The physiology and control of crown bud formation and development in gentians

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Abstract

The spread in time to flower harvest maturity and an inability to produce an adequate number of crown buds during crop establishment, were perceived as problems by commercial growers of gentians for cut flowers. By identifying and investigating plant and environment related factors that influence growth and development, the current thesis aimed to investigate both the causes of these problems and identify potential solutions.

Crown buds which appeared during the previous growth cycle were the source of floral shoot production and, in both true seedlings and clonally propagated plants, these buds typically originated on the transition zone between shoots and roots. When formed on the transition zone the first and, as a consequence, apical bud of the crown bud cluster, originated adventitiously. The individual crown buds which subsequently initiated within the cluster developed as axillary buds from that apical bud. As evident from the appearance of two to five times more crown buds and shoots, and crown bud clusters, the naturally occurring short photoperiodic regime promoted the crown bud formation process, compared to a long photoperiodic regime. With the application of Ethephon, or Ethephon followed by either Thidiazuron or Paclobutrazol, it was possible to achieve a similar increase in the number of crown buds under the long photoperiodic regime. In contrast to the strong photoperiodic response, exposure to the cooler temperatures experienced outside did not influence the crown bud formation process.

Once formed, crown buds were under different types and degrees of dormancy (i.e. endo, para or eco). Dependent on both the developmental stage of plants and the hierarchical position of buds within a crown bud cluster, shoot emergence and subsequent development to flowering varied in the quantity, quality of shoots, and timing in response to dormancy breaking treatments (chilling, gibberellic acid (GA_3), or clipping), Hence when applied prior to shoot emergence, application of chilling to plants of the cultivar ‘Diva’ resulted in an eight-fold increase in the number of floral shoots, along with a 83 day reduction in the spread in time to harvest maturity. The requirement of chilling could be substituted by GA_3. In contrast, when applied following the start of shoot emergence, only application of GA_3 was effective in increasing shoot emergence and narrowing the spread in time to harvest maturity.

Within a bud cluster, a positive relationship between duration to harvest maturity and a negative relationship with floral shoot length was evident with hierarchical position of the crown bud from proximal to the distal end. This influence of hierarchical position was most evident following application of chilling or GA_3, potentially explaining up to 28 days spread in duration to harvest maturity in 'Diva'. While the aforementioned factors related to shoot emergence, development and hierarchical position contribute to the sources of variability within a plant or an individual cluster, both the sources and extent of variation in time to harvest maturity, varied within cultivars, with wider spread in time to harvest maturity in 'Starlet' (41 days) than 'Diva' (35 days) and 'Spotlight' (29 days).

The present study has increased the understanding of factors contributing to growth and development of gentians as an ornamental crop. Treatments have been developed to allow manipulation of the formation of crown buds, subsequent emergence and compactness of flowering duration.

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List of Abbreviations

1-MCP	1-methylcyclopropene
ABA	Abscisic acid
AO	Acridine orange
BA	Benzyladenine
CRD	Completely randomised design
DNMRT	Duncan's multiple range test
ethephon	2-chloroethylphosphonic acid
FAA	Formaldehyde: glacial acetic acid: ethanol
FI	Flower initiation
GA	Gibberellins
GA3	Gibberellic acid
IAA	Indole-3-acetic acid
LD	Long photoperiodic regime
LSMEANS	Least square means
NAA	Naphthalene 1-acetic acid
NPA	Naphthalphthalamic acid
NZ	New Zealand
PAR	Photosynthetically active radiation
PBZ	Paclobutrazol
SAM	shoot apical meristem
SAS	Statistical Analysis System
SD	Short photoperiodic regime
T	temperature
TDZ	Thidiazuron

List of Publications

- Samarakoon, U.C., K.A. Funnell, D.J. Woolley, E.R. Morgan**. "Sources of Variation in Time of Harvest Maturity of Flowering Shoots in Three Cultivars of Gentian"** submitted for publication in New Zealand Journal of Crop and Horticultural Science. (Chapter 2)

- Samarakoon, U.C., K.A. Funnell, D.J. Woolley, E.R. Morgan. 2012 **"Temperature Impacts Changes in Crown Buds and Flowering of Gentian 'Spotlight'"** Scientia Horticulturae vol. 143(49-55)

- Samarakoon, U.C., K.A. Funnell, D.J. Woolley, G. Burge, E.R. Morgan. **"Initiation, Emergence and Development of Crown Buds in Gentians"** accepted for publication in Proceedings of International Symposium of Ornamental crops, IHC 2010. (Part of Chapter 3; included Appendix VI)

Chapter 1 General introduction and literature review

1.1 Introduction

The value of exports of cut flowers and foliage from New Zealand (NZ) was around NZ$ 35.1 million in 2009 (Aitken and Hewett, 2010). NZ is among the top twenty countries of flower exporters, and the main importer of cut flowers from NZ is Japan (NZ$ 19.1 million). The NZ export industry was initially developed around *Cymbidium* orchid, and has now diversified to include novel cut flowers like *Zantedeschia*, *Sandersonia*, *Nerine*, *Paeonia* and *Gentiana* species. Japan, the world's largest market for gentians (≈ 125 million stems per annum) (Anonymous, 2011a), is also the biggest importer of gentians from NZ. As production of gentians in NZ coincides with winter in Japan, there is a strong demand for high quality flowers produced at this time as natural production is low. Demand for gentians as a cut flower in other international markets is also growing. Hence there is potential to increase export earnings from gentians provided that flowers are supplied according to the required quality, quantity, and time.

Originally most of the gentians for cut flower production were either blue or white. Recently however a breeding program within the NZ Institute for Plant & Food Research has successfully produced new cultivars, derived primarily from *Gentiana triflora* Pall and *Gentiana scabra* Bunge, with an extended range of colours (Morgan et al., 2003). The intention of this breeding was that this increased diversity would assist further expansion of the market. So as to get higher prices for their cut flowers, gentian growers are looking for opportunities to:

1. better target specific market times, and
2. extend the flowering season.

This thesis therefore, was undertaken to assist in providing scientific knowledge that might underpin the industry's strategies to take advantage of these opportunities. Given the focus of this thesis on the recently developed cultivars, the term "gentian(s)" was primarily intended to refer to these cultivars and their respective parental lines, i.e. not necessarily all species.

1.2 Morphology, ecology and growth cycle

1.2.1 Morphology

Gentiana L. belongs to the family Gentianaceae, and the genus *Gentiana* is reported to comprise 362 species distributed worldwide, mainly associated with an alpine/mountain habitat (Ho and Liu, 2001). In horticulture, some species and hybrids are used as landscape or potted plants, and some as a cut flower. Plants flower in spring to autumn (albeit species of interest in this thesis flower in late summer through autumn), and have characteristic trumpet-shaped flowers on leafy stems.

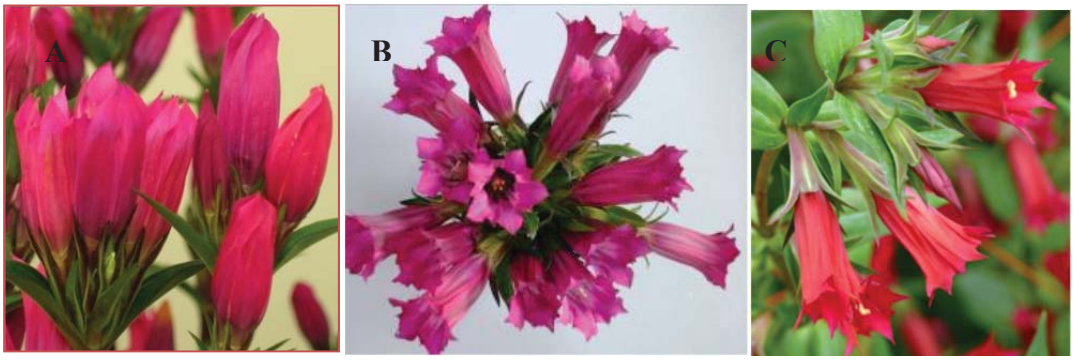


Figure 1.1 Recently developed cultivars of gentians; (A) 'Showtime Spotlight', (B) 'Showtime Diva' and, (C) 'Starlet'.

Source of images; courtesy of NZ Institute for Plant & Food Research

The inflorescence is a cyme, which can be simple with 1-3 flowers or compound with flower numbers ranging between *"few to many"* (Ho and Liu, 2001). Within the naturally occurring species, deep blue and sky blue are the most common flower colours but, white, cream, yellow, and pink coloured flowers are also found (Bartlett, 1975). Hybrids developed recently by the NZ Institute for Plant & Food Research, including 'Showtime Spotlight' ('Spotlight'), 'Showtime Diva' ('Diva') and 'Showtime Starlet' ('Starlet') (Figure 1.1), were predicted to have market potential as they comprise novel colours. These hybrids have *G. scabra* and *G. triflora* as dominant parental lines. Both these species belong to section Gentiana of the taxonomic classification of *Gentiana* spp (Ho and Pringle, 1995). This section consists of perennial plants with short, thick, fleshy, horizontal to vertical rhizomes underground (Figure 1.2), with stems branching sympodially with one to a few erect, simple flowering shoots (Ho and Liu, 2001). While taxonomic descriptions refer to the storage organ in these species as rhizomes (Ho and Liu, 2001; Ho and Pringle, 1995), other articles on gentians refer to a crown with dormant buds (Ohkawa, 1983). The term "crown" can be defined as comprising the

compressed stem tissue (i.e. rhizome), the associated buds from which new shoots emerge, storage roots and feeder roots (Figure 1.2). Both published (Ohkawa, 1983) and anecdotal comments by growers refer to these buds present within the crown as "crown buds", "winter buds", "dormant buds" or "buds arising from an underground stem".

