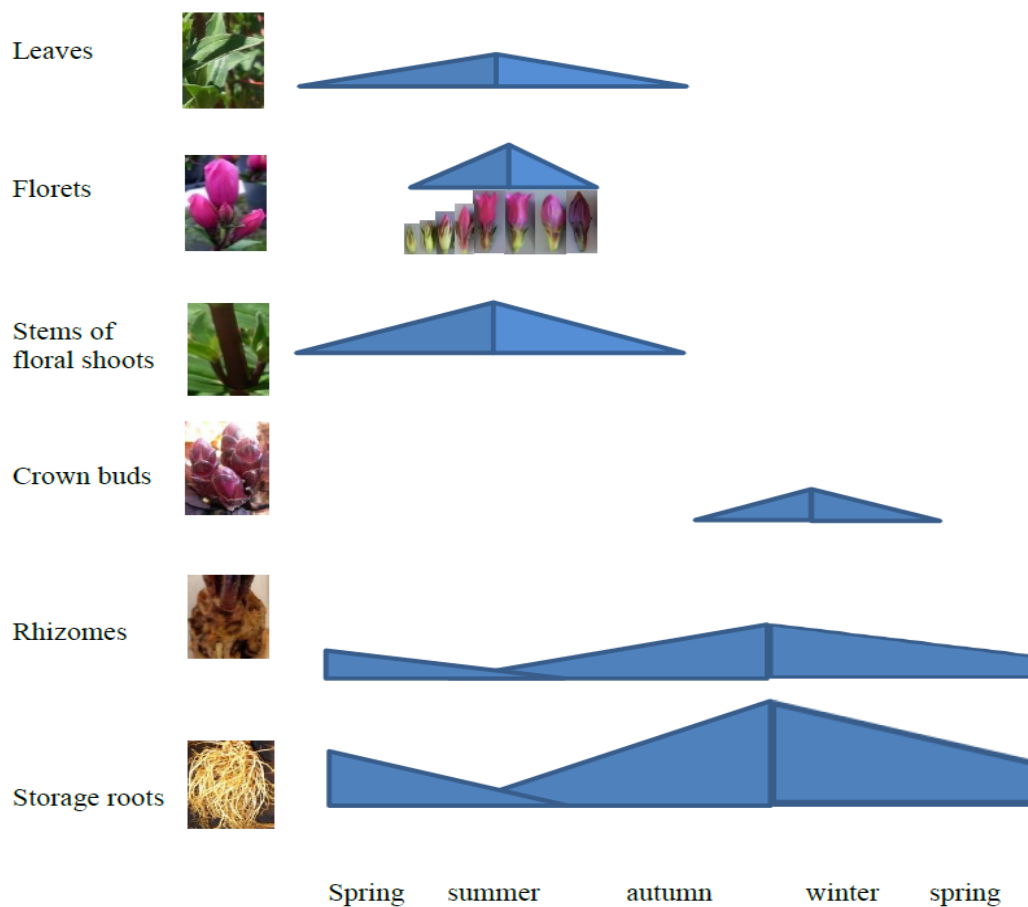
### 9.3 Seasonal fluctuation

Partitioning of carbohydrates includes complicated physiological processes such as synthesis, transport, storage, remobilization, and utilization, and these process can be affected environmentally and intrinsically (Ho & Rees, 1976; Beck & Hopf, 1982; Grange, 1985; Miller, 1992; McCormick et al., 2006; Gesch et al., 2007; Mohapatra et al., 2009; Rennie & Turgeon, 2009). As very limited information is available in terms of carbohydrate partitioning in gentians, this thesis, via monitoring the seasonal changes of NSCs in various organs, provides a start in understanding the partitioning of the unique carbohydrates and, their physiological function in this crop.

The distribution of NSCs in various parts of other perennial plants has been reported to fluctuate with seasonal changes, being tightly related to the phenology of plant growth, development and reproduction (Bradbury & Hofstra, 1977; Pressman et al., 1993; Wyka, 1999; Lee & Sugiyama, 2006; Walton et al., 2007; Roca et al., 2008; Slewinski, 2012).

However, little of such information is available within the literature pertaining to gentians. Data obtained in the current study has, for the first time, quantified the distribution and the fluctuation of the concentration of NSCs in various organs at different developmental stages and/or seasons over the annual growth cycle. This data has been linked to the transition between sink and source (i.e. storage and utilization) of NSCs, occurring in the whole plant (Chapters 3, 4, 5, 6, and 7). Taking ‘Showtime Spotlight’ as an example, in spring and summer above ground organs (leaves, stems and florets) were the main sinks where NSCs accumulated. In contrast in autumn, after flowering, the accumulation of NSCs moved down to the underground crowns (crown buds, rhizomes and storage roots), while in winter and early spring, NSCs stored in crowns were the carbon source for overwintering and initiation of plant re-growth. These transitions also provide important information as to the dynamics of supply and demand for carbohydrates in various organs during the growth and development of gentians. The specific physiological function of NSCs and their correlation with the growth and development of the gentian plant are discussed in detail in the following sections.

Storage roots and rhizomes were the main storage organs for NSCs, presenting much higher concentration of TNC than any other organs (Figure 9.1). Based on the measurements of NSCs during two growth cycles using ‘Showtime Spotlight’ in the current study, it was found that at least 30% and 40% of the accumulated TNC within storage roots and rhizomes, respectively, were not consumed in the normal annual growth cycle (Figure 6.4 and 6.5). As gentianose was the main storage NSC in both the rhizome and storage roots, it may act as a long-term storage carbohydrate, similar in functionality to starch or fructans in other plant species (Woolley et al., 1999; Wyka, 1999; Walton et al., 2007). The results in the defoliation experiment (Chapter 4) indicated that the reduction in source size, and subsequent depletion of carbohydrate reserves within the underground crowns, potentially resulted in plant mortality (Table 4.2 and Figure 4.4). This long-term storage of gentianose therefore, can be explained as an evolutionary adaption in the pattern of carbon partitioning in response to adverse conditions. Such adverse conditions may not only be limited to cold winters, but also other unexpected occurrences, such as animals grazing on gentian plants during the growing season (Miller et al., 1999).



**Figure 9.1** Diagram of the seasonal changes of the comparative content of total non-structural carbohydrate (TNC) in various organs (y-axis) over an annual growth cycle (x-axis) of 'Showtime Spotlight'. ▲: increase of TNC content over time; ▼: decrease of TNC content over time.

The phloem is the primary pathway for carbohydrate transport, particularly long distance transport from source (e.g. leaves) to sink (e.g. storage organs) (Thorne & Giaquinta, 1984; Van Bel, 2003). In most plant species, the carbohydrates transported in the phloem are non-reducing carbohydrates, such as sucrose, raffinose or polyalcohol (Reidel et al., 2009; Rennie & Turgeon, 2009), although van Bel (2008) reported a widespread occurrence of hexoses, i.e. reducing monosaccharides, in the phloem sap in two plant families, i.e. Ranunculaceae and Papaveraceae. Due to the existence of unique carbohydrates (gentianose and gentiobiose) in significant quantities in gentians, the forms of carbohydrate(s) transported in the phloem of gentians need to be identified. At the

beginning of this thesis both the stylectomy technique and EDTA-induced exudation, were attempted in experiments to analyse the carbohydrates in gentian phloem. As failing to establish a suitable plant-aphid feeding system prevented the stylectomy technique from being established, and EDTA in the exudate sample resulted in an abnormal chromatogram from the initial analysis by HPLC (Appendix III), these techniques were not developed further within this thesis. However, Keller and Wiemken (1982) reported that in storage roots of *G. lutea*, both gentianose and gentiobiose were located exclusively in vacuoles. This result seems to imply that gentianose and gentiobiose are not the carbohydrates for transport in phloem of gentians. In addition, although in the current experiments the HPLC chromatogram of EDTA exudates was abnormal (Appendix III), the three peaks present in the chromatogram looked to be derived from sucrose, fructose and glucose. However, to better understand the transport of carbohydrates in gentians, experiments using aphid stylectomy to obtain pure phloem sap would provide more accurate information.

Carbohydrate synthesis and degradation as well as the activity of associated enzymes are deeply involved in carbohydrate partitioning. For example, continuous hydrolysis of sucrose by acid invertase in cell walls at the unloading site increases the steepness of the sucrose gradient, leading to a faster unloading of sucrose from the phloem (Zhang et al., 2001). Sink or source strength are also related to the activity of many enzymes such as invertase, sucrose synthase, amylase and ADPglucose pyrophosphorylase (Sung et al., 1994; Mohapatra et al., 2009; Kaur et al., 2012). The results of preliminary experiments within this thesis indicated that the activity of GHs was closely related to the fluctuations of carbohydrate concentration, revealing a possible regulation mechanism, i.e. control of the hydrolysis of carbohydrates, for carbohydrate storage and utilization in gentians (Chapter 8).

Temperature is regarded as the major environmental factor influencing the timing of phenology in geophytes of temperate origin (Rees, 1992; Le Nard & De Hertogh, 1993b). In the current study with the seasonal changes in both 2007-2008 and 2010-2011 growth cycles, 'Showtime Spotlight' not only presented similar patterns in terms of phenological events (Figure 6.2), but also changes in concentrations of NSCs. For example, the concentration of TNC in storage roots increased through summer to their maximum concentration in autumn, and then declined during winter and spring, followed by gradual

replenishment over summer and autumn (Figure 6.4A and B). However, such replenishment of NSCs in storage roots occurred earlier in 2010-2011 than 2007-2008, and flowering time of 'Showtime Spotlight' also occurred one month earlier in the 2010-2011 season compared to the 2007-2008 season (Figure 6.2). Given the correlation between environmental temperature and ontogeny in other crops (Le Nard & De Hertogh, 1993b; Yin et al., 2005; Funnell, 2008), these differences may be attributed to monthly mean daily air temperatures in spring, since the 2010-2011 season was, in average, 1.0 °C higher than the 2007-2008 season (Figure 6.10). However, the mechanism underlying the relationship between temperature and partitioning of NSCs in gentian plants needs further studies of growth patterns, photosynthesis and changes in enzyme activity, ideally under conditions of controlled temperatures.

While the present study based on net concentration of extracted NSCs has provided a valuable framework of information in terms of the distribution, storage and utilization of NSCs in various organs over seasons, further studies on the following aspects will help to provide a more complete picture of the partitioning of NSCs in gentians:

- Effects of environmental factors, such as temperature, light intensity and photoperiod, on the carbohydrate acquisition (photosynthesis), storage and utilization;
- Correlation between photosynthesis and accumulation of NSCs;
- Using radioactively labelled carbon, tracking carbon flux (translocation, storage and remobilization) and determining how much of photo-assimilate is used for respiration, storage or remobilization.

#### **9.4 Physiological functions**

Acting as main carbon and energy sources, NSCs play a vital physiological role for plant growth and development (Lewis, 1984a). In addition, NSCs are also involved in many other physiological processes and functions, such as tolerance to adverse condition (Miller et al., 1999; Palonen, 1999; Patton et al., 2007), morphogenesis (Wyrzykowska et al., 2002), flowering (Corbesier et al., 1998; El-Lithy et al., 2010), bud dormancy (Anderson et al., 2005; Gesch et al., 2007), and signalling (Rolland et al., 2006). Based on the results in the current study, the potential physiological functions related to the development of

crown buds, overwintering, spring re-growth and, flowering, are discussed in the following sections.

#### **9.4.1 Development of crown buds**

Perennial gentian plants carry out vegetative regeneration via the re-growth of crown buds in spring. Based on the fact that all flowering shoots originate from crown buds initiated and developed in the preceding year's growing season, and the size of crown buds may influence the length of flowering shoots (Yamanaka, 1978; Ohkawa, 1989; Samarakoon, 2012), crown buds, therefore, are a determinant influencing the yield and quality of flowering shoots in gentian. As explored further within this section, understanding the development of crown buds and related influencing factors becomes, therefore, important both for understanding the physiology of plants and improving commercial horticultural practice.