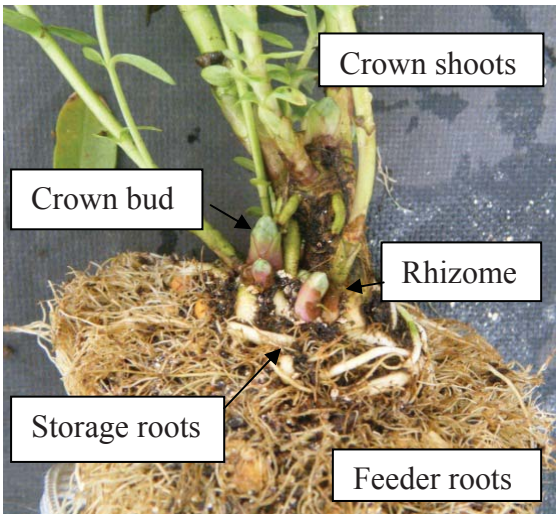


Figure 1.2 Typical features of the crown present in cultivars of gentian derived primarily from the parental line of *Gentiana triflora*. Morphological features include; rhizome, crown buds, crown shoots, storage roots and feeder roots.

In this thesis, unless stated otherwise, use of the term "stem" is reserved for reference to the rhizome, with the term "shoot" used to refer to the leaf-carrying structures arising annually from buds during the growing season (Figure 1.2). So as to enable comparisons to existing literature, within some experiments involving histological examination, both structures are referred to as "stem" (refer Chapter 5).

1.2.2 Natural habitat of parental lines of recently introduced hybrids

As one of the dominant species used in the development of new cultivars, *G. scabra* is native to Korea, Manchuria, Northern China and Japan (distributed across the approximate latitudes between 30°N to 54°N), "*being found in thickets, grassy places and wet meadows in lowlands and low elevations within mountains*" (Ohwi, 1965). The said areas have corresponding photoperiods of 14:00 h:min to 17:00 h:min in June, and a minimum of 7:00 h:min to 10:00 h:min in December (Lammi, 2005). The other dominant species, *G. triflora*, is native to eastern Siberia, Korea, Sakhalin and Japan (distributed across the approximate latitudes between 34°N and 54°N) being found in alpine meadows (Ohwi, 1965). For this species, the said areas have corresponding

photoperiods of 14:25 h:min and 17:00 h:min in June, and a minimum between 7:22 h:min and 10:00 h:min in December (Lammi, 2005).

At the commencement of the current research, the critical photoperiod for attainment of different developmental stages in parental lines or presently cultivated varieties were not known. Assuming the hybrids used have inherited characteristic responses for the photoperiod from the parental lines, the above photoperiodic ranges were utilized as the basis in determining potential short and long photoperiodic regimes to be investigated within some of the experiments (refer Chapters 6 & 7).

With regards to the naturally occurring temperature, in parts of Japan where some of the parental lines are naturally found, and commercial cut flower production has been carried out historically, the average monthly temperature ranges from -4 to 18 ºC (Anonymous, 2011b). These plants in Japan experience a summer with ambient temperatures ranging from 22.5 to 13.7 ºC and, in winter, from -1.4 to -7 ºC, during which plants are covered with snow. In comparison, commercial fields in NZ experience a milder winter climate (e.g. between 1 and 10 ºC) (Anonymous, 2011c). Along with diverse temperature ranges in areas of current cultivation, at the commencement of the current research, the critical temperature for attainment of different developmental stages in parental lines or presently cultivated varieties, were not reported.

1.2.3 Growth cycle of cultivars used in cut flower production

Due to the lack of peer-reviewed articles related to the growth cycle of gentians, only one main article is cited in this section, i.e. Ohkawa (1983). Based on that article gentian plants mature in 2 years, but commercial cut flower production is from the fourth year. The details of the annual growth cycle presented (Ohkawa, 1983), can be adapted to NZ conditions as follows: Gentian plants undergo *"dormancy"* in winter (June-August), with shoot emergence and vegetative development occurring in spring (September to October; Figure 1.3). Flower initiation is reported to occur in early summer and anthesis in summer to late autumn. During autumn through winter the above-ground portion progressively dies off (i.e. natural senescence), with the plant overwintering as an underground crown, bearing buds. Based on this growth habit, gentians can be categorised as perennial geophytes, i.e. plants with perennial buds

situated below ground on a storage organ, and show active growth in a limited number of seasons (Dole, 2003).

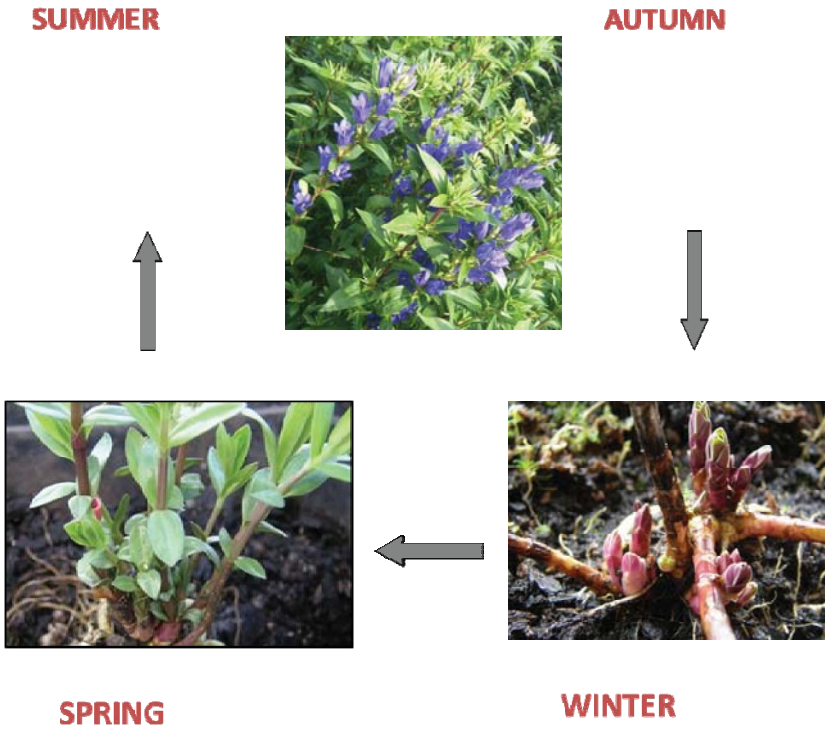


Figure 1.3 Diagram depicting typical seasons corresponding to the stages of growth and development of mature gentian plants during an annual growth cycle, i.e. dormant crown buds in winter, shoot emergence in spring, and flowering in summer through autumn.

In Japan, historical cultivars (predominantly derived from *G. triflora*) are categorised as early or late flowering, with the timing and requirements for flower initiation and flower development presented based on conditions that are typically occurring during flowering in these regions (Table 1.1). To which category the cultivars recently developed by the NZ Institute of Plant & Food Research, or their parents, belong, is however unknown. With the timing of flowering noted as one of the primary research opportunities noted for this thesis (refer Section 1.1), development of an understanding of the annual growth cycle with relevance to these new cultivars was, therefore, seen as worthy of investigation.

As an additional reason for questioning the relevance of existing information, most of the cultural recommendations presently used for gentians were developed for cultivars and climatic situations available in Japan. As noted above (Section 1.2.2), when compared to Japan, temperature changes within seasons are comparatively mild in NZ. Hence, how the timing of the various developmental phases relates to the annual growth

cycle in Japan (Table 1.1) may not be directly applicable to NZ conditions, nor to the cultivars of interest. This questionable relevance of information available also prompted the need for further investigation within the current thesis.

Table 1.1 Timing, morphological changes and conditions typically occurring during flowering in historical cultivars of gentians (i.e. selections of *G. triflora)*. (Calendar months have been modified to equate to those occurring in the southern hemisphere).

Requirement	Early flowering cultivars	Late flowering cultivars
Time for flower initiation (FI)	November -December	Late December
Stem length at FI	30-40 cm	60-70 cm
No. of unfolded leaves at FI	15-17	24-28
Temperature at FI	17 ºC (max 22, min12 ºC)	
Conditions for flower development	Long day & high temperature (20-25 ºC)	Short day & cool temperature. But delayed at long day & high temperature (20-25 ºC)
Anthesis	January	March

Source: Ohkawa (1983).

1.3 Problematic situation

1.3.1 Spread in time to flower harvest maturity

At present, NZ's export-oriented gentian growers are faced with the problem of a wide spread in timing of individual shoots reaching flower harvest maturity, which makes it difficult to target specific markets with a specific cultivar. For growers not targeting specific markets, the natural spread in timing of harvest maturity among multiple cultivars of gentians is however beneficial, as growers can get production throughout a wider period of the year. It is the former scenario therefore, not the latter, that provides the industry's motivation for the current research.

As presented within Figure 1.4, based on data from a growers' field, when Cultivar 2 and 3 are considered, flower production was less at the beginning and end of each of their individual flowering seasons, and thus created a wide spread, i.e. between 6 and 9

weeks (Figure 1.4). This is in contrast to the peak in flowering with Cultivar 1 which was spread over a period of only 4 weeks.

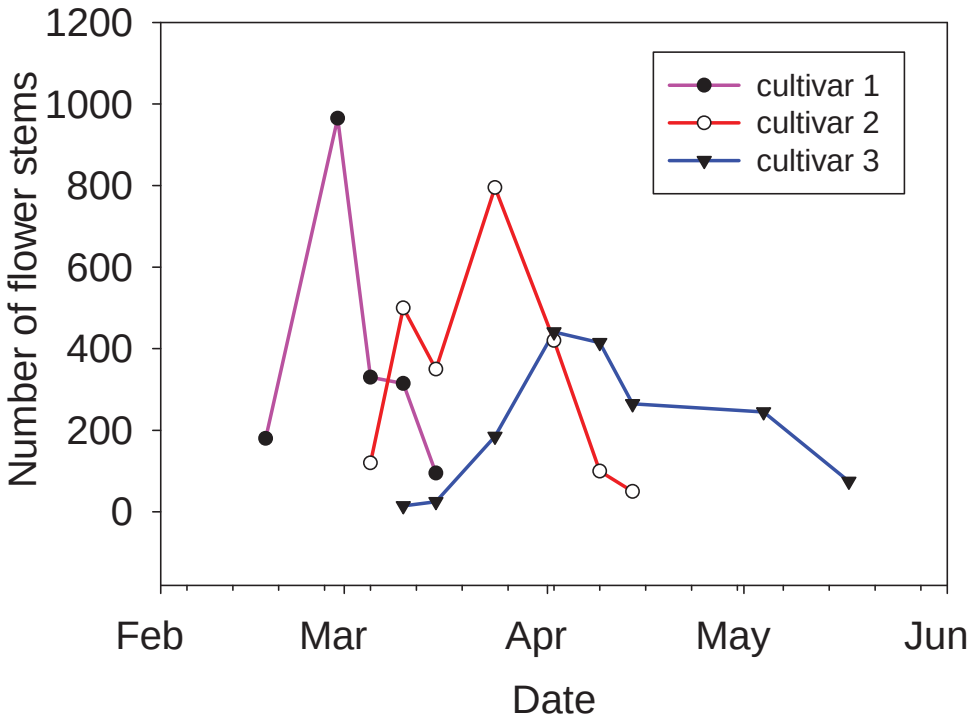


Figure 1.4 Number of floral shoots harvested per week from 3 cultivars in a commercial field in New Zealand (Feb-May 2007).

Data: Courtesy of NZ Ashiro Gentian Growers.

Having a wide spread in the timing of flower harvest maturity, with few floral shoots to harvest on any one day, is not considered to be economical to the growers, as the labour cost for flower pickers is less likely to be compensated by the sales from production. For a given cultivar therefore, a few weeks of flowering with a high number of floral shoots is most desirable. The existing interpretations of spread in time to flower harvest maturity however, are not based on statistically validated data, hence their accuracy is questionable. At the commencement of this thesis therefore, it was considered necessary to quantify the spread in time to flower harvest maturity of some recent cultivars (Chapter 2).

Although research related to plant breeding (Morgan, 2004) and post-harvest management (Eason et al., 2004) has been published, research based on the growth and physiology of gentians is limited, or published in technical reports in Japanese. Peer-reviewed literature with statistically validated information did not exist pertaining to the

influence of plant or environment-related factors on the timing of flower harvest maturity in gentians. Hence at the beginning of this thesis there was no accurate data quantifying the spread in time to flower harvest maturity or identification of the potential sources of such variation. As explored within this thesis, it was therefore considered necessary to identify and quantify the potential factors, both plant and environment-related, that could influence time to flower harvest maturity.

1.3.2 Vegetative propagation – crown buds

Gentians can be propagated from seeds, cuttings or through tissue culture. In commercial cultivation, vegetative propagation is desired so as to get a uniform population of true-to-type plants. Thus cuttings and tissue cultured plants are common methods of propagation in both NZ and Japan. Based on anecdotal information however, plants propagated through cuttings and tissue culture either take longer than desired to reach commercial levels of productivity or fail to regenerate through to the next growing cycle when first transplanted (Ed Morgan personal communication; Takashi Hikage personal communication). It was hypothesized that these limitations could be related to the failure of initiation and development of an adequate number of crown buds. Crown buds were presumed to contribute to the floral shoots harvested and, without these buds, plants would not survive beyond the first growth cycle so as to contribute to commercial yields. It was considered necessary therefore, to identify the factors that influence the initiation and development of crown buds in gentians, with the ultimate view to develop strategies to achieve earlier plant regeneration, and to reduce the duration to start commercial flower production.

1.3.3 Potential commercial implications of the thesis

In order to address the problematic situation mentioned above, the broad horticultural goal of the current research programme, of which this thesis forms a part, was to identify;

- firstly the potential factors for spread in the time to flower harvest maturity within a season and,
- secondly the potential strategies to manipulate initiation, development and emergence of buds.

The former would enable development of strategies for growers of gentians to improve their ability to schedule production of flowers. Commercial technologies to control timing of flower harvest maturity may increase profitability both in export and domestic markets. The latter would enable early initiation, development and emergence of buds in plants, which would subsequently potentially aid in early and uniform flowering and spread, as well as prevent the loss of plants following transplanting.

In addition to the industry-orientated goals noted above, since there is minimal scientific information published about the physiology of gentians, any information developed would contribute to the advancement of understanding of the environmental requirements and physiology of this crop, as well as herbaceous perennials in temperate climates in general. The current research therefore has the potential to benefit the NZ floriculture export industry, domestic gentian growers, and the international scientific community keen on understanding the environmental requirements and physiology of temperate plant species.

1.4 The research strategy for the thesis

Based on the brief description of the growth cycle of gentians presented above (Section 1.2.3), it was concluded that the timing of flower harvest maturity could depend upon various development stages, and factors that influence these different stages. At the commencement of the current thesis it was assumed that the main stages defining the developmental process would start from initiation and development of a bud, followed by its emergence and subsequent development as a shoot, through to reaching anthesis. In terms of the factors that might influence these developmental stages, both plant-related and environmental factors can be influential (Koornneef et al., 1998; Reeves and Coupland, 2000) and, therefore, were considered appropriate for investigation in this thesis. Available peer-reviewed articles however, only provided summary data of some cultural information related to temperature and photoperiodic influences on growth, with no provision of statistical validation (Ohkawa, 1983). Due to this lack of adequate data related to the developmental process, it was first considered necessary to identify and quantify the timing of the developmental stages and their correlations (Chapter 2 and 3). It was envisaged that this would provide an understanding of how key developmental stages that occur earlier in the developmental process would subsequently influence flowering. Subsequently these key developmental stages could be investigated further in detail, to identify the potential influence of any plant or

environmental related factors, and potential to manipulate them, in order to achieve better floral productivity (Chapters 4, 6-10).

As applied to other plant species, the timing of developmental stages can be described with chronological days (Funnell et al., 2003), degree-days (Funnell, 2008), and also using morphological features (Sachs, 1999). These have not been described however, for new cultivars of gentians. Within the context of the current study with gentians, following identification of the stages of the developmental process and corresponding morphological changes leading to flowering (Chapter 2, 3 & Appendix I), the time to reach these stages could be quantified among cultivars, plants or shoots. Any differences detected following such quantification would enable identification of any sources of variability in duration to reach these developmental stages (Chapter 2 & 3).

1.5 Plant form and the developmental process

1.5.1 Types of buds

In the developmental process to flowering, the initiation and development of buds was seen as being the starting point of the process. In order to determine the factors influencing this initial stage, as described below, it was considered necessary to describe the types of buds present in gentians.

1.5.1.1 Apical, axillary and adventitious buds

Plants can be viewed as consisting of banks of different types of buds, that can give rise to vegetative regeneration (Vesk and Westoby, 2004). These banks of buds can be utilized following termination of dormancy, to supplement developing shoots during the growth cycle or, to replace growing shoots lost (Stafstrom, 1995). Types of meristems/buds can be categorised based on their location of origin on plants and their initiation, as being apical, axillary, or adventitious (Evert, 2006; Kerstetter and Hake, 1997). The apical bud is located at the apex of the shoot, is derived from the apical meristem, and lays the foundation for the subsequent development of the whole shoot via cell division. Axillary buds typically develop from the axils of the leaves, and adventitious buds arise *de novo* from any plant tissue. Hence adventitious buds may appear on leaves, stems or roots.

With gentians possessing a determinate shoot growth habit and sympodial branching (Ho and Liu, 2001), the involvement of the apical meristem in the first flowering of a

gentian plant from seed is known. In subsequent growth cycles however, in gentians, the types of buds that develop into floral shoots (continuation buds) have not previously been described, i.e. whether they are axillary or adventitious in origin. If both axillary and adventitious buds are present in gentians, they could both potentially give rise to floral shoots. In contrast to axillary buds, leaf expansion and shoot development rates are typically slower in adventitious buds, as noted in other species (Del Tredici, 2001; Vesk and Westoby, 2004). If, therefore, different types of buds are present in gentians, their growth and development may differ, giving rise to different times at which shoots reach anthesis in any given season (refer Section 1.3.1). It was therefore necessary to identify which types of buds exist, and the morphological and physiological changes during growth and development of shoots originating from the differing bud-types present (Chapters 3, 4 & 5).

Morphology, anatomy and ontogeny of buds could be used in determining whether these buds in gentians are axillary or adventitious. Axillary meristems, which give rise to axillary buds, are typically associated with a leaf primordium. This axillary meristem could be derived from the primary shoot apical meristem (SAM), which arises during embryogenesis (Evert, 2006; Kerstetter and Hake, 1997) or from groups of meristematic cells which originate directly from a detached part of the primary SAM in the axils of leaf primordia (Shimizu-Sato et al., 2009). In contrast, adventitious buds develop from endogenous (i.e. tissues deep within the parental axis) or exogenous tissue (i.e. tissue positioned at a relatively superficial depth) of any plant organ (Evert, 2006). In order to distinguish between axillary and adventitious buds present in gentians, characteristic features relative to the origin, associated tissues, and vascular connections from the bud to the tissue of origin could be utilised as a research tool when investigated both macroscopically and microscopically (Chapter 5).

In herbaceous perennials, vegetative propagules (including stems, rhizomes, tubers, bulbs, stolons etc) contain axillary and adventitious buds capable of producing new shoots under permissive environments (Anderson et al., 2001). Most of the cultivars of gentians used in the current research programme have been developed from *G. scabra* and *G. triflora*, as dominant parental lines. As mentioned previously, the published literature (Ohkawa, 1983), and growers, refer to buds present in gentians as “crown buds”, “winter buds”, “dormant buds” or “buds arising from an underground stem”. The origin of these particular buds or their function however, has not previously been

described. As it was considered important for the current study to develop a more appropriate descriptive terminology for crown tissue and crown buds, anatomical studies were conducted in order to identify the types, ontogeny and role of the buds in the gentians of interest (Chapter 3, 4 and 5). Further to that, it was considered necessary to understand the developmental sequence of any bud type from initiation till anthesis, in order to investigate what if any correlations might exist between developmental stages and, therefore, what potential might exist for manipulation of initiation, development, emergence of buds and, development of shoots.