The formation of clusters of crown buds, arranged in a spiral pattern (Figure 3.3 to 3.6), was an evident feature in the development of crown buds. Macroscopic observations within the current study *in vivo* (Figure 3.3) and *in vitro* (Figures 5.2 and 5.8) indicated that a cluster of crown buds commonly appeared as an axillary bud from a leaf axil at the base of a shoot. In addition, recently published anatomical investigations of seedlings (Samarakoon et al., 2013) illustrated that initiation of the first bud of a cluster of crown buds can also be adventitious, arising on the transition zone (between shoots and roots) of gentians. Taken together, these results illustrate the plasticity of response in gentian plants, i.e. the initial bud from which a crown bud cluster is derived may be adventitious or axillary. This plasticity may enhance the capability of gentian plants to produce crown buds, e.g. forming crown buds adventitiously when there are no sufficient leaf axils due to browsing of shoots. The total number of crown buds was determined by the number of clusters (CN) and the number of crown buds per cluster (CBNC). Positive correlations between shoot number, cluster number and the total number of crown buds in the same growing season (Figures 3.10 to 3.12), indicated that the shoot number might, via influencing the number of clusters, influence the total number of crown buds. To confirm the causality between shoots and crown buds, further information in terms of the physiological effects of photo-assimilate acquisition and/or hormonal regulation on the morphogenesis of crown buds are needed.

Carbohydrates are the essential carbon and energy source for bud development and maintenance (Vesk & Westoby, 2004). However, little literature is available as to the effect of carbohydrate on the development of crown buds in gentians. Similar to a previous report (Samarakoon et al., 2012b), the current observations showed that new crown buds were macroscopically visible from December (i.e. the beginning of summer) with the total number of crown buds per plant (TCBN) increasing through to the end of autumn for the genotype 03/04-114 (Figure 3.9). During this period, the rate of increase in TCBN presented a peak soon after the peak of flowering (Figure 3.9). Given the strong evidence for the substantial demand for carbohydrates associated with floret development (Figure 7.5), it may be interpreted that the increased rate of appearance of crown buds after the peak in flowering is related to the change of partitioning of carbohydrates at that time, i.e. the main sink of carbohydrates transfers from the floral shoots to underground organs (it is worth noting here that 03/04-114 is a sterile genotype; Ed Morgan, personal communication, hence carbohydrates are not required for seed development). This interpretation is also supported by the fact that the increased formation of crown buds (Figure 3.9) coincided with the increase in concentration of TNC, gentianose and sucrose in crown buds, rhizomes and storage roots (Figure 6.4A and B; Figure 6.6B). This coordination of timing therefore, further supports the evidence from the defoliation experiment (Chapter 4), mentioned in the next paragraph, that the NSCs in crown buds, storage roots and/or rhizomes may be involved in the morphogenesis of crown buds. The continuous increase in both the size of crown buds and NSC concentration within them through winter, when natural senescence of above ground shoots had already completed (end of May 2009), supports the hypothesis that the carbohydrate reserves in the rhizome and/or storage roots, may also be remobilized to support crown bud development during this time.

The results of the defoliation experiment (refer Chapter 4) showed a positive correlation between the NSCs reserves in crowns in autumn and both crown bud number and size (Table 4.4 and 4.5) and, therefore, supports the hypothesis that carbohydrate supply is likely to be a limiting factor influencing the development of crown buds. Although all the above data support the hypothesis that carbohydrates may be involved in the development of crown buds, due to the lack of direct evidence they could not, alone, confirm the physiological functional of carbohydrates in the morphogenesis of crown buds. However, further evidence supporting their function in morphogenesis of crown buds was provided

by experiments using an *in vitro* system (Chapter 5). Sucrose concentration between 6% (175.3 mM) and 12% (350.6 mM) in the medium, compared with 3% (87.6 mM) sucrose, resulted in an increase in the proportion of plantlets of ‘Showtime Diva’ forming crown buds, and even an increase of endogenous NSCs in plantlets (Figure 5.4). It is tempting to interpret these results as supporting earlier reports that carbohydrates are involved in plant morphogenesis as an osmotic regulator (Brown et al., 1979; Kolarovic et al., 2006; Twumasi et al., 2010). Additionally, as demonstrated in the development of florets in gentian (Chapter 7), the metabolism and/or mobilization of NSCs in petals were closely related to the osmotic regulation pertaining to pressure potential. However, to confirm if carbohydrates act as an osmoticum in enhancing crown bud formation, further experiments *in vitro* should be conducted, using a carbohydrate-replaceable osmotic agent such as polyethylene glycol (PEG), sorbitol or mannitol (Sawwan et al., 2000; Hongbo et al., 2005). While the increase of sucrose concentration in the medium *in vitro* promoted the formation of crown buds, the maximum percentage of plants forming crown buds was comparatively low, i.e. only less than 20% at best (Table 5.1). This result implies that other potential factors, in addition to carbohydrates, may be involved in the morphogenesis of crown buds.

Photoperiod and temperature have been reported as factors affecting the morphogenesis of plants *in vitro* (Ronse et al., 1997; Sengar et al., 2011). However, in the current experiments *in vitro*, within the range of temperatures between 15 to 25 °C, there was no significant effect of temperature on the formation of crown buds, and no interaction between temperature and photoperiod (Table 5.3). While a long photoperiod of 18 h (compared with a short photoperiod of 7 h) resulted in a limited increase (significant at the 10% confidence interval) in the formation of crown buds, only a 20% proportion of plants formed crown buds (Fig 5.5 and 5.6). In contrast *in vivo*, Samarakoon (2012), using the same cultivar as in the current experiments, i.e. ‘Showtime Diva’, recently reported that short photoperiods significantly increased the number of crown buds per plantlet than achieved under a long photoperiod. It is worth noting that exposure to a short photoperiod increases carbohydrate allocation to the underground organs in other genera (Shillo & Halevy, 1981; Wallace et al., 1993). If similarly acting in gentians as well, the short photoperiod used in Samarakoon’s study might not be a direct signal increasing crown bud number, but act indirectly by increasing the partitioning of photo-assimilate to underground organs within the crowns, which in turn increases the formation of crown

buds. To confirm this hypothesis, the analysis of carbohydrate partitioning within plantlets under different photoperiodic regimes may provide some useful information. In the current study *in vitro* however, a sufficient supply of carbohydrates, i.e. high sucrose concentration of 9% (262.9 mM) in the medium, may remove any effect due to carbohydrate limitation; therefore the results may reflect the real role of photoperiod, i.e. long photoperiod enhances crown bud formation.

Given that the influences of carbohydrates (sucrose), photoperiod and temperature on the formation of crown buds *in vitro* were limited; the role of ethylene, as a potential factor influencing the formation of crown buds (Samarakoon, 2012), was examined *in vitro* in the current study. The result indicated that the application of 10-20 ppm ethephon (2-chloroethylphosphonic acid) in the presence of a high sucrose concentration (9%), was approximately four times more effective in forming crown buds than high sucrose concentration (9%) alone (Figure 5.10 and Table 5.1). However, it needs to be noted that the application of ethephon alone without high sucrose concentration (i.e. under a comparatively lower sucrose concentration of 3%) resulted in an increased number of axillary buds/shoots per plantlet, with no dormant crown bud-like structures. This result implies that ethephon may play an important role in enhancing the differentiation of axillary buds/shoots by altering gene expression both transcriptional and post-transcriptional processes (Bhat et al., 2010). In contrast, while the effect of the increased sucrose concentration on increasing the number of crown buds *in vitro* was limited, a high concentration of sucrose (9% sucrose) in the medium effectively produced the dormant crown buds, i.e. swollen axillary buds with protective scales (Figure 5.2 and 5.8). This dormancy was quickly removed once plants were transferred to a low (3%) sucrose medium (Figures 5.3 and 5.11). This fact emphasizes the interaction between carbohydrates and plant-hormones in regulating the morphogenesis of crown buds. In addition to this finding *in vitro*, the pattern of seasonal change of TNC in crown buds *in vivo* (Figure 6.6) was consistent with the changes in dormancy status of crown buds, which also supports the hypothesis that carbohydrates may be directly or indirectly involved in regulating the dormancy status of vegetative buds (Anderson et al., 2005; Chao & Serpe, 2010; Richardson et al., 2010). Such involvement in bud dormancy may be related to osmotic regulation (as evident in the development of floret buds in Chapter 7), or potentially signal transduction (Anderson et al., 2005; Chao et al., 2007). The

physiological role of the dormancy of crown buds in the growth cycle of gentians is also discussed in the following section.

In summary, with regard to the development of crown buds the current series of experiments indicate that the morphogenesis of crown buds is influenced by sucrose supply, ethephon, photoperiod and their interactions. However, to fully understand the mechanism of morphogenesis of crown buds in gentians, future studies *in vitro* or *in vivo*, should examine:

- Whether different carbohydrates, particularly gentianose and/or gentiobiose, have different effects on morphogenesis of crown buds of gentians (Petersen et al., 1999; Fuentes et al., 2000);
- Whether carbohydrates act as a carbon source, signal (Sairanen et al., 2012) and/or osmoticum (Roycewicz & Malamy, 2012), in terms of the morphogenesis of crown buds;
- the physiological mechanism of ethylene action in the morphogenesis of crown buds and its interaction with carbohydrates, environmental factors, and other plant hormones (Dolan, 1997; Pierik et al., 2006).

### **9.4.2 Overwintering and re-growth**

A large number of plant genera and species exhibit the geophytic habit that enable them to survive adverse environmental conditions, such as dry, hot summers or cold winters, by dying back to special underground storage organs in their perennial life-cycles (Le Nard & De Hertogh, 1993a; Kamenetsky & Okubo, 2012). These underground organs such as crowns, bulbs, corms, rhizomes, roots, etc., store energy (commonly carbohydrates), nutrients, and even water, for initiating re-growth of vegetative buds in favourable seasons. For gentians, crowns (comprised of crown buds, rhizomes and storage roots) are the perennating underground storage structure of horticultural relevance. In the current study, the distribution of NSCs, based on both concentration and biomass, in various organs within the crown, indicated that storage roots were the main storage organ for NSCs, and gentianose was the main storage NSC in crowns (Table 3.1; Figure 4.4; Figure 6.4 to 6.6). As many other geophytes originating from temperate regions (Woolley et al., 1999; Walton et al., 2007; Uleberg et al., 2009), it seems plausible that perennial gentians for cut

flower production will need sufficient accumulation of NSC reserves within underground structures (crowns) by autumn to survive through the cold of winter and initiate re-growth in spring. Consistent with such a hypothesis, TNC, especially gentianose, in storage roots and rhizomes of ‘Showtime Spotlight’ gradually increased from summer to their maximum concentration at the end of autumn, before they then declined during winter and spring (Figure 6.4 and 6.5). The defoliation experiment in Chapter 4 presented significant positive correlations between carbohydrate reserves in crowns and both winter survival and re-growth of crown buds in spring (Table 4.4 and 4.5), therefore, supporting the hypothesis that carbohydrate reserves may be a limiting factor influencing the winter survival and re-growth of gentian plants.

The ability of gentian plants to survive the cold of winter generally depends on the ability of crowns to tolerate low temperatures at that time of year. It has been recognized that cold tolerance is normally associated with the concentration of NSCs in plant tissue (Koster & Lynch, 1992; Svenning et al., 1997; Patton et al., 2007; Uleberg et al., 2009). It was found that compared with storage root and rhizomes within crowns, TNC concentration in crown buds was the lowest (Figure 6.4 to 6.6). Carbohydrate supply to crown buds therefore, is possibly a key or limiting factor determining the ability of gentians to survive winter. The concentration of TNC in crown buds showed a sharp increase after entering winter, and reached its maximum in the middle of winter, i.e. July (Figure 6.6). Compatible with the lowest temperature that occurred in July in Palmerston North, NZ, (Figure 6.3), the peak of TNC concentration (mainly due to the increase of sucrose) in crown buds, was most likely positively related to the cold tolerance for crown buds. Consistent with this hypothesis, the analysis via stepwise regression indicated that sucrose concentration in crown buds contributed the most to winter survival (Table 4.5). In addition, the significant increase of L-bornesitol concentration in crown buds in winter may also be related to the increased cold tolerance of crown buds (Figure 6.11A). However, it needs to be noted that the high peak of sucrose concentration in crown buds occurred in winter when no photosynthetic activity was occurring and, therefore, the increase of sucrose in crown buds can be presumed to have come from the conversion and remobilization of gentianose (main storage NSC) in storage roots and/or rhizomes. If so, gentianose in storage roots may play a key role as a carbohydrate source involved in cold tolerance of crown buds.

For perennial gentians, it is necessary to maintain the dormancy of crown buds over the long period from summer through the cold of winter, so as to provide a reservoir of crown buds for re-growth and also prevent cold damage to tender new growth until the arrival of warmer weather in spring. During summer and autumn, apical dominance of shoots may have a key role in maintaining the dormancy (paradormancy) of crown buds. With the senescence of shoots in winter, other internal physiological factor(s) are required to control crown bud dormancy, i.e. endodormancy (Lang et al., 1987; Chao et al., 2007). It has been recognized that carbohydrates may be directly or indirectly involved in the dormancy status of vegetative buds as a signal (Chao & Serpe, 2010) or osmoticum (de Fay et al., 2000; Morisset et al., 2012). *In vitro* experiments (Chapter 5) indicated that a high concentration of sucrose (9%) in the medium effectively maintained the dormancy of crown buds formed *in vitro* (Figure 5.3 and 5.11) and, at the same time, significantly increased the concentration of endogenous NSCs (Figure 5.4). *In vivo*, the concentration of TNC (mainly sucrose) in crown buds increased and reached its highest level in the middle of winter (July), and then decreased in spring when re-growth was evident (September; Figure 6.6). The changing pattern of TNC in crown buds, therefore, appears to have coincided with the changes in dormancy status of crown buds. These results support the hypothesis that, in addition to cold tolerance, the increase in concentration of NSCs in crown buds, derived from the high concentration of NSCs in storage roots and rhizomes, is also involved in maintaining the dormancy of crown buds. As evident in the development of florets (Chapter 7), the changes of NSC concentration play an important role in regulating the osmotic potential in petals; therefore, the metabolism and remobilization of NSCs in crowns may also be potentially involved in cold tolerance and/or bud dormancy as an osmoticum. To confirm this hypothesis, further experiments are required to examine the relationships between the cold tolerance, dormancy status, osmotic potential and TNC molality in crown buds.

In the current study, *in vivo*, crown buds started growing and developed new leaves in spring (September). During this period, the concentration of TNC (mainly sucrose and gentianose) rapidly decreased in crown buds and subsequently increased in the newly developed leaves (mainly sucrose) and stems (mainly gentiobiose). These results imply that stored carbohydrates in crown buds were utilized for the initial growth of crown buds, until new leaves became net carbohydrate exporters (Danckwerts & Gordon, 1989; Baur-Hoch et al., 1990; Woolley et al., 1999). In the defoliation experiment (Chapter 4), the

proportion of buds sprouting in spring was positively correlated with NSC concentration in crowns in the preceding autumn (Table 4.3 and 4.4). This result supports the hypothesis that availability of NSCs that are stored in the preceding autumn, is a limiting factor to the initiation of re-growth of crown buds in spring; the more carbohydrate reserves available, then the higher the proportion of crown bud sprouting achieved.

The comparison between the changes in the concentration of NSCs and the activity of associated GHs (Chapter 8) provided more information to understand the function and mechanism of carbohydrates involved in overwintering and spring re-growth of gentians. The results showed that the decrease of gentianose concentration in storage roots and, increase of sucrose in crown buds during winter, were accompanied by the increased activity of gentianose GH in storage roots (Figure 8.8 B). In addition, the rapid decrease of sucrose and gentianose (main NSCs) in crown buds in spring (September) were accompanied by a rapid increase of GH activity for both sucrose and gentianose (Figure 8.7). Comparing the changes of GHs with related carbohydrates in both crown buds and storage roots further supported this hypothesis, i.e. gentian plants accumulate gentianose in storage roots over summer and autumn and, in winter, when no new carbohydrate is available from leaves, a portion of the stored gentianose is hydrolysed by gentianose GHs for remobilization to crown buds. These carbohydrates, once moved to the crown buds, presumably may then be used to restore the carbon source for cold tolerance and retaining crown bud dormancy. In spring the GHs hydrolyse those carbohydrates stored within the crown buds again, thereby providing the necessary carbon source and energy for re-growth.

In summary, in order to successfully survive winter and initiate re-growth in spring, with their geophytic growth habit gentians rely on the sufficient accumulation of NSCs and, subsequent conversion and remobilization of NSCs under the control of the activity of different enzymes. This interrelationship reflects the importance of gentianose as the main storage NSC in crowns, especially in storage roots (Figure 9.2). These dynamic changes of NSCs in the crowns of gentian also confirm that physiological and biochemical changes, never completely stop in underground storage organs during the period of dormancy (Lang, 1995). However, to further understand the physiological function of NSCs and their mechanism(s) of action in terms of cold tolerance, bud dormancy and carbon remobilization during overwintering and spring re-growth, future studies should focus on:

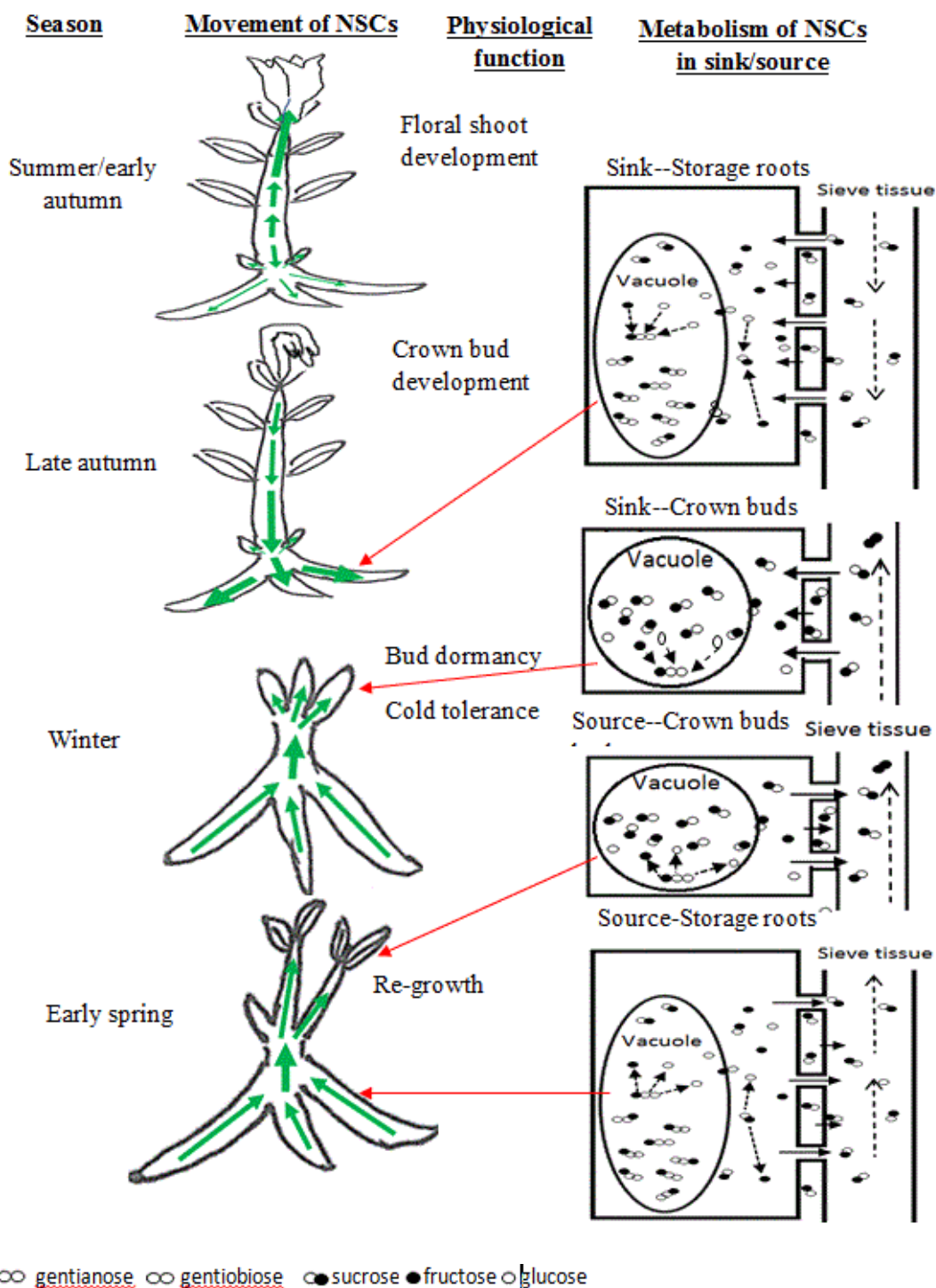


Figure 9.2 Schematic diagram illustrating the seasonal changes in storage and utilization (transition between source and sink) of non-structural carbohydrates (NSCs), their physiological function, within the crown and floral shoots of a perennial gentian plant. Green-coloured arrows indicate net direction of flow of NSCs.

- the role of NSCs in the mechanism of regulating bud dormancy, cold tolerance, and re-growth, either as an osmoticum (de Fay et al., 2000), signal (Chao et al., 2007), and/or involvement in gene expression (Chao & Serpe, 2010);
- using labelled carbon for tracking the carbon flux, and quantify if gentianose, in the roots and/or rhizomes is remobilized for cold tolerance and spring re-growth of crown buds, as are oligosaccharides in asparagus crowns (Woolley et al., 1999);
- determining if the cold tolerance of various cultivars or genotypes of gentians is related to the level of TNC, individual NSCs or related enzymes such as gentianose and gentianose GHs in crowns, as in other plant species (Jacobsen et al., 2007; Patton et al., 2007).

### 9.4.3 Development of floral shoots

The floral shoots of gentian are spike-type inflorescences bearing a number of florets. As presented by a large increase of dry weight of petals from when they were small buds to when the florets were just opening (Figure 7.3), it is evident that the fast growth of florets requires a large amount of carbohydrate supply as both carbon and energy sources. In addition to the import of newly produced photo-assimilate from leaves (Yamane et al., 1991; van Meeteren et al., 1995), the current study indicated that both substantial gentiobiose stored in stems of floral shoots (Figure 6.8 B) and gentianose in petals before floret opening (Figure 7.5), are potentially the important sources of carbohydrates to meet such demand during the development of florets. From an ecological perspective, such storage of NSCs is also valuable for unexpected stressful conditions, such as shading or drought, when photosynthesis is restricted or reduced (Stamp, 2003; Slewinski, 2012). This storage of NSCs is also important for the continued growth of undeveloped florets after the harvest of flowering shoots, such as continued petal expansion and development of pigmentation (Zhang & Leung, 2001; Eason et al., 2004; Eason et al., 2007).

It has been hypothesized that carbohydrates may act as major osmotica involved in regulating osmotic potential, promoting water influx for cell expansion and flower opening during flower development (Reid, 2005; Kumar et al., 2008b; Norikoshi et al., 2013). However, little direct evidence has been provided in terms of the interrelationships between carbohydrates and water, osmotic, and pressure potentials. The current study showed the osmolality of TNC (all NSCs are soluble in gentian) in petals increased with

the development of florets until fully opened (Stage 5) reached its maximum level (Figure 7.4), and then decreased with the senescence of florets until finally senesced (Stage 8). As the osmotic potential derived from carbohydrates only accounted for approximately one fourth of total sap osmotic potential in petal tissue (Figure 7.7), there were other factors contributing to the osmotic potential, such as proteins, organic and inorganic compounds, etc (Nilsen & Orcutt, 1996b; Norikoshi et al., 2013). Rather than being correlated with changes of water potential and osmotic potential in petal tissue, the changes of osmolality of TNC (soluble) was extremely significant in its correlation to the changes of pressure potential during the development and senescence of florets (Figure 7.8). Not only this, the absolute value of the osmotic potential derived from TNC was slightly higher than that of the pressure potential in petal tissue (Figure 7.7). This indicated that TNC has the capability to contribute to the level of pressure potential in petal tissue. These results provided direct evidence, supporting the hypothesis that the regulation of pressure potential in petal tissue of gentian is mainly contributed by the activity of carbohydrate metabolism involved in osmotic regulation (Equation 2 in Chapter 7), which is physically the key driving force involved in cell expansion and opening of florets (Vergauwen et al., 2000; Yamada et al., 2009; Kwon et al., 2010; Norikoshi et al., 2013).