1.5.2 Methods for morphological and anatomical investigations

As detailed in Section 1.5.1.1, in order to identify what if any association existed between types of buds and their developmental process, as explored below, both macroscopic and microscopic techniques were used during the current thesis.

1.5.2.1 Macroscopy

Tracking of changes occurring within the crown of geophytes, such as *Asparagus officinalis* L., at regular intervals, has been successfully used to identify the types of buds as well as the timing of their emergence and subsequent fate (Danningsih, 2004). It was envisaged during the current thesis that by using a similar procedure the emergence of buds and subsequent shoot development could be traced over time, to identify and quantify the different bud types and the influence of timing of their initiation, emergence and development, on the timing of harvest maturity (Chapter 3). Macroscopic examinations could also be useful in determining the origin of buds relative to the associated tissue, and their progressive development (Chapters 4 and 5). Further to this, dissections (Chapter 3, 4 & 5) could be used to reveal the progressive arrangement of structures such as buds, and leaf or floral primordia (Inouye, 1986; Sabatier and Barthélémy, 2001).

1.5.2.2 Microscopy

Different microscopic techniques such as light, confocal, fluorescence or electron microscopy could be usefully applied in identifying anatomical features of plant tissues. Histological studies have been undertaken successfully using light microscopy to identify the origin and development of buds on seedlings of herbaceous perennials like *Euphorbia esula* L. (Myers et al., 1964), *Cardopatum corymbosum* L. (Chiatante et al.,

2008), as well as many woody perennials like *Quercus* species (Pascual et al., 2002), *Myrtaceae* species (Burrows, 2000) and *Araucariaceae* species (Tomlinson and Huggett, 2011). Light microscopy was therefore considered as a possible technique to use in the histological studies presented in this thesis (Chapter 4 & 5). Laser confocal microscopy has been used as a method in studying anatomical features of plant tissues (Pawley, 2006), including SAMs (Lemon and Posluszny, 1998), young shoots (Kitin et al., 2005), and xylem vessels (Kitin et al., 2004). This technique offers the advantage of enabling observation of tissues, regardless of thickness of the section. Given the wide divergence of tissue types/sizes that could be evident in gentians, it was considered that in the current study laser confocal microscopy might also offer a possible alternative, if light microscopic techniques were found to be limiting.

Proper sample preparation is a key requirement for any microscopic technique. During the current study it would be necessary to achieve progressive sections of the samples in order to identify the vascular connections to the associated tissues. Sample preparation could be conducted using protocols such as wax (Eaton et al., 2010; Spence, 2001) or resin embedding (Busse and Evert, 1999), as this facilitates sequential sectioning of tissues using a microtome. Based on the facilities available at Massey University, wax embedding protocols were considered to be economical and feasible for the current study. The success of sectioning following wax embedding however depends upon the softness of tissues. In contrast to light microscopy, confocal microscopic techniques do not require having thin sections, therefore could be used to handle even fibrous tissues (Chapter 5).

1.5.2.3 Staining of the tissues for microscopy

1.5.2.3.1 Light microscopy

Various stains reacting to components of different cell types can be used to examine a particular tissue during light microscopy. Toluidine Blue has been used in numerous studies of buds and their associated tissues (Burrows, 2000; Burrows et al., 2008; Burrows, 2002; Foster et al., 2007; Mibus and Sedgley, 2000; Waters et al., 2010). Toluidine blue stains lignified walls of xylem vessels in a blue-green colour (O'brien et al., 1964), therefore could be used as a stain that would enable identification of vascular connection of a bud to the associated tissue. By identifying the vascular connection of

the buds to the tissue of origin, and the morphological identity of this tissue (i.e. stem or root), an understanding could be achieved of the types of buds present.

1.5.2.3.2 Confocal microscopy

In confocal microscopy various fluorescent dyes can be used to identify the tissue or, in some cases, cells naturally give an autofluorescence without a stain. Autofluorescence of lignin in secondarily thickened cell walls, has been used to detect the presence of vascular bundles (Pawley, 2006). Stains such as Acridine Orange and Ethidium Bromide have been used in combination, to distinguish between lignified and non-lignified cells (Yang et al., 2007). Safranin and Acridine Orange have been used in identifying structures such as the cambium (Kitin et al., 2000). During the preliminary stages of the current study, autofluorescence of the tissues and the potential use of several stains, individually or in combination, were therefore investigated in order to identify the suitable protocol for buds and associated tissues in gentians (refer Chapter 5).

1.6 Influence of plant and environment related factors during the developmental process

1.6.1 Initiation and development of buds

Most of the herbaceous geophytes in temperate regions utilize buds produced in the previous season for the current season's shoot production (i.e. preformation of continuation buds) (Vesk and Westoby, 2004). Whether buds which form the flowering shoots in gentians are preformed was not clear, hence if floral shoots do arise from preformed buds, growth conditions during the previous season may influence the current season's growth and, ultimately, timing of flower harvest maturity. Potentially any delays in timing of initiation or lack of uniformity of timing could lead to differences in timing of flowering. In line with the goals of the research programme (refer Section 1.3.1) it may, therefore, also be possible to control the spread in time to flower harvest maturity via manipulation of the timing of initiation and development of continuation buds. Although possibilities exist for the control of timing of anthesis (Funnell, 2008), with gentians neither the physiology of growth and development of these buds, nor their timing of emergence, has previously been described. This therefore was investigated within the current study (refer Chapters 2 & 3).

As applied to woody temperate plant species, the process of initiation and development of buds can be separated into bud formation, acclimatization to cold, and dormancy (Ruttink et al., 2007). Subsequent to their formation, or concurrently, in temperate plant species buds become dormant to survive through winter. In gentians the process of initiation and development of buds has not previously been interpreted progressively as separate stages, but only that when dormancy exists it can be broken following exposure to low temperature (Ohkawa, 1983). It was therefore not clear whether initiation and development of buds and the development of dormancy occur concurrently or separately in gentians. If the process of initiation and development of buds in gentians follows a series of developmental stages (i.e. initiation and development of buds, induction of dormancy and breaking of dormancy) as noted in woody temperate species, many plant and environmental related factors (Reeves and Coupland, 2000) could influence the plants at different stages of the developmental process.

As introduced before (Section 1.3.2), the timely initiation of buds during plant establishment is perceived as a potential problem in gentians. Investigation of factors influencing initiation and development of buds therefore, would also potentially aid plant establishment of gentians. In order to achieve this, it was considered important to first determine the stages in the bud formation process (Chapter 5), and then the potential involvement of both plant-related and environmental-related factors that might influence this process (refer Chapters 6 - 9).

1.6.2 Bud dormancy

Dormancy in plants involves the temporary suspension of growth (Chao et al., 2007), which in some species occur due to inactivation of the meristem followed by a cessation of primordia initiation (Rohde and Bhalerao, 2007). Dependent upon the external signal, and where the perception occurs, bud dormancy can be categorised as either; endo, eco or para (Figure 1.5) (Horvath et al., 2003; Lang et al., 1987). The duration of the different types of dormancy can be specific or overlapping (Faust et al., 1997), therefore any given type of dormancy could have different degrees of depth at different stages of the annual growth cycle.

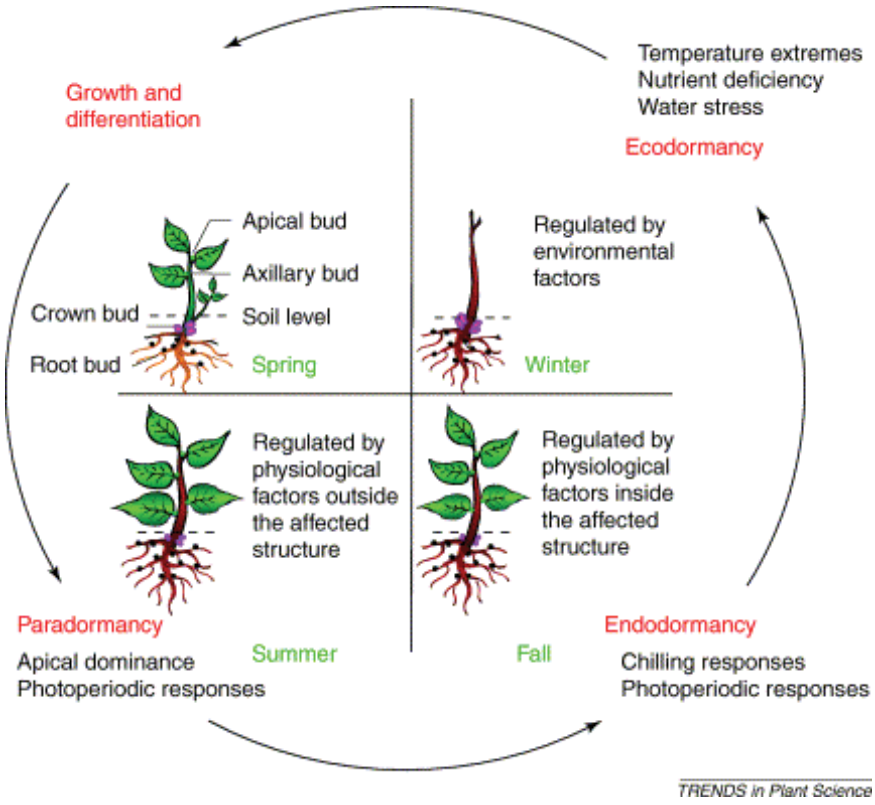


Figure 1.5 Diagram depicting signals and typical seasons corresponding to the different types of dormancy that is associated with dormancy of herbaceous geophytes and woody plants. Within the parts of the annual growth cycle, details are presented of the three types of bud dormancy (red text), the general seasons in which they might predominantly occur (green text) and relevant signals.