The dynamics of concentration of individual NSCs and the activity of related GHs during the development of florets, further revealed the metabolic mechanism underlying carbohydrate involvement in osmotic regulation. Similar to storage roots and crown buds, dramatic metabolism of carbohydrates was found in petal tissue accompanying development through to senescence (Table 7.1), and were controlled by the activity of relevant GHs (Figure 8.10). The most evident was the substantial accumulation of gentianose in petal tissue before floret opening that resulted in the decrease of osmotic potential, and subsequently the rapid hydrolysis of gentianose into glucose and fructose (Figure 8.5) during floret opening, which further decreased the osmotic potential of petal tissue. At the same time, both the decrease of gentiobiose GH and increase of sucrose GH together resulted in the increase of concentrations in gentiobiose, fructose and glucose. All these metabolic activities lead to the highest molar concentration of total carbohydrates when florets were just opening at Stage 5 (Figure 7.4). Similar to previous reports relating to petals of rose (Yamada et al., 2007), the current study showed sucrose concentration did not decrease with the increase of sucrose GH activity during floret opening (Figure 8.10A). Similarly therefore in the current study, it was hypothesized that at the same time as

sucrose GH hydrolysed sucrose to fructose and glucose, sucrose was continuously imported to petals, thereby achieving a balance in sucrose concentration (Yamada et al., 2007). In addition, with the senescence of florets, all NSCs decreased quickly through to Stage 8 when florets had completely senesced (Figure 7.5). As demonstrated in *Gladiolus*, which has a similar spike-type inflorescence as gentians (Yamane et al., 1995), this reduction in NSCs may be not only due to carbohydrate consumption for respiration, but also a carbon-saving phenomenon, i.e. re-exporting from senescing petals to other developing florets. To confirm this hypothesis in gentians, further investigation using labelled carbon to track the carbon transport and metabolism is recommended. This GHs-involved carbohydrate metabolism pertaining to osmotic regulation in florets, also now provides useful information for better understanding the possible role of these same carbohydrates as osmotica in crown bud morphogenesis, cold tolerance and dormancy. Future research into the formation of crown buds may benefit, therefore, from utilising GHs activity as a research tool.

The preceding paragraphs within the current section have discussed the metabolism of carbohydrates and their possible physiological roles during the development of florets from small buds through to senescent florets. In these experiments petal tissue at various stages (Table 7.1) were collected at the same time at mid-day (Section 7.2). Within this experimental system it was noted that the developed florets of gentians (opening florets between Stage 5 and 6; Table 7.1) exhibited diurnal changes between open and closed, i.e. flowers open during the day and close during night (E. Morgan, personal communication, 2011). It has been suggested that the repeated movement of flower opening and closure may be regulated by many factors depending plant species, such as light, temperature, humidity, hormones and endogenous rhythms etc. (van Doorn & van Meeteren, 2003). Although it was reported in *Gentiana kochiana* that the epidermis cells on the inner side of petals expanded during the day and contracted at night, the outer epidermis in contrast did not show a change in length ((Claus, 1926), as cited by (van Doorn & van Meeteren, 2003)), the mechanism controlling these movement is still not clear. Given the current findings reported in this thesis, whether or not carbohydrate metabolism or other factors are involved in the repeat movement of opening and closure in developed florets of gentian, is worthy of further investigation in a future study.

Ethylene plays an important role in flower development and senescence (Pun & Ichimura, 2003). It has been recognised in other flowers that carbohydrates can inhibit both production of ethylene and sensitivity to ethylene (Ichimura & Suto, 1999; Verlinden & Garcia, 2004). Eason *et al* (2007), using the gentian cultivar ‘Showtime Starlet’, showed that inhibiting either ethylene production or ethylene perception prevented petal senescence. Although ethylene was not measured in the current series of experiments, the pattern of NSC fluctuation in petals (Figure 7.5A) seems to be consistent with the logic that the increase and high level of NSC in petals during the early stages, up to the opening of florets, may inhibit the synthesis and/or perception of ethylene. In contrast, this inhibition will be subsequently reduced following the decrease of NSC with the aging of florets. To confirm this hypothesis, further experiments using the treatment of carbohydrates, ethylene and/or ethylene inhibitors, as well as detection of ethylene synthesis are needed. As gentianose and gentiobiose are predominant within petals, and presented extensive fluctuation with the changes of floret development, the influence of these unique carbohydrates is particularly worthy of future investigation.

In summary, the current study on the storage, utilization and mobilisation of carbohydrates and their relationship to flower development in gentians:

- 1) extended the understanding of how these may be related to the physiology of development of floral shoots and postharvest performance;
- 2) supported the hypothesis of the activity of different enzymes in the role of regulating carbohydrate concentration to meet the requirements of floral shoot development;
- 3) provides a valuable experimental system for the future study of carbohydrate metabolism and their physiological functions in gentians, such as:
  - using labelled carbon to track and quantify the carbohydrate import, export and the conversion between various NSCs (Yamane *et al.*, 1995; Yamada *et al.*, 2007);
  - study the regulation mechanism of the activity of GHs (Hirsche *et al.*, 2009; Zhang *et al.*, 2013), particularly the GH of gentianose as a main storage NSC in gentians;

- examining what, if any, effect carbohydrates have on the production of ethylene and the sensitivity to ethylene during the development and senescence of gentian florets, particularly the unique and predominant carbohydrates, gentianose and gentiobiose (Ichimura & Suto, 1999; Verlinden & Garcia, 2004).

## 9.5 Commercial application

The current study has provided not only a better understanding of the physiological function of NSCs, but also valuable information for horticultural practice during the cultivation of gentians. For example:

- When harvesting flowering shoots in gentians, to achieve sufficient NSC reserves in crowns, retaining a neough number of whole shoots or enough lower parts of shoots until their natural senescence is the optimum strategy. This will benefit crown bud formation, overwintering and re-growth next spring. Within a commercial context however, to determine how much/many shoots, should be left on the plants when harvesting, further experiments under the condition of commercial production are required. The experiments would compare the effect of different severities of harvest on NSCs reserves, crown bud formation, over-wintering, re-growth the next spring, development of crown buds into shoots, and subsequent commercial yields.
- For commercially harvested flowering shoots, NSCs stored in both leaf and stem tissue are likely to be an important source of carbohydrates for the continued development of unopened florets after harvesting. Thus management strategies during cultivation that can increase the accumulation of NSCs in floral shoots, before their harvest, will be valuable. For example, optimizing shoot density of gentian plants can allow leaves on floral shoots to intercept sufficient photosynthetically active radiation for photosynthesis. In addition, the current study demonstrated a clear diurnal pattern of change of NSCs in leaves when floral shoots were at harvest maturity, i.e., TNC reached its maximum at sunset (Figure 6.8A). It is inferred, therefore, that the harvest of flowering shoots of gentian later in the day should be considered, in order to maximise the concentration of NSCs within floral shoots for improving postharvest quality of flowering shoots. However, the effectiveness of late-day harvesting on actual postharvest performance, still needs to be examined in practise.

- Tissue culture is a common method of propagation of gentian. The crop, however, currently requires a relatively long period of cultivation (i.e. 2 to 3 years) to establish mature plants for commercial production. It has been noticed that young clonal plants of some genotypes in gentian are ready to commence reproductive growth *in vitro*, which would reduce vegetative growth of young plants after deflasking, and in turn prolong the period to establish of mature plants. The effect of ethephon (10 to 20 ppm) on the inhibition of reproductive growth of gentian plantlets *in vitro* (Chapter 5) could be used to increase the vegetative growth of young plants, and then reduce the period for gentian plants to reach commercial production. Additionally, in some instances, it has been anecdotally reported that clonal plants from tissue culture have died once deflasked, due to the lack of crown bud formation. Hence forming crown buds while plantlets are *in vitro*, via the regulation of carbohydrate and ethephon concentration in the medium may provide an alternative strategy to optimise crop establishment during vegetative propagation.

### **9.6 Conclusion**

Experiments in this thesis quantified the distribution of NSCs in various organs of gentian plants with seasonal changes, and determined the effect of carbohydrate supply on their development and growth. The current results identified the existence of unique carbohydrates in all organs of gentian, i.e. gentianose, gentiobiose and L-bornesitol, and confirmed their important roles associated with the development of crown buds, overwintering, spring re-growth and floral shoot development. Clearly, although carbohydrates are an important factor affecting crown bud formation, the morphogenesis of crown buds is influenced by multiple factors including, carbohydrates, photoperiod and ethylene; the effects and mechanism of influence of these factors need to be clarified in future studies. Sufficient accumulation of gentianose in storage roots before winter and subsequent enzyme activity involved in the conversion and remobilization, are essential for the plant to successfully survive a cold winter and initiate re-growth in spring. This also reflects the importance of gentianose as the predominant storage NSC in gentian. During the fast development of florets, the stage-specific storage, conversion and mobilization of carbohydrates under the control of different enzymes extended the understanding in terms of the role carbohydrates have as osmotica involved in plant

development. This study also provided a framework of information to improve carbohydrate management in gentian plants for increasing yield and quality of flowering shoots in commercial production, and opened new opportunities for further studies in physiology and metabolism of the unique carbohydrates, gentianose, gentiobiose and L-bornesitol.

## Appendices

### **Appendix I; Identification of gentianose, gentiobiose and L-bornesitol in gentians**

In preliminary work to confirm the identity of unusual carbohydrates (subsequently determined to be gentianose, gentiobiose and L-bornesitol) in gentian, carbohydrate samples extracted from gentian cultivars were separated and collected using High-performance Liquid Chromatography (HPLC) and then identified using nuclear magnetic resonance (NMR). The gentian plant samples from which gentianose and gentiobiose were identified were collected from gentian cultivar ‘Showtime Spotlight’ by Jocelyn Eason in 2007 (Section I.I.D); plant samples for identifying L-bornesitol were collected from ‘Showtime Spotlight’ by Yugu Wang in 2008 (Section I.I.II). NMR analysis was conducted by John van Klink (Sections I.I and I.II).

#### **I.I identification of gentianose and gentiobiose**

##### **I.I.I Sample collection**

Carbohydrate samples were extracted from floral shoots (comprising leaf, floral stem and floret tissue) of gentian ‘Showtime Spotlight’ at mature harvest stage (refer to Section 6.2.2.2), using 62.5% methanol at 55 °C. Gentianose and gentiobiose were separated by HPLC on a Rezex 8 µm (particle size), 8% Ca monosaccharide column (Phenomenex, Torrance, CA) in water at 85 °C with a flow rate of 0.6 ml min<sup>-1</sup>, and detected using an evaporative light scattering detector (ELSD 1000, Polymer Laboratories, Church Stretton, Shrop., UK), with a gas flow 1.5 SLM (standard litre per minute), nebulising temperature 90 °C, evaporating temperature 110 °C. Unidentified gentianose (Sample A) and gentiobiose (Sample B) were collected utilising the same retention times of their standards (gentianose 8.34 min, Extrasynthese; gentiobiose 9.06 min, Sigma), and evaporated to dryness using centrifugal vacuum concentrators (High Capacity Savant SpeedVac<sup>®</sup> plus SC210A, RVT4104, and VPOT100, HiTechTader, USA). The identities of gentianose and gentiobiose were confirmed, using 1H-1H 2D NMR analysis, by John van Klink (refer I.I.II).

##### **I.I.II NMR analysis report of gentianose and gentiobiose**

## $^1\text{H}$ NMR analyses of Sugars from *Gentiana triflora*

Plant Extracts Research Report, March 2007

John van Klink

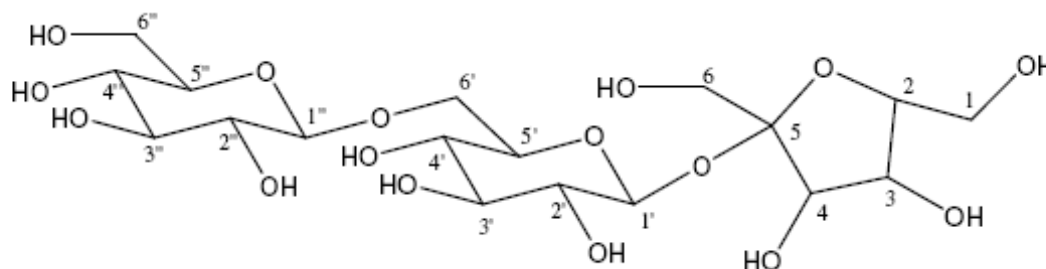
### *Samples received:*

Two samples were received from Jocelyn Eason (CFR Palmerston North), with suggested structures presented below.