Source; (Horvath et al., 2003)

As reported previously, in gentians the over-wintering buds on the crown develop into shoots in late spring, after overcoming dormancy (Ohkawa, 1983; Takahashi et al., 2006). The type, depth or duration of these dormant stages however, has not previously been published in peer-viewed literature. In contrast however, the various types of dormancy have been defined for another herbaceous perennial *E. esula* (Horvath et al., 2003), which has some similarities to gentians in terms of the morphology of buds in the crown and seasonality of bud/shoot development. Applying the same types of dormancy as used for *E. esula* to gentians provided a basis for investigation of potential types of dormancy during the growth cycle of gentians, at the beginning of this thesis. Such identification and description of stages of bud dormancy in gentians would help in the development of strategies for induction, maintenance and release of dormancy of buds, and subsequent manipulation of flowering. During the current study, it was considered important to investigate the types of dormancy and stages of their potential existence during the growth cycle and, the potential influence of plant and

environmental conditions for induction and breaking of dormancy (Chapters 4, 6, 7, 8 & 9).

1.6.2.1 Paradormancy

Paradormancy (Correlative inhibition) is the cessation of growth controlled by physiological factors external to the affected structure (Anderson et al., 2001; Chao et al., 2006; Lang et al., 1987). Paradormancy on an axillary bud could therefore be from the apical bud (apical dominance) (Cline and Deppong, 1999), from developing leaves (Horvath, 1999), other axillary buds (Zieslin and Halevy, 1976) or from bud scales (Schneider, 1968). Apical dominance is the most studied type of paradormancy, and is defined as the control exerted by the shoot apex over the growth of the lateral buds into shoots (Cline, 1997; Dun et al., 2009; Müller and Leyser, 2011; Shimizu-Sato et al., 2009; Yamaguchi and Kyozuka, 2010). Apical dominance and its subsequent release can be divided into four developmental stages (Figure 1.6):

(I) lateral bud formation,

(II) imposition of inhibition on lateral bud growth,

(III) release of apical dominance following decapitation, and

(IV) branch shoot development (Cline, 1997).

These stages can overlap, but involve different processes, which involve regulation by plant hormones such as auxin. Apical dominance however, depicts the relation between apical and axillary buds, whereas in gentians, the types of buds present or their hierarchical arrangement had not previously been determined. In order to identify the potential factors influencing the paradormancy status of a bud from initiation through to emergence, in the current study, it was considered important to determine the hierarchical arrangement of buds (Chapter 5).

The occurrence of paradormancy during development of buds or shoots has not been published in gentians. In *E. esula*, a herbaceous perennial with development of buds similar to that found in gentians, auxin produced in apices of growing shoots, and another signal linked to sugar produced in mature photosynthesizing leaves, regulate paradormancy in buds located underground (Horvath et al., 2002). Hence if paradormancy exists in gentians, the emergence of buds could be similarly influenced

and, therefore, any delays in emergence could also influence timing of flower harvest maturity. During the current study therefore, it was considered plausible to design and implement experiments to examine whether buds were under paradormancy during the growing season (Chapter 4, 8, 9 & 10).

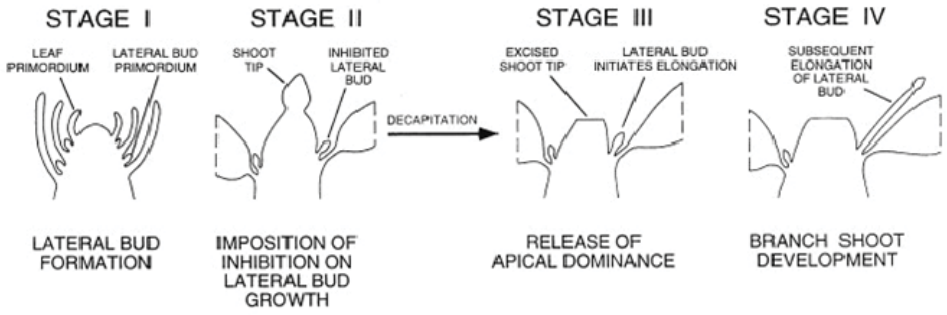


Figure 1.6 Diagram presenting the developmental stages of apical dominance before and after release of apical dominance of the shoot apex.

Source ; (Cline, 1997)

1.6.2.2 Endodormancy

The induction, maintenance and release of endodormancy is mainly environmentally controlled (Tanino, 2004). With initiation of endodormancy in plants, formation of bud scales and cessation of cell division in the meristem was observed (Horvath et al., 2003), both in embryonic leaves and in the sub-apical domain (Ruttink et al., 2007). Once endodormancy is induced, even if the environmental cue for initiation is not there, it continues until the specific environmental cue for breaking dormancy is present (Shimizu-Sato and Mori, 2001), and this was observed even if buds were removed and grown separately (Lang et al., 1987). Although endodormancy has not specifically been previously identified in gentians, based on anecdotal observations of thick, scaly, purple-coloured buds visible on the crown during winter (Figure 1.2 & Figure 1.3) and the requirement of a period of cold to emerge (Ohkawa, 1983), it was hypothesized that gentian plants undergo a period of endodormancy during winter.

Within the literature a diversity of environmental signals, primarily the change to low temperature and short days, influence initiation of endodormancy in buds (Atwell et al., 1999) or the transition from para to endodormancy (Arora et al., 2003). Some plants exhibiting endodormancy respond to shortening day length (photoperiod) as a cue to induce the process of growth cessation in meristems and cambium, and formation of protective scales in buds (Lagercrantz, 2009; Ruttink et al., 2007). Some deciduous

plants, such as *Malus pumila* Mill (apple) and *Pyrus communis* L. (pear), are insensitive to photoperiod, and low temperature (less than 12 ºC) alone is the main signal for induction of dormancy (Heide and Prestrud, 2005). Similarly, short periods of cold temperature enhance initiation of endodormancy in herbaceous perennials like *E. esula* (Horvath et al., 2003). In contrast, such environmental changes were not required for the induction of endodormancy in the temperate herbaceous perennial *Zantedeschia* (Funnell et al., 2002b). While environmental conditions could potentially be important for induction of endodormancy and development of dormant buds in gentians, there is limited published information relative to critical photoperiod or temperature. Given the geographic distribution of different parent species in the genetic lines of interest in gentians however (refer Section 1.2.2), signals associated with induction of dormancy could vary between species. Identifying the exact signals associated with the induction of dormancy in gentians if any therefore, remained to be examined (Chapter 6 and 7).

1.6.2.3 Ecodormancy

In most temperate plant species, experience of a certain amount of chilling ends endodormancy and buds may subsequently undergo a period of ecodormancy before bud break. Ecodormancy is due to the growing conditions not permitting growth, such as periods of cold or drought (Horvath et al., 2003). In such a case this cold-induced ecodormancy can be broken by warm temperature (Lang et al., 1987). Optimum temperature for growth and development of gentians is 15-18 ºC, and growth is retarded above 25 ºC (Ohkawa, 1983). One possible factor for breaking ecodormancy in gentians therefore, could be warm temperatures of 15-18 ºC, but the specific environmental conditions for breaking ecodormancy have not yet been published for gentians. Although it was not the intention to undertake a detailed analysis of the duration of ecodormancy, and the environmental cues inducing and breaking it, potential environmental conditions for emergence following breaking dormancy would need to be investigated within this thesis (Chapter 3).

1.6.2.4 Involvement of growth regulators in initiation and development of buds and bud dormancy

Exogenous hormones have been used in the commercial floriculture industry for a long time, as most plant responses to external or plant-related stimuli occur via the involvement of endogenous growth regulators. With the exception of an analysis of

hormones inferring the presence of gibberellins during vegetative growth (Koshioka et al., 1998), the influence of exogenous or endogenous hormones on initiation and development of buds in gentians, has not been published in peer-reviewed articles. It was not the intention during the current study however, to quantitatively analyse the endogenous growth regulators. Before anyone analyses the endogenous growth regulators, the research strategy used in the current thesis was to identify exogenous growth regulators, which may supplement inadequate levels of environmental stimuli required for the bud formation process or emergence. Some technical reports however, present usage of exogenous growth regulators in promoting initiation of buds (Morgan and Debenham, 2008; Okayama-Ken and Nogyo, 2003; Sato, 1988). During the preliminary investigations therefore, in order to identify the potential exogenous growth regulators to be applied for initiation and development of buds, this information from technical reports was utilized as a starting point.

As discussed above (refer Section 1.6.2.2), during the later stages of the bud formation process, endodormancy may develop. The potential involvement of exogenous growth regulators in the process of initiation and development of buds (Chapters 6 & 7) could therefore also be investigated in conjunction with environmental stimuli such as photoperiod and cold temperature, which can induce endodormancy. During the development of endodormancy, the endogenous content of abscisic acid (ABA) increased at the beginning, and the levels were enhanced by decreasing temperature, which is known to induce endodormancy (Faust et al., 1997). Although the type of dormancy was not specified, elevated levels of ABA prior to development of dormancy were noted in *Betula pendula* Roth (Rinne et al., 2001) and *Allium wakegi* Araki (Yamazaki et al., 1999). Recently however, the influence of ethylene and auxin on regulation of ABA has also been noted in some species (Tanino, 2004), which infers the existence of cross-talk between growth regulators in determining endodormancy. As described in *Populus tremula* × *Populus alba* (poplar), with exposure to short days (inductive for endodormancy), down regulation of gibberellins (GA) occurred in the first week, followed by ethylene biosynthesis triggered after 2 weeks (Ruttink et al., 2007). This proceeds to cessation of internode elongation and the first visible signs of bud formation. Supporting this involvement of ethylene, in ethylene insensitive mutants of *B. pendula*, endodormancy was delayed and ABA was not accumulated in response to short day conditions (Ruonala et al., 2006). The role of ethylene in the formation of dormant buds therefore, was proposed to be through ABA synthesis (Ruonala et al.,

2006; Suttle, 1998). Supporting the involvement of GA, in temperate perennials a reduced concentration of endogenous GA during endodormancy, led to reduced shoot elongation and cell division (Horvath et al.), with increased GA following shoot emergence (Atwell et al., 1999). Gibberellic acid GA_3 (Okayama-Ken and Nogyo, 2003) and ethephon (2-chloroethylphosphonic acid; an ethylene source) (Morgan and Debenham, 2008; Sato et al., 1988) have been used in increasing the number of buds in preliminary technical reports. Based on these reports, and their potential involvement in endodormancy, ethylene and GA_3 were identified as potential plant growth regulators to be explored during the current thesis (Chapters 6, 7, 8 & 9).