### *NMR analyses:*

NMR spectra of  $\text{D}_2\text{O}$  solutions at  $25^\circ\text{C}$ , were recorded at 500 MHz on a Varian Inova 500 spectrometer. Chemical shifts are given in ppm on the  $\delta$  scale. A PRESAT pulse sequence was used to suppress the water signal. 2D proton-proton (COSY) experiments were undertaken, using both gradient (gCOSY) and double filter quantum (dfqCOSY) pulse sequences. The residual water peak was referenced to 4.8 ppm, though the position of this can vary with sample pH and is therefore only a relative measure. No attempts were made to reference any of the other sample signals observed.

*Sample A* (3mg), thought to be gentianose (PERU label P140-826-1)



### *Sample A analysis:*

A search of the Chemical Abstracts found 112 references to gentianose, though most of these were not in English. Published  $^1\text{H}$  NMR data for gentianose seemed to be limited to a single paper,<sup>1</sup> that only lists 2 signals:  $\delta$  5.44 ( $J=3.2\text{Hz}$ ), and 4.49 ( $J=7.2\text{ Hz}$ ). Analysis of sample A by  $^1\text{H}$  NMR (Figure 1) showed signals in the same region with similar coupling constants. This combined with 2D analysis (Figure 2) and comparison to published NMR data for sucrose,<sup>3</sup> the structure of gentianose was able to be confirmed.

Figure 1: P140\_826\_1 proton spectra of sample A

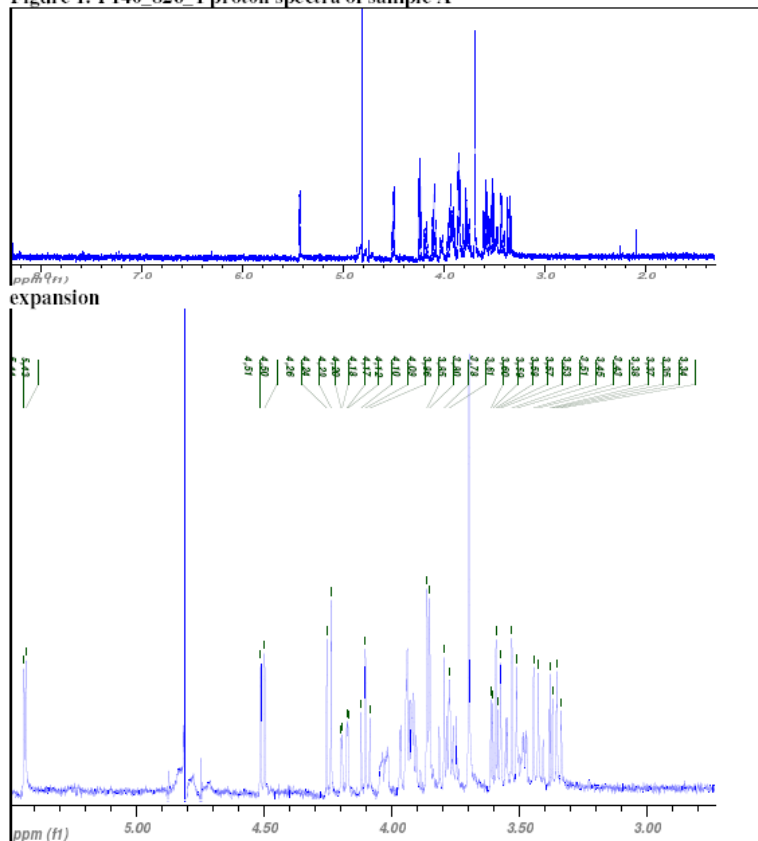
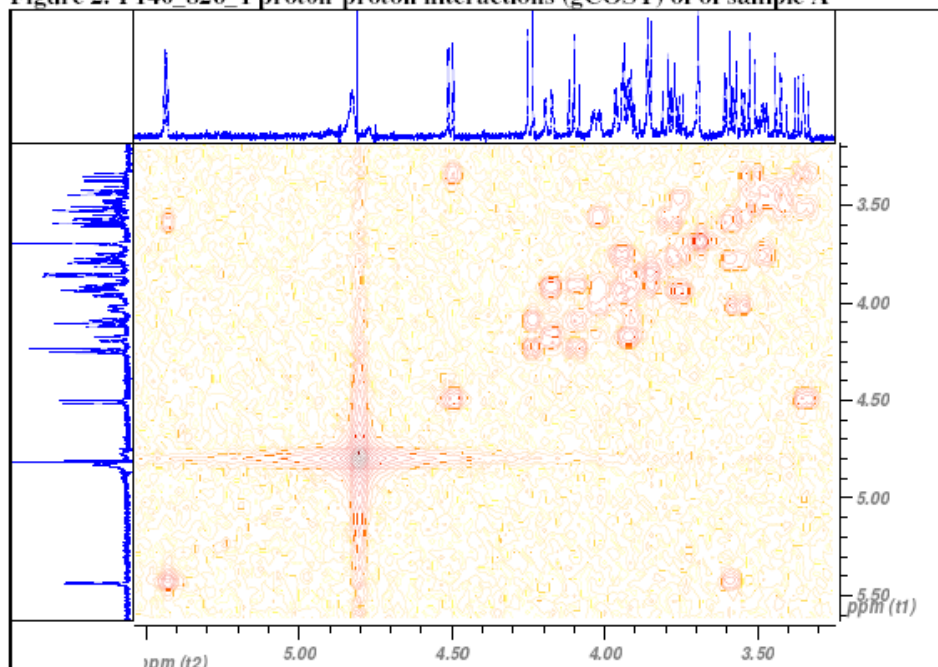
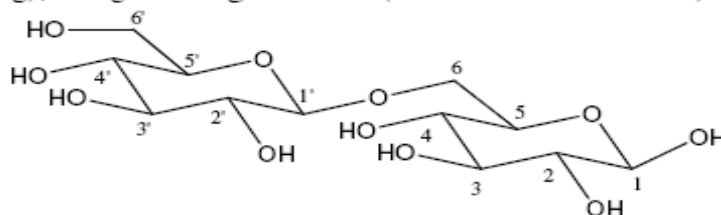


Figure 2: P140\_826\_1 proton-proton interactions (gCOSY) of of sample A



**Sample B** (10 mg), thought to be gentiobiose (PERU label P140-826-2)



A Chemical Abstracts search of gentiobiose found numerous references (>1500), many of which included structural studies of naturally-occurring sugars with some NMR data. Examples of published (see references below)  $^1\text{H}$  NMR data for gentiobiose include a paper by Majak and Benn (1988)<sup>2</sup> (see Table 1) and a web-based database.<sup>3</sup> Analysis of sample B by  $^1\text{H}$  NMR (Figure 3) indicated the presence of sucrose at about the same level as the other major compound which is most likely gentiobiose. The mixture of compounds makes it difficult to unequivocally assign the signals. 2D proton-proton (COSY) analysis (Figure 4) supports the structure of gentiobiose. However, there are some signals that did not show clear correlations, for example the anomeric doublet at 5.24 ppm may be due to the presence of gentiobiose anomers or sucrose anomers.

**Table 1:** NMR Data for gentiobiose from Majak and Benn (1988).<sup>2</sup>

$^1\text{H}$	gentiobiose
1	4.66 $J_{1,2} = 7.8$ Hz
2	3.26 $J_{2,3} = 9$ Hz
3	(3.50) too strongly coupled for analysis
4	(3.50)
5	3.63 $J_{5,6A} = 1.9$ Hz, $J_{5,6B} = 5.5$ Hz
6	4.21(A) and 3.85(B) $J_{6A,6B} = -12$ Hz
1'	4.51 $J_{1',2'} = 7.8$ Hz
2'	3.33 $J_{2',3'} = 9$ Hz
3'	3.51 $J_{3',4'} = 9$ Hz
4'	3.39 $J_{4',5'} = 10$ Hz
5'	(3.47) $J_{5',6A'} = 2$ Hz, $J_{5',6B'} = 5.5$ Hz
6'	3.93(A) and 3.73(B) $J_{6A',6B'} = -11.4$ Hz

Figure 3: P140\_826\_2 proton of sample B

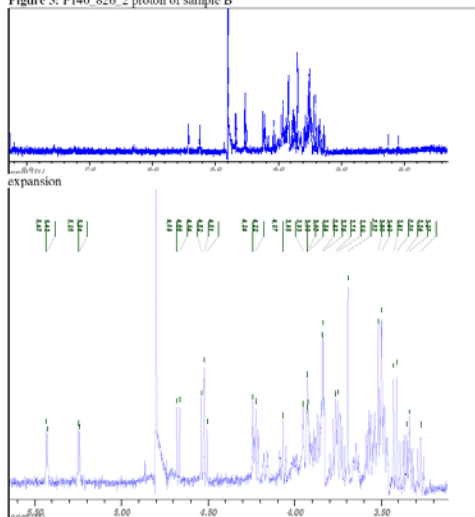
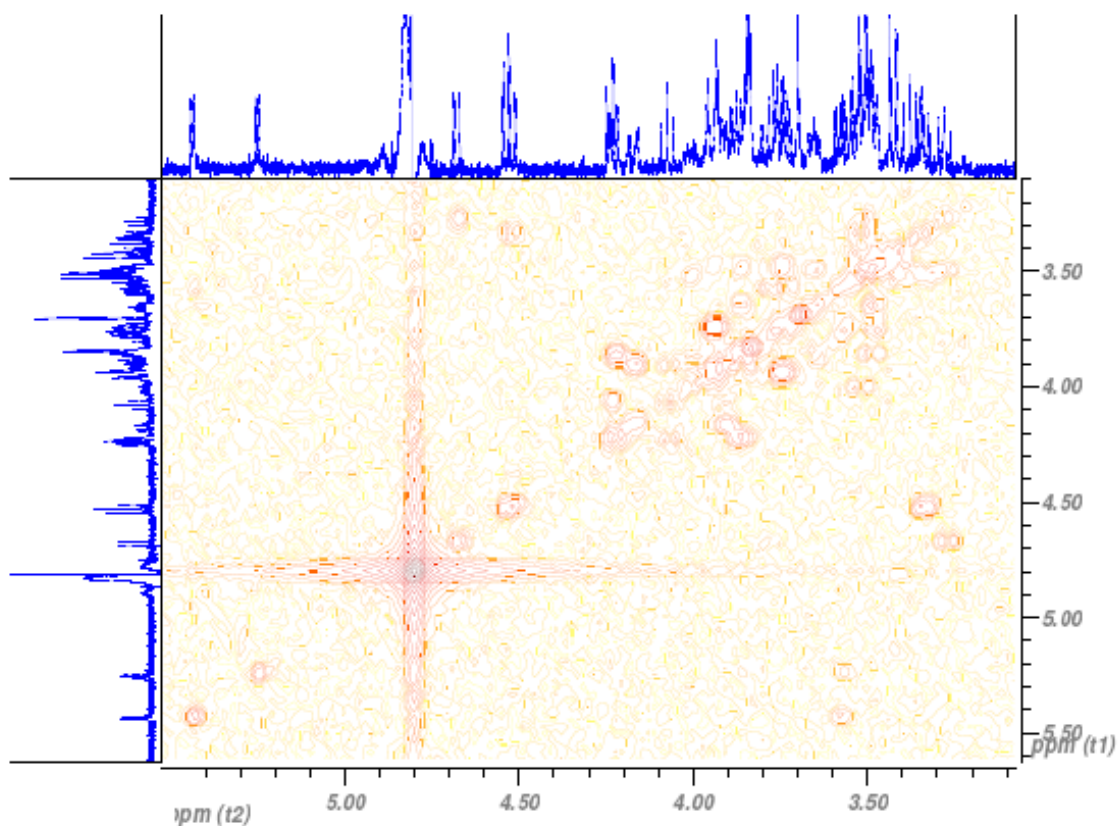


Figure 4: P140\_826\_2 proton-proton interactions (gCOSY) of sample B



**References:**

1. Suami, T., Otake, T., Kato, N., Nishimura, T. and Ikeda, T. 1972. Synthesis of gentianose. *Carbohydrate Research*, 21: 451-454.
2. Majak, W and Benn, M. H. (1988). 3-Nitro-1-propyl- $\beta$ -D-gentiobioside from *Astragalus miser* var. *serotinus*. *Phytochemistry*, 27(4): 1089-1091.
3. Spectral database for organic compounds (<http://www.aist.go.jp/RIODB/SDBS>). However the data for gentiobiose in this database was a mixture of anomers, partially assigned.