The hormonal physiology underlying initiation and control of apical dominance are well documented in terms of the involvement of hormones. As such, auxin from apical buds inhibited growth of axillary buds whereas direct application of auxin to axillary buds promoted outgrowth (Shimizu-Sato and Mori, 2001). Due to the interaction of auxin with cytokinin (Woolley and Wareing, 1972) in apical dominance, cytokinin was also identified as the commonly known paradormancy breaking agent (Shimizu-Sato and Mori, 2001). Although GA are not reported to be involved in paradormancy exerted by the apex, it has been reported to be involved in paradormancy exerted by leaves, as GA_3 partially broke this type of paradormancy exerted on root buds of *E. esula* promoting their initiation and development (Horvath et al., 2002). The involvement of auxin was also found with paradormancy of root buds of *E. esula*, as paradormancy was promoted by naphthalene 1-acetic acid (NAA) and indole-3-acetic acid (IAA), but inhibited by auxin inhibitors (Nissen and Foley, 1987). During the current study with gentians therefore, it seemed justified that a preliminary screening of the use of auxin, cytokinin and GA, be explored in relation to the initiation and development of crown buds due to their involvement in removal of paradormancy.

Mechanical methods of removal of paradormancy including clipping, defoliation and terminal bud removal (Faust et al., 1997), can lead to rapid promotion of cell division in axillary buds (Shimizu-Sato and Mori, 2001), as well as promotion of adventitious shoots from roots (Wan et al., 2006). Given the ease with which they can be applied as treatments, in order to study the existence of paradormancy within gentians, some of the mechanical (Chapters 4, 8 and 9) and exogenous hormonal applications (Chapters 6, 7, 8 and 9) could be considered for utilization within the current thesis.

1.6.3 Shoot emergence, development and flowering

1.6.3.1 Vernalization and breaking endodormancy

Cold temperature (chilling) is required for flower initiation (vernalization) as well as to break endodormancy in some plants (Anderson et al., 2005; Iversen and Weiler, 1994). These are two separate processes regulated by chilling (Horvath et al., 2003) and, therefore, both the duration and the specificity of the temperature regime required for completion, may vary (Sung and Amasino, 2005). Inadequate exposure to chilling (i.e. to break endodormancy) can cause a delay in bud break, a decrease in percentage of buds emerging, as well as slow or weak shoot growth (Lang et al., 1987). As also reported in some herbaceous perennial species like *E. esula* (Harvey and Nowierski, 1988) and *Asparagus officinalis* L. (asparagus) (Ku et al., 2007), the subsequent growth rate of shoots was also found to increase as a function of increased duration of cold temperature. In asparagus, slow growth rates due to inadequate chilling delayed both harvesting of spears as well as the release of the next bud in the cluster (i.e. paradormancy). The types of dormancy occurring in gentians have not yet been identified but, if endodormancy, paradormancy and/or a vernalization requirement is evident, insufficient chilling may influence emergence of buds, shoot development and, subsequently, timing of flower harvest maturity. As presented in the current thesis therefore, it was envisaged that identification and quantification of the influence of any chilling requirement on breaking dormancy and/or vernalization, would potentially enable control of timing of harvest maturity and other qualitative and quantitative aspects of flower production in gentians (Chapters 8 & 9).

Depending on species, vernalization can be quantitative (i.e. plants remain vegetative without cold exposure) or qualitative (i.e. cold hastens flower induction and initiation) (Iversen and Weiler, 1994). The involvement of vernalization has not been well described in gentians. As noted with historical cultivars of gentians (predominantly derived from *G. triflora*), when plants which were partially chilled were forced in a greenhouse, anthesis was delayed (Ohkawa, 1983). This delay occurred even though endodormancy was broken due to partial chilling. During forcing of gentians, application of GA_3 (100 ppm) without chilling resulted in shoot emergence without flowering, but GA_3 combined with low temperature resulted in both emergence and flowering (Ohkawa, 1983). It is possible that this is indicative of a cold temperature requirement for vernalization in these gentian cultivars. The specific temperature

requirements have not been quantified or identified for vernalization or endodormancy, for the recently introduced cultivars of gentians nor for their parental lines. Since a cold temperature requirement seems to play a role in flowering of herbaceous perennials like gentians, within this thesis it was considered worthy of examination in terms of the potential to control timing of flower harvest maturity, as well as floral stem yield. Use of different durations of chilling was considered as a possible research strategy to identify the requirement of cold temperature to break endodormancy and/or vernalization in the recently introduced cultivars (Chapters 8 & 9).

1.6.3.2 Development of floral shoots

Timing of floral initiation is controlled by multiple environmental and endogenous cues (Battey, 2000; Henderson and Dean, 2004; Simpson and Dean, 2002). Literature associated with historical cultivars of gentians (predominantly derived from *G. triflora*) infers that flower initiation occurs within the current growth cycle (Ohkawa, 1983). In contrast, studies conducted under natural habitats with *Gentiana pneumonanthe* L. report flowering frequency and number of flowers were related to the temperature of the growth season of the previous year/season (Rose et al., 1998). Their hypothesis was that flower initiation in *G. pneumonanthe* takes place in the previous year's growth cycle. Since these ecological studies were not based on the species identified as the parental lines used in the current research, and were not conducted under controlled environmental conditions, their relevance to present cultivars is questionable, albeit leading to an important conclusion that gentian species and cultivars may differ. It was therefore considered important to identify the environmental factors that influence floral initiation in cultivars of current interest. With reference to the developmental process already introduced, it was also acknowledged that integration of such factors at different developmental stages could potentially influence floral initiation and timing of shoots reaching harvest maturity and its spread.

Depending upon the cultivar, the process of flower initiation and development in historical cultivars of gentians seems to require, or be optimised at, either high or low temperatures (Table 1.1). In early-flowering cultivars, flower development was faster and anthesis found to be earlier at temperatures between 20 and 25 ºC (Ohkawa, 1983). In late-flowering cultivars, flower development seems to be promoted by cool temperature and short days, i.e. anthesis delayed at 20 to 25 ºC. For example, in *Gentiana makinoi* Kusnetz, the combination of short days (8 h) and night temperatures

of 15 ºC reduced the number of days to flower (Ohkawa, 1983). While these findings were only reported in a review article, and were not accompanied by statistical data, it seems reasonable to suggest that the influence of temperature on timing of flower harvest maturity seems to be highly species/cultivar specific. Given the genetic diversity introduced in the recently developed hybrid cultivars utilised in the current thesis, it was considered important to acknowledge that their response to the growing environment had not yet been determined. During the current study therefore, the influence of temperature of the growing environment on the timing of flower harvest maturity and shoot development was considered as worth investigating (Chapters 2 & 3).

1.6.4 Concept of chill units

The amount of exposure to cold temperature (chilling) required to break dormancy in plant species is conveniently measured in terms of chill units(Dole, 2003). While specific reference to quantified chill units for breaking dormancy in gentians has not previously been published, crowns should be kept at 0 ºC for a "*long time*" for late-flowering cultivars, and for a "*short time*" for early-flowering cultivars (Ohkawa, 1983). In contrast however, according to industry-orientated information from NZ, the chilling requirement is met by *"temperatures below 5 ºC for 50 days"* (Anonymous, 2004). Chill unit recommendations for Japan are less likely to be applicable to NZ because NZ's regions of cultivation have a mild winter where low air temperatures could range from 1-10 ºC (Anonymous, 2011c), whereas in Japan plants are exposed to -1.4 to -7 ºC, (Anonymous, 2011b) including snow, for a significant part of the winter. The variation in temperatures among different regions could lead to variation of chill unit accumulation over a period of time. Further to that, as indicated with the historical cultivars of gentians (Ohkawa, 1983), and in other plants (Wall et al., 2008), chill unit requirements are cultivar specific. During the current study therefore, what if any chilling requirement for some of the key new cultivars for the NZ gentian industry, was investigated (refer Chapters 3 and 8).

The most commonly used model for high-chill cultivars of *Prunus persica* (L.) Batsch was the Utah model, in which one hour at 6 ºC is used as a unit for optimum chilling (Richardson et al., 1974). The Dynamic Model (Fishman et al., 1987) was subsequently proposed for use, so as to improve some difficulties encountered with the Utah Model, especially when applied in mild-winter climates as occurs in NZ (Erez et al., 1989). As gentians originated in temperate regions, often mountainous, where low temperatures

persist, the Utah model may suit quantification of chill units. But in NZ, gentians are grown under warm-temperate conditions and, therefore, the Dynamic Model should also be assessed for suitability.

In other species like apple and pear, breaking bud dormancy required exposure to cold temperature, specifically 6 to 9 ºC for 6 weeks (Heide and Prestrud, 2005), with temperatures above 12 ºC increasing the required duration up to 12 weeks or more. In *E. esula*, a herbaceous perennial with dormant adventitious buds on a crown, temperatures of 0 to 6 ºC for more than 2 weeks promoted growth (Harvey and Nowierski, 1988). A minimum temperature during forcing of 15 ºC is considered to involve no negation or promotion of chilling for a wide range of plants (Dole, 2003). Assuming this would also apply to the new hybrid gentian cultivars used in this thesis, this temperature was used as a targeted minimum within a greenhouse environment, so as to avoid any accumulation of chill units during the experiments to be undertaken. Similarly, when chilling was required, a temperature of 5 ºC was considered suitable, as also recommended for initial studies with geophytes with temperate origins (Dole, 2003).

Criteria to measure bud break (completion of endodormancy) in other crops, has been based on; the presence of unfolded leaves, growth of shoots after dormancy, the percentage of flowers in full bloom, or leaves fully open (Dennis, 2003). The speed of shoot emergence (time to reach a given stage of development) was considered to be a better variable to study bud break, compared to the percentage bud break at a given time, as certain buds may have dormancy broken but may not fully develop due to other physiological reasons. Within the studies presented in this thesis therefore, time taken for shoot emergence, and other variables describing shoot development, were considered suitable to quantitatively describe bud break in gentians.