## **I.II Identification of L-bornesitol**

### **I.II.I Sample collection**

Carbohydrate samples were extracted from leaf tissue of ‘Showtime Spotlight’ using 62.5% methanol at 55 °C, and separated by HPLC on a Prevail™ carbohydrate column using acetonitrile and water as a mobile phase. An unidentified sample of L-bornesitol was collected during the retention time between 8.0 to 8.7 minute (detected using ELSD 1000; refer Section 4.2.7; Figure 5). The collected sample was dried using centrifugal vacuum concentrators (High Capacity Savant SpeedVac® plus SC210A, HiTechTader, USA). The dry sample was sent to Dunedin: Plant Extracts Research Unit, Plant & Food Research NZ, and the unknown compound were identified by John van Klink (refer I.II.II).

### **I.II.II NMR analysis report of L-bornesitol**

#### **Analysis of a Gentian “unknown” compound by NMR spectroscopy**

John van Klink, August 2010

Plant Extracts Research Unit Report

#### **Sample**

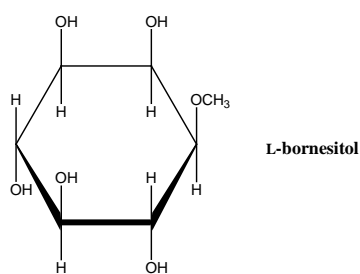
The pale amber sample (1-2 mg) was supplied in a plastic Eppendorf tube by Yuguang Wang (Plant & Food Research (PFR), Palmerston North) with the chromatogram attached (Figure 5). The sample had been extracted from the leaves of a PFR *Gentiana triflora* cultivar using methanol and then purified by HPLC on a Prevail™ carbohydrate column using acetonitrile and water as a mobile phase.

#### **Analyses**

The sample was dried and flushed with dry nitrogen since it appeared to contain some residual solvent. For nmr analysis it was dissolved in D<sub>2</sub>O and run on a Varian AR shielded 500 MHz instrument. In addition to <sup>1</sup>H and <sup>13</sup>C nmr analyses, a suite of 2D experiments (COSY, NOESY, HSQC and HMBC) was undertaken to determine the structure of the compound.

## Results and discussion

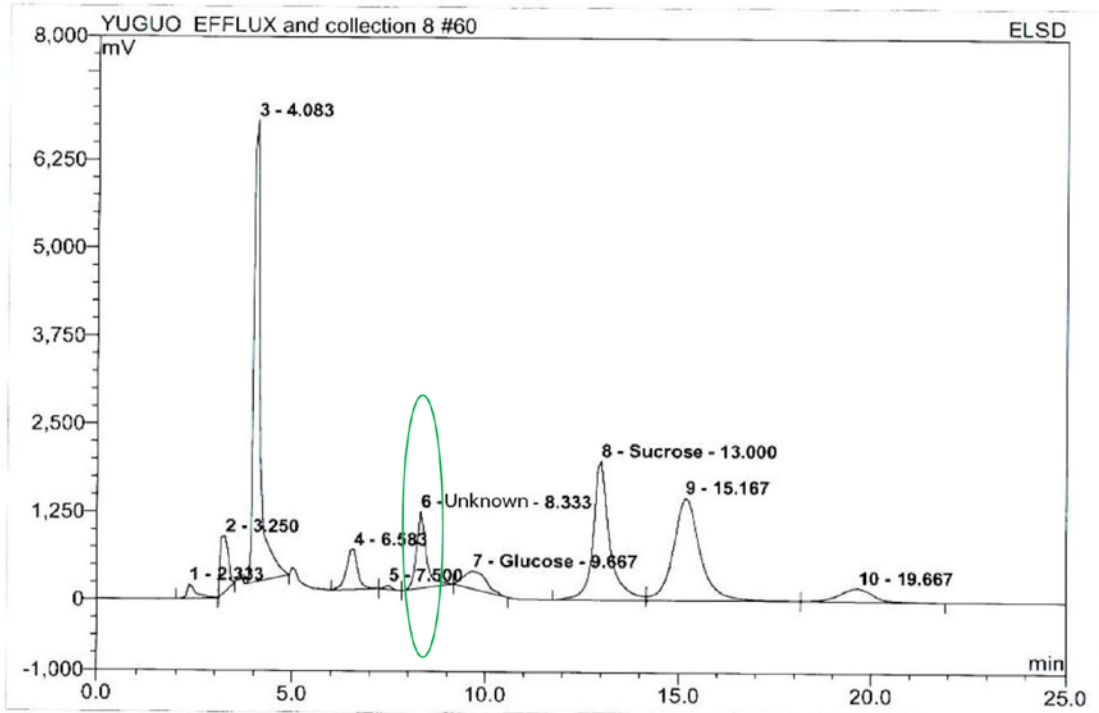
The  $^1\text{H}$  nmr spectrum (Figure 6) showed a signal (3.4 ppm) indicative of a methoxy group and this led to the initial idea (subsequently discounted) that the sample may have been an artefact produced via methanolysis from the extraction procedure. Subsequent data analysis suggested the structure to be an inositol derivative and this was confirmed with further detailed analyses and literature searching. The structure was determined to be **L-1-O-methyl-*myo*-inositol**, a cyclitol methyl ester commonly known as **L-bornesitol**. The  $^1\text{H}$  and  $^{13}\text{C}$  (Figure 7) nmr data matched that reported for bornesitol in the literature,<sup>1</sup> in a paper describing its changes during flower bud development. Cyclitols and their methyl esters such as bornesitol and its isomer pinitol are thought to act as osmolytes.<sup>1</sup> Bornesitol has also been reported as a constituent of several *Gentiana* species, though not specifically from *G. triflora*.<sup>2</sup> Therefore, it seems likely that the compound occurs naturally in the PFR *G. triflora* cultivar and it is not an artefact of the extraction process.



## References

1. Ichimura, K.; Kohata, K.; Mukasa, Y., Yamaguchi, Y.; Goto, R. and Suto. K. (1999). Identification of L-Bornesitol and changes in its content during flower bud development in sweet pea (*Lathyrus odoratus* L.) *Biosci. Biotechnol. Biochem.* 63: 189-191.
2. Schilling N. (1976). Distribution of L-(+)-bornesitol in the Gentianaceae and the Menyanthaceae. *Phytochemistry.* 15: 824-826.

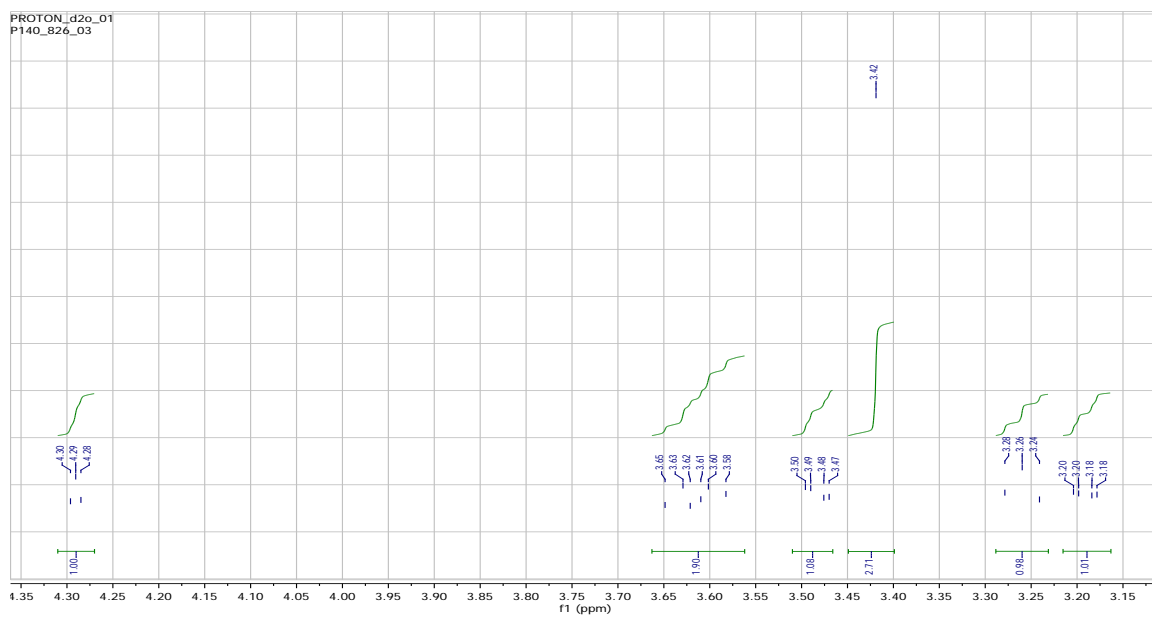
60 Day Night Leaves*4 collection			
Sample Name:	Day Night Leaves*4 collection	Injection Volume:	20.0
Vial Number:	RA5	Channel:	ELSD
Sample Type:	unknown	Wavelength:	n.a.
Control Program:	Sugars_ELSD (Prevail Col) 30min	Bandwidth:	n.a.
Quantif. Method:	SUGARS_ELSD_prevail	Dilution Factor:	1.0000
Recording Time:	26/7/2010 10:27	Sample Weight:	1.0000
Run Time (min):	25.00	Sample Amount:	1.0000



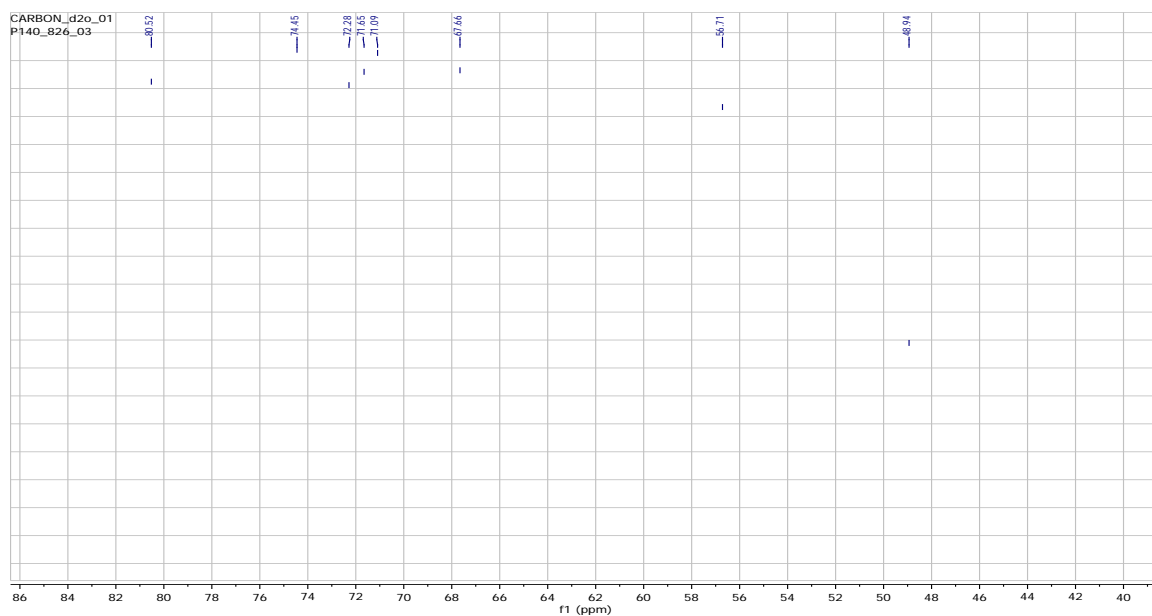
No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount ug	Type
1	2.33	n.a.	202.489	55.427	1.17	n.a.	BMB
2	3.25	n.a.	794.770	184.691	3.89	n.a.	bMB
3	4.08	n.a.	6557.688	1445.780	30.47	n.a.	BMB
4	6.58	n.a.	568.873	193.367	4.08	n.a.	BMB
5	7.50	Fructose	59.123	11.994	0.25	n.a.	bMB
6	8.33	Unknown	1091.298	323.812	6.83	17.207	bMB
7	9.67	Glucose	248.413	175.972	3.71	21.975	bMB
8	13.00	Sucrose	1977.159	981.046	20.68	63.332	BM
9	15.17	n.a.	1461.079	1185.494	24.99	n.a.	Mb
10	19.67	n.a.	187.459	186.902	3.94	n.a.	bMB
<b>Total:</b>			#####	4744.486	100.00	102.514	

Figure 5 HPLC chromatogram from evaporative light scattering detector (ELSD) and associated details of retention times for unknown Peak 6 from ‘Showtime Spotlight’ leaf samples.