1.6.5 Concept of thermal time

For horticultural crops, temperature response curves and degree-day calculations help in describing the time to reach developmental stages, but are typically cultivar specific (Dennis, 2003). Applicability of growing-degree-days (GDD) is considered more reliable than calendar days with regard to developmental stages of plants. If the attainment of development stages are under the control of temperature, then it was expected that shoot emergence occurs after a fixed amount of chilling time and subsequent exposure to a fixed amount of degree-days after that (Dennis, 2003). In

asparagus, both chilling (5 ºC for 5 or 10 weeks) as well as increasing temperature (10 ºC to 30 ºC) in spring, were found to increase bud break and subsequent growth rate (Ku et al., 2007). Thus timing of flower harvest maturity of gentians could also be influenced by such growth conditions, especially temperature during and after chilling. In order to study the variation in timing of flower harvest maturity of gentians therefore, calculation of heat units or GDD could be used (Chapter 2). Methods for determining the degree-day requirement have been developed and used for other *species*, like *Scadoxus multiflorus* subsp. katharinae (Baker) Friis & Nordal (Funnell, 2008) and *Thalictrum delavayi* Franch. 'Hewitt's Double' (Huang et al., 1999), to estimate the expected duration to various stages of development including anthesis. Degree-day and chill unit requirements have not yet been defined for gentians, and it is not considered possible to determine this for all cultivars during the current research program. Hence in the current study both chill units and GDD were used as indicators of growth and development in relation to a limited number of cultivars, which are considered important to the NZ industry.

1.7 Overview of the thesis

As a whole, based on the analysis of both published and anecdotal information on gentians, variation in timing of flower harvest maturity could be due to differences in types of buds, their time of initiation and emergence, or shoot development. Factors influencing these key developmental stages and their durations could be both plant-related and environment-related. In order to provide the grower with the information to enable accurate scheduling of flowers, so as to reduce spread of time to flower harvest maturity, as well as achieve successful crop establishment, it was considered essential to identify and quantify these factors for the cultivars of interest. Within the given timeframe of this thesis, it may not be possible to identify all the factors that influence flower timing. It was therefore proposed that the main focus of the current investigations would involve; anatomical and histological studies of bud types, environmental and hormonal physiology of their initiation, emergence and subsequent development.

As a PhD research programme, the research strategy developed was to first identify the types of buds and their pattern of growth and development. Thus the initial experiments of this PhD research programme would mainly focus on studying growth and development of recent cultivars of gentians in terms of the timing of developmental

stages and their correlations (Chapters 2 & 3). Subsequently, detailed morphological investigations would be used to identify types of buds and some of the plant-related factors (Chapters 3, 4 & 5). This would then be followed by investigating the factors influencing each developmental stage (i.e. bud initiation, development, emergence, and subsequent shoot development), and potential growth manipulations (Chapters 6-9). To assist the reader in gaining an overview of this PhD thesis, Figure 1.7 presents a schematic illustration of the thesis and each of the research objectives noted below, in relation to the developmental process of the gentian cultivars of interest.

Overall aim

Quantitative description of physiological changes of growth and development of buds, in selected gentians, in relation to plant-related and environmental factors.

Specific Objectives

1. Quantification of variation of seasonal timing in floral shoot production and correlation between developmental stages among the commercially cultivated gentian cultivars: ‘Spotlight’, ‘Diva’ and ‘Starlet’.

2. Identification and quantification of the influence of bud types and the time of their appearance, emergence and development, on the timing of flower harvest maturity.

3. Identification of origin, anatomy and ontogeny of buds in seedlings and vegetative propagules.

4. Identification of potential involvement of environmental factors (temperature and photoperiod), and exogenous growth regulators, on the process of initiation and development of crown buds in gentians.

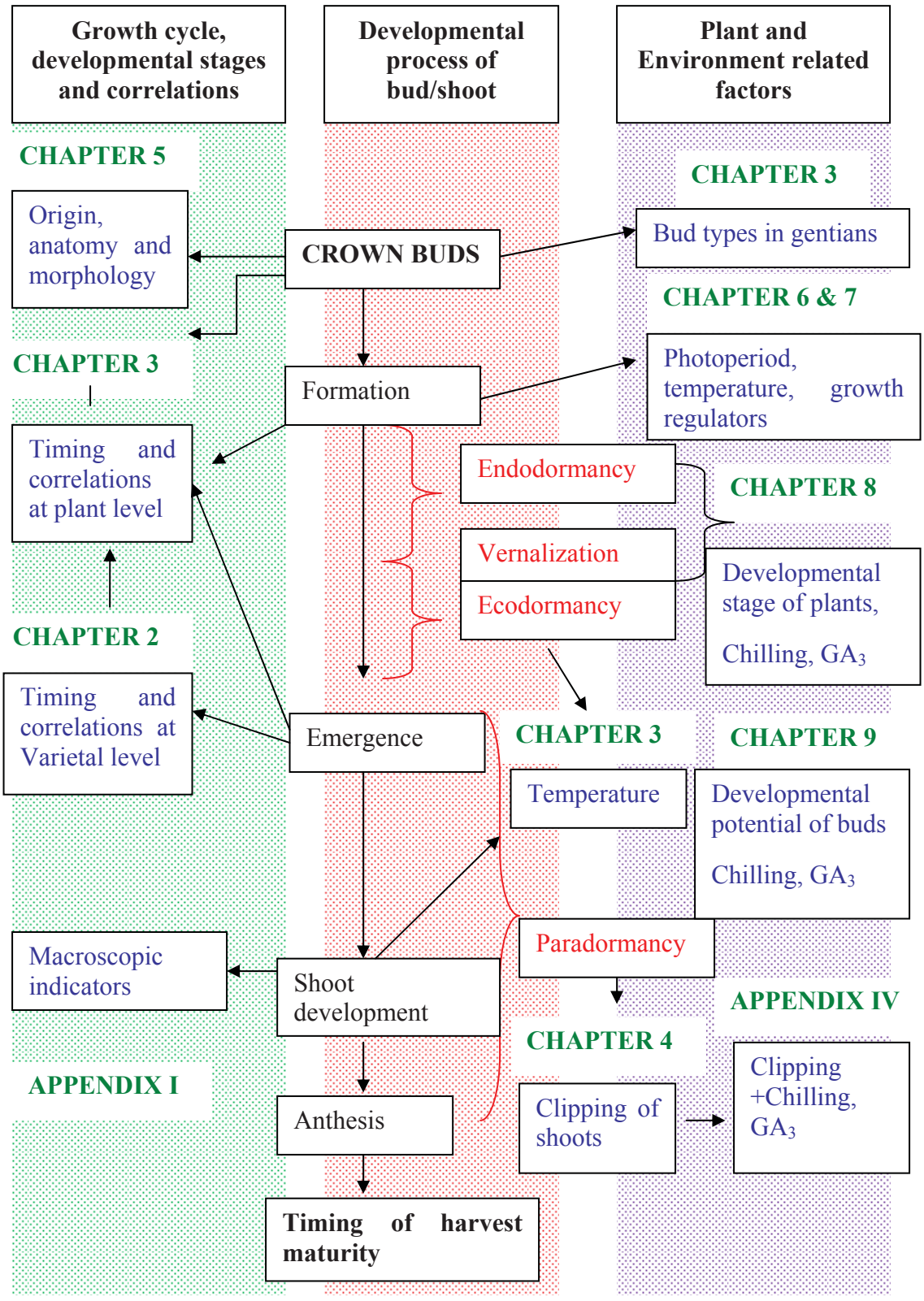


Figure 1.7 Schematic presentation of chapters within this PhD thesis according to their relevant objectives along a developmental pathway from bud through to anthesis for gentian plants.

Chapter 2 Sources of variation in time to harvest maturity of flowering shoots in three cultivars of gentian

2.1 Abstract

Flowering shoots of gentian reach harvest maturity over an extended period of time. The dates which shoots reached harvest maturity, and the correlation between duration to harvest maturity with duration to shoot emergence, were investigated in three cultivars. The duration to harvest maturity and the extent and source of variability in maturity dates differed among cultivars, with a wider spread in time to harvest maturity in 'Starlet' (41 days) than 'Diva' (35 days) and 'Spotlight' (29 days). Despite being vegetatively propagated, cultivars also differed by more than twice as much in duration to harvest based on their plant to plant variability. Strategies to control the spread in late maturing cultivars such as 'Starlet' should focus on uniform shoot emergence and controlling temperature during growth. In earlier maturing cultivars such as 'Spotlight' however, strategies will primarily require a greater understanding of the factors influencing the variability in maturation of shoots within individual plants prior to, and after, emergence.

2.2 Introduction

The different cultivars making up New Zealand's outdoor-grown gentian (*Gentiana* sp.) crop can be quite variable in timing of flower harvest maturity and, additionally, can have a long harvest window. Anecdotally, the variation has been observed between cultivars, and between plants of the same cultivar (Chapter 1). This variation in harvest maturity makes it difficult to schedule harvest operations and target high value markets. A further difficulty of the long window of flowering means there are periods when few shoots are at harvest maturity, so the economics of harvesting stems can sometimes be marginal. Existing information about the spread in time to harvest maturity for this crop is based on anecdotal observations, with no published information available. Hence it was considered useful to quantitatively describe variation in duration to harvest maturity, both between and within cultivars, so as to identify potential opportunities to manipulate flowering time.

Gentian plants are winter dormant, sprouting in spring and growing through the summer period. Flower initiation is thought to occur in early summer, and anthesis is observed

to occur in summer to late autumn. There are usually many flowering shoots produced per plant. During autumn and winter the above-ground organs naturally senesce, with the plant overwintering as an underground crown, bearing buds. Gentian cultivars are broadly categorised as either early or late flowering (Ohkawa, 1983). Although details of breeding (Morgan, 2004; Morgan et al., 2003) and post-harvest research on this crop (Eason et al., 2004) have been published, peer-reviewed articles related to growth and physiology are limited. Detailed descriptions of flowering time and reasons for the spread of harvest maturity among cultivars, among plants or even among individual shoots on the same plant, have not previously been reported.

In addition to cultivars differing in flowering time, as observed in *Scadoxus multiflorus* subsp. *katharinae* (Baker) the time of emergence of individual shoots varies, influencing both time to, and spread of, harvest maturity (Funnell, 2008). A differential temperature response of flower development between early and late flowering gentian cultivars was postulated by Ohkawa (1983), with flowering of early cultivars promoted at 20 to 25 °C, whereas late flowering cultivars were delayed by this temperature range. Thus while effects of temperature have not been quantified, they are likely to have an influence on the duration to harvest maturity of individual shoots.

Most research related to flowering in ornamental plants deals with the phenological event (e.g. average time to anthesis or harvest), whereas in commercial reality, the spread in timing of these events is also important. As illustrated with other crops, the time required to reach anthesis or harvest maturity can be described using chronological days, growing-degree-days (GDD), and/or phenological scales (Fisher et al., 1997; Funnell et al., 2003). The temperature response curve and parameters for calculation of GDD have not been defined for any gentian cultivars. In the research presented here, therefore, the duration to harvest and other related phenological events were analysed based on calendar days and a GDD model that assumed a linear response curve and a base temperature of 0 °C (Arnold, 1959).

Many of the cultural recommendations for gentians were developed for Japanese cultivars and conditions. It would be useful therefore, to describe growth and development of the crop in the warm-temperate climate of New Zealand (NZ). Spread in time to harvest maturity of a given cultivar can be due to variation in maturity dates of individual plants or of individual shoots within a plant. Seedling plants are generally more variable than clonally propagated cultivars, but with many new cultivars being

clonally propagated, genetic variation is minimised. In this chapter the spread in time to harvest maturity was described of three clonally propagated *Gentiana* cultivars; 'Spotlight', 'Diva' and 'Starlet', grown under field conditions in NZ. To identify possible sources of variation among and within cultivars, spread in the time to harvest maturity and its correlation with shoot emergence was analysed.

2.3 Materials and methods

Clonally propagated plants of the cultivar 'Diva' were established as a commercial crop during spring of 2004, with 'Spotlight' and 'Starlet' established in spring 2003. Five single-plant replicates, each producing 20 or more flowering shoots per year, were selected from established plots in an open field located at The New Zealand Institute for Plant & Food Research Limited (Palmerston North, New Zealand; 40°37'S 175°60'E). Data collection started on 10 September 2007 when buds first emerged after overwintering, and continued until 30 June 2008. Daily average air temperatures during this period were recorded (Figure 2.1). At this latitude, day length varied from 11:51 h:min in September to a maximum of 15 h in December, before declining to a minimum of 9:16 h:min in June.

When a shoot reached 5 cm above the soil surface, it was considered emerged. The emergence of individual shoots on each plant was recorded monthly. As each shoot reached harvest maturity (i.e. top-most flower bud was not open but had developed colour (Eason et al., 2004)), its length and date of harvest maturity was recorded. Date of harvest maturity and length of axillary shoots, which emerged from the proximal end of already developed flowering shoots prior to their harvest, were also recorded.

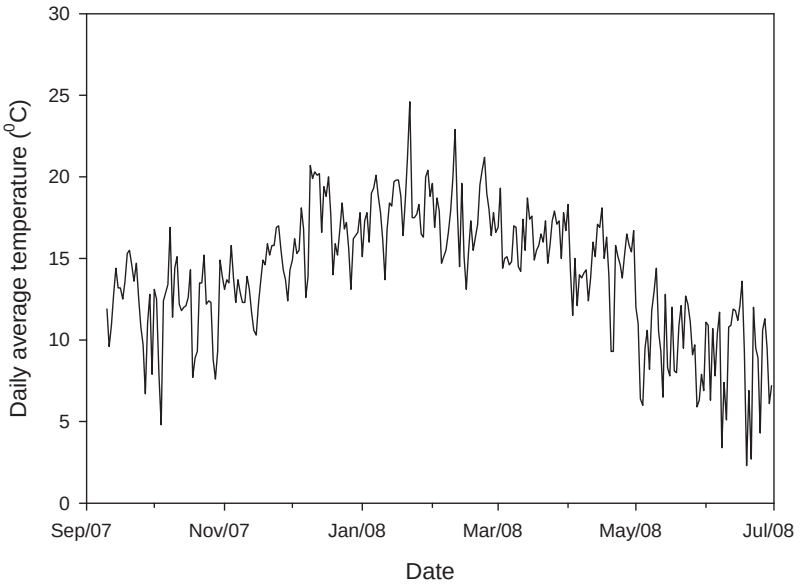


Figure 2.1 Daily average temperature encountered in the open field during September 2007 to July 2008.

For each shoot, the date of shoot emergence (*a*), and harvest maturity (*b*) were recorded as calendar days. The duration from emergence of the first shoot through to the emergence of subsequent shoots *(te)* and harvest maturity *(th)*, and the duration from emergence of an individual shoot to harvest maturity of that shoot *(teh)* were determined as calendar days (Figure 2.2).

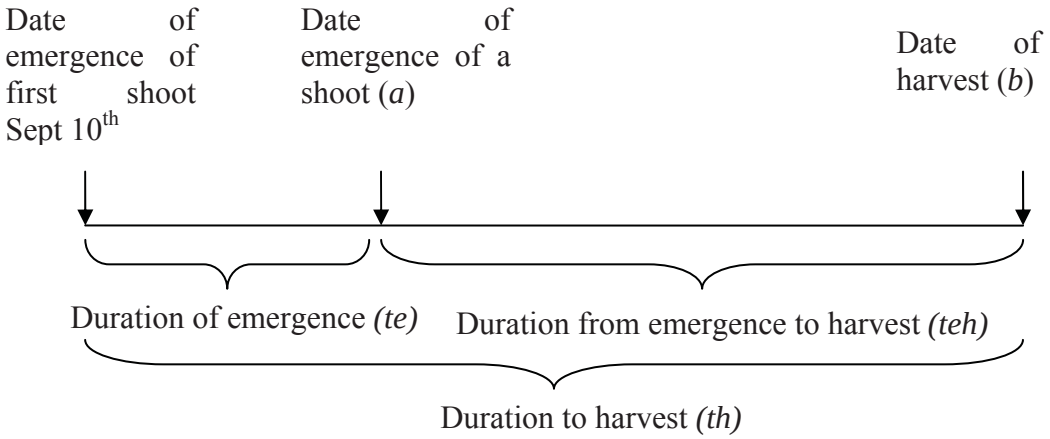


Figure 2.2 Variables recorded and calculated related to shoots reaching emergence and harvest maturity during the current study.

Times of emergence and harvest for each shoot were also calculated using a linear GDD model (Funnell, 2008) with a base temperature (T_{base}) of 0 °C. Daily maximum and

minimum air temperatures were sourced from the AgResearch meteorological station (AgResearch, Palmerston North; 40°38'S 175°61'E). Accumulated GDD from the date of emergence of the first shoot (*t*) until attainment of each of the phenological stages of development (*a* and *b*) were calculated utilizing Eq. (1):

$$\mathbf{GDD} = \sum_{t}^{a \to b} \left[\left(\frac{(\text{maximum temperature} + \text{minimum temperature})}{2} \right) - \mathbf{T_{base}} \right] \qquad \textbf{Eq. (1)}$$

Data were analysed using the General Linear Model's procedure and Regression procedure in the Statistical Analysis System software version 9.13 (SAS Institute, Cary, N.C, USA). Mean comparisons were conducted using Tukey's test. Data were either normally distributed or approached normality to the extent that transformations did not alter the statistical outcome. Duration of emergence and harvest, and from emergence to harvest, were analysed using both calendar days and GDD.

Spread in time to harvest maturity among cultivars and within (i.e. among plants) were quantified with box and whisker plots in Sigma Plot (version 10.0, Systat software Inc., San Jose, CA, USA) using data pertaining to multiple shoots within a plant. Together with the calculated mean values, the 10th, 25th, 50th (median), 75th and 90th percentiles from the box and whiskers plots were used. Both standard deviation (SD) and the number of days representing the central 80% spread, as indicated by 10 to 90th percentile distribution in harvest maturity for each plant, were used to describe the spread in time to harvest maturity among and within cultivars. For variation within a cultivar, data for shoots arising from each of the five individual plant replicates were analysed separately. Outliers, present before the 10th and after the 90th percentile were used to describe the 20% of shoots available for harvest prior to and after the end of the commercial harvest season. The relationship between duration of emergence and harvest maturity, using both calendar days and GDD, was evaluated using regression analysis within SAS.

2.4 Results

2.4.1 Spread in time to harvest maturity among cultivars

2.4.1.1 Distribution of time to harvest maturity

All shoots that emerged flowered. The average duration from emergence of the first shoot (10 September 2007) to harvest maturity *(th)* differed among the cultivars (Table

2.1), with 'Spotlight' maturing first, followed by 'Diva' and then 'Starlet'. The same sequence was observed with duration from shoot emergence to harvest maturity *(teh)*, with 'Spotlight' requiring fewer days than both 'Diva' and 'Starlet' (Table 2.1). When time was expressed as GDD, the same sequence of harvest maturity *(th)* was observed (Table 2.2); 'Spotlight' required fewer GDD than either 'Diva' or 'Starlet'. GDD from date of emergence to harvest *(teh)* was shortest for 'Spotlight', followed by 'Diva' and 'Starlet', respectively (Table 2.2).

The standard deviation of duration (days) from the emergence of first shoots to harvest maturity *(th)* was not significantly different among cultivars ($P > 0.05$). It was different ($P < 0.01$) however for duration from shoot emergence to harvest *(teh)*, and the greatest variation was observed for duration to harvest maturity of 'Diva' (Table 2.1). As