**Figure 6:**  $^1\text{H}$  nmr spectrum of unknown fraction

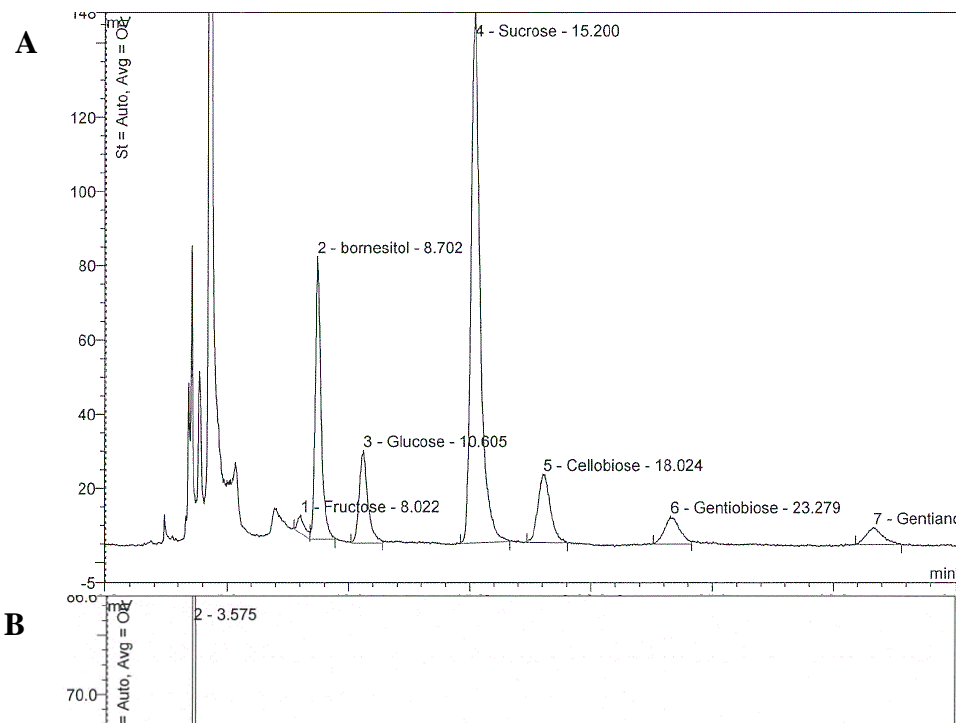


**Figure 7:**  $^{13}\text{C}$  nmr spectrum of unknown fraction

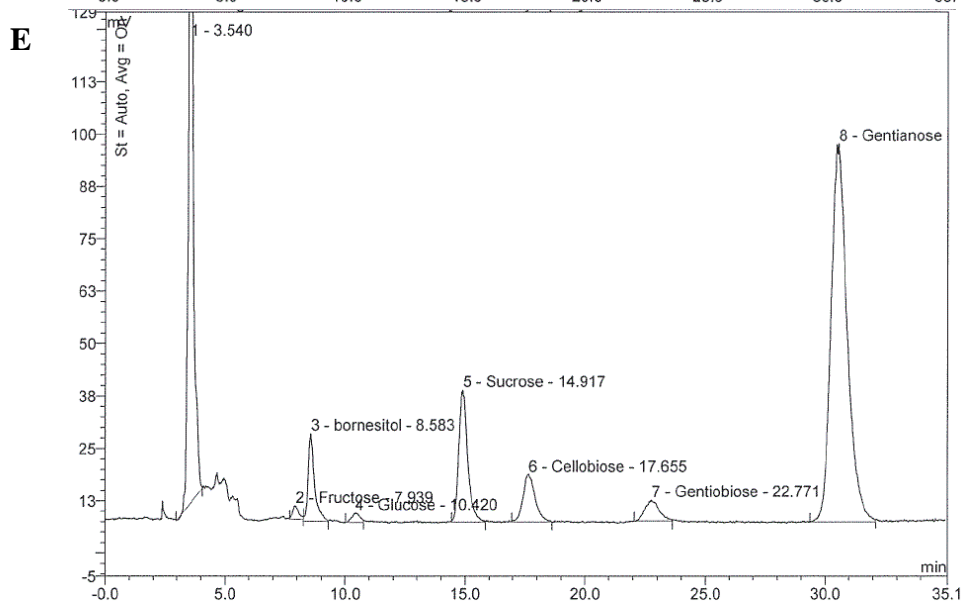
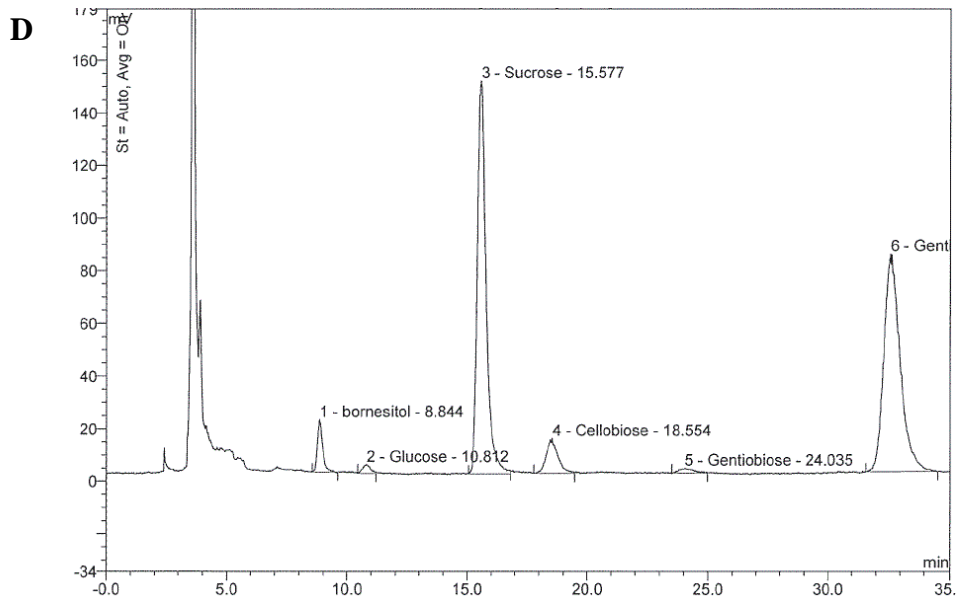
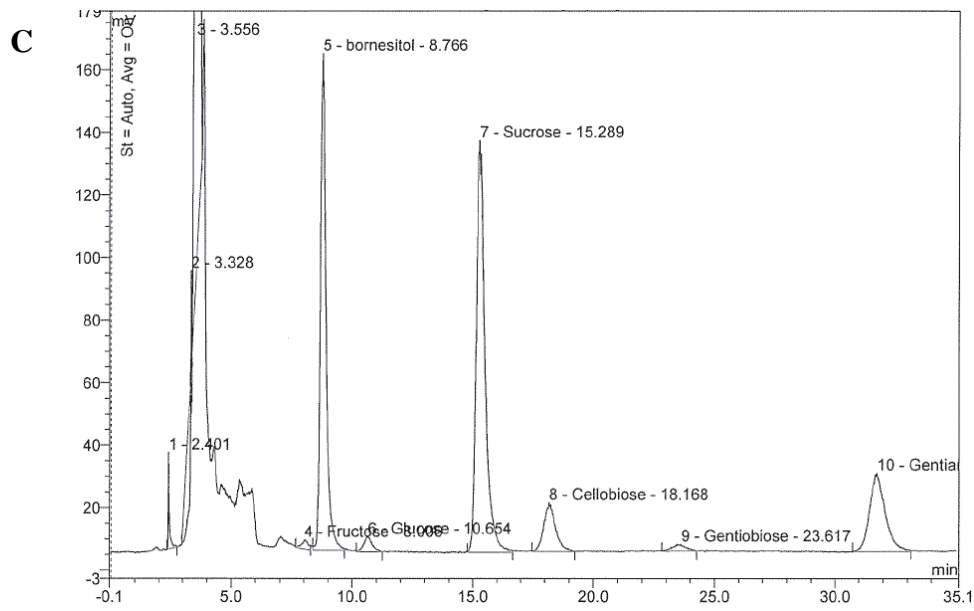


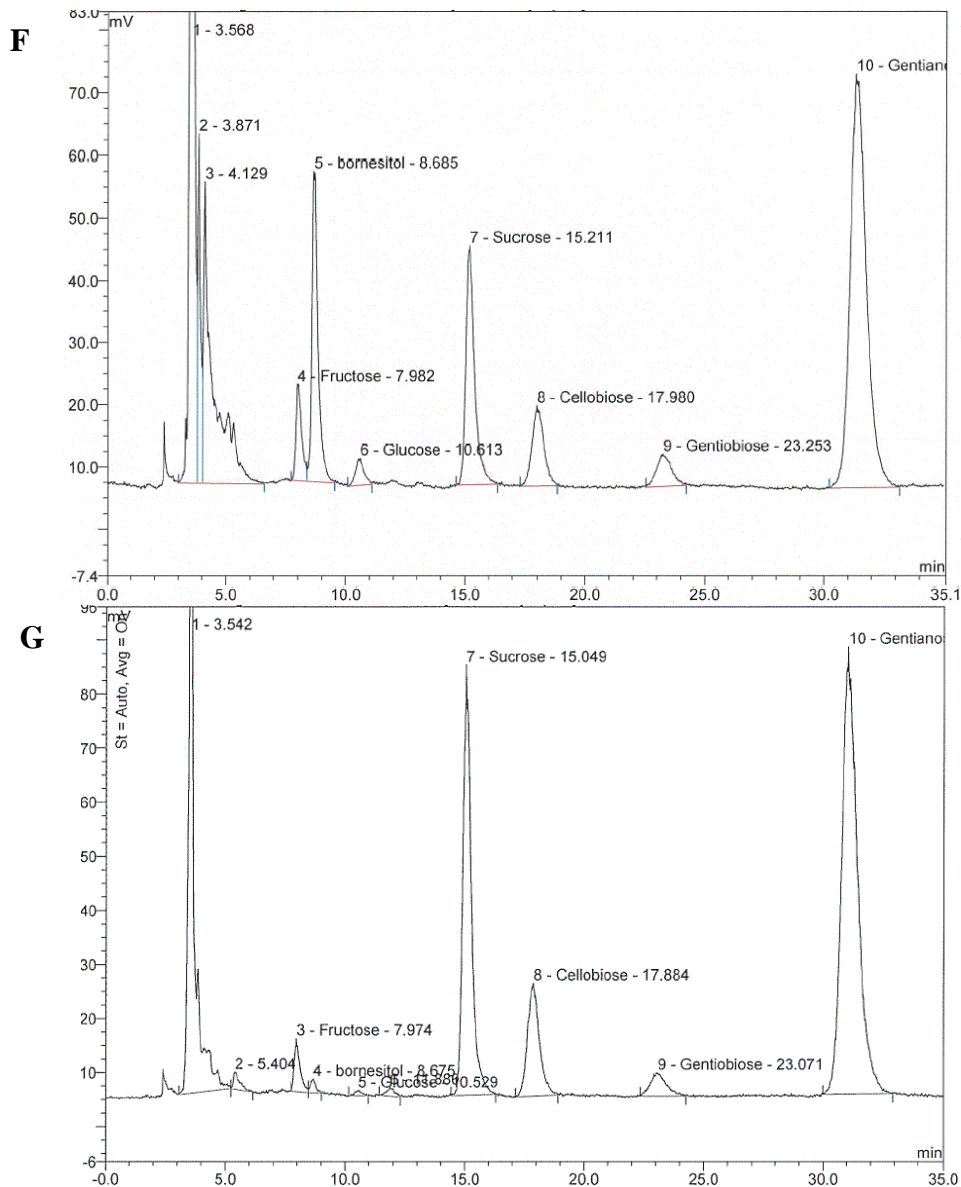
## Appendix II; HPLC chromatograms of non-structural carbohydrates in various organs of gentians

Non-structural carbohydrates (NSCs) in gentian plants are mainly soluble carbohydrates, genianose, gentiobiose, sucrose, fructose, glucose and, potentially, L-bornesitol. These carbohydrates were well separated using a High-performance Liquid Chromatography (HPLC), and detected by an evaporating light scattering detector (ESLD; refer Section 4.2.7). Some representative examples of HPLC chromatograms of the NSCs in various organs of gentian plants are presented (Figure 1A to E) for plants grown *in vivo* and, *in vitro* (Figure 1F and G).



Appendix II – HPLC chromatograms of NSCs

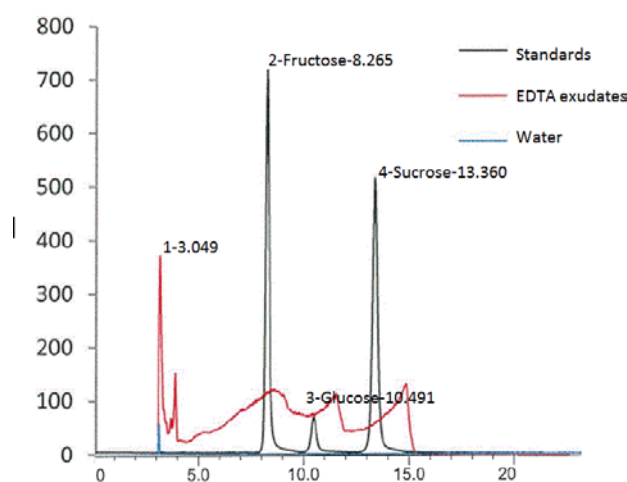




**Figure 1** HPLC chromatograms of non-structural carbohydrates in various organs of gentian plants including, ‘Showtime Spotlight’ cultivated *in vivo* during the 2010 to 2011 growth season, (A) leaves, (B) floral stem tissue, (C) rhizomes, (D) storage roots and, (E) flowers; ‘Showtime Diva’ cultivated *in vitro* for 16 weeks on basal medium containing 9% sucrose (refer Section 5.2.1 and 5.3.4), (F) shoots, (G) roots. HPLC analyses used a column: Alltech® Prevail™ Carbohydrate ES 5 $\mu$  column (250 x 4.6 mm; Grace Davidson Discovery Science), at 30 °C; mobile phase: 25% water: 75% acetonitrile (v:v); injection: 20  $\mu$ l; detector: evaporating light scattering detector (ELSD 1000, Polymer Laboratories, Church Stretton, Shrop, UK) with a gas flow 1.0 SLM (standard litre per minute), nebulizing temperature 40 °C, evaporating temperature 90 °C.

### Appendix III; Abnormal chromatogram of EDTA exudates from the phloem of gentian shoots

Both the stylectomy technique and EDTA-induced exudation were used to analyse the carbohydrates in the contents of the phloem of gentian plants. Poor establishment of aphid feeding prevented the stylectomy technique from being used. In addition, from the initial analysis by High-performance Liquid Chromatography (HPLC), the existence of EDTA in the exudate samples resulted in abnormal chromatograms (i.e. wide tailing peaks, with retention times differing for the standards of the expected carbohydrates; Figure 1). Due to time being limiting, these techniques were not developed further within this thesis. Looking to the future however, it could be suggested that the abnormal HPLC chromatogram was probably a result of the interactions with acetonitrile (the solvent), or the sorbent of the analytical column, and the problem could be resolved with further examination. Similarly the methodology of stylet-exudation may be used in future experiments to obtain pure phloem sap, once a suitable insect/plant combination was established. While this information will be helpful to understand carbohydrate partitioning and sink-source interactions, time did not permit more extensive exploration of either technique in this thesis.



**Figure 1 HPLC chromatogram of EDTA exudates from the phloem of shoots of gentian ‘Showtime Spotlight’; red coloured line: EDTA exudate, black: standards of fructose, glucose and sucrose, blue: water. HPLC analyses used: Alltech® Prevail™ Carbohydrate ES 5 $\mu$  column (250 x 4.6 mm; Grace Davidson Discovery Science), at 30 °C; mobile phase: 25% water: 75% acetonitrile (v:v); injection: 20  $\mu$ l; detector: evaporating light scattering detector (ELSD 1000, Polymer Laboratories, Church Stretton, Shrop, UK), with a gas flow 1.0 SLM (standard litre per minute), nebulizing temperature 40 °C, evaporating temperature 90 °C.**

### Appendix IV; Effect of sucrose concentrations on flowering of gentian plantlets *in vitro*

Experiments conducted *in vitro* (refer Section 5.2.2) showed that the concentration of sucrose in the basal medium (BM) significantly affected flowering of ‘Showtime Diva’ while *in vitro* (Figure 1; Table 1). Plantlets started to present open florets in the 10<sup>th</sup> week while *in vitro*, with no new florets opening by the 16<sup>th</sup> week. The highest percentage of plantlets flowering occurred with the BM containing 6% sucrose (87.5 % and 65 % for tips and nodes, respectively). Plantlets derived from tip cuttings developed florets on BM containing 3% to 15% sucrose. In contrast, plantlets derived from nodal cuttings only developed florets on BM containing 3% to 9% sucrose. While the florets on plantlets derived from shoot tips on BM containing 12% and 15 % sucrose were visibly evident, they did not open. Additionally, the plantlets that developed florets did not develop crown buds.



**Figure 1** Flowering of plantlets of ‘Showtime Diva’ after 12 weeks growing *in vitro* on basal media containing 9% sucrose, cultured under a 18 h photoperiod and 20 °C.

**Table 1** Effect of different sucrose concentrations within the basal media *in vitro* on flowering of gentian ‘Showtime Diva’. Data was collected after 16 weeks cultivation.

Sucrose concentration (%)	Total proportion of plantlets flowering (%) <sup>Z, Y</sup>	
	Tips	Nodes
0	-	-
3	62.5 ± 8.8 b	52 ± 4.7 ab
6	87.5 ± 3.9 a	65 ± 7.3 a
9	70 ± 7.5 ab	40 ± 5.5 b
12	20 ± 3.1 (not open)	0
15	16 ± 6.0 (not open)	0
20	-	-

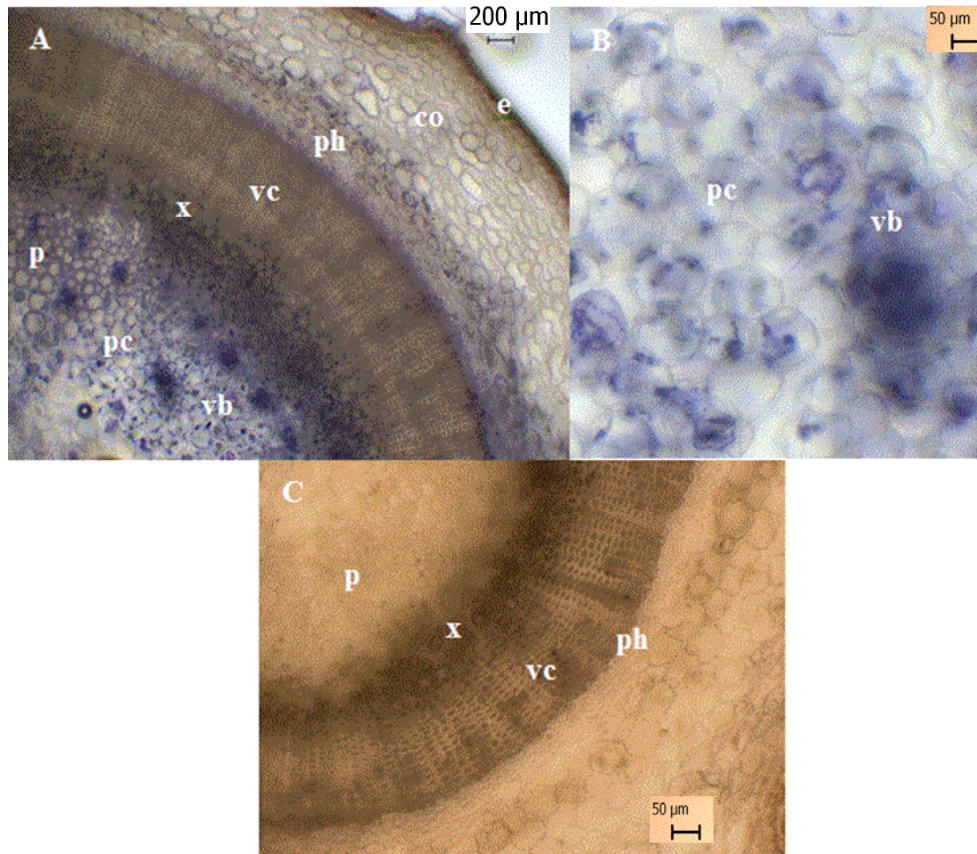
<sup>Z</sup> Mean values (± SE). Treatments within which cuttings did not develop are represented by ‘-’.

<sup>Y</sup> Within the same column, mean values followed by different letters were significantly different (Turkey test:  $P < 0.05$ ).

## **Appendix V; *in situ* staining of glycoside hydrolase activity involved in carbohydrate metabolism in gentian tissue**

As an extension to protocols developed within Chapter 8, glycoside hydrolase (GH) activity of sucrose, gentianose, and gentiobiose were attempted to be stained *in situ*, using the method described by Doehlert and Felker (1987) and Sergeeva et al. (2002). Fresh floral shoot stem tissue of gentian ‘Showtime Spotlight’ at harvest maturity (refer Chapter 7), were cross-sectioned by hand with a razor blade at 120 – 200  $\mu\text{m}$  thickness. The sections were immediately fixed in 2% paraformaldehyde with 2% polyvinylpyrrolidone 40 (PVP) and 0.001 M dithiothreitol (DTT), pH 7.0, at 4°C for 1 h. After fixation, sections were rinsed overnight in water at 4 °C and refreshed at least five times to remove soluble carbohydrates. To detect the activity of enzymes, sections were incubated in 1 ml of incubation medium in a water bath at 30 °C for 30 min. For sucrose and gentianose GHs the incubation medium contained 38 mM sodium phosphate buffer (pH 6.0), 25 U glucose oxidase (Ceusters et al.), 0.024% nitroblue tetrazolium (NBT), 0.014% phenazine methosulphate, and 10 mM sucrose and gentianose. For gentiobiose GH, sodium phosphate buffer (pH 7.0) was used. In the controls, sucrose, gentianose, and gentiobiose were omitted, respectively. After the incubation period, all enzyme reactions were terminated by rinsing the sections in distilled water. The sections could be stored in distilled water at 4 °C for at least 1 month without loss of staining.

In this preliminary experiment, sucrose GH activity was successfully stained using fresh floral shoot stem tissue, while gentianose and gentiobiose GH activity were not established. In the stem tissue, a clear staining of sucrose GH activity was found in the phloem, vascular bundles, and parenchyma cells in the pith (Figure 1). The methods for successful staining of gentianose and gentiobiose GHs appear to require further optimization, probably by adjusting pH, substrate concentration, and time of collecting samples, etc.



**Figure 1** *In situ* staining of sucrose GH activity in floral shoot stem tissue of 'Showtime Spotlight'; (A) a successfully stained cross section identifying cell types within stem tissue, (B) a magnified image of stained parenchyma cells within the pith and, (C) the control with sucrose substrate omitted. Samples were collected in autumn (April) 2011. e - epidermis, co - cortex, ph - phloem, x - xylem, vb - vascular bundles, p - pith, pc - parenchyma cells.

Ceusters, J., Borland, A. M., Ceusters, N., Verdoort, V., Godts, C., & De Proft, M. P. (2010). Seasonal influences on carbohydrate metabolism in the CAM bromeliad *Aechmea* 'Maya': consequences for carbohydrate partitioning and growth. *Annals of Botany*, 105(2), 301-309

Doehlert, D. C., & Felker, F. C. (1987). Characterization and distribution of invertase activity in developing maize (*Zea mays*) kernels. *Physiologia Plantarum*, 70(1), 51-57

Sergeeva, L. I., & Vreugdenhil, D. (2002). *In situ* staining of activities of enzymes involved in carbohydrate metabolism in plant tissues. *Journal of Experimental Botany*, 53(367), 361-370

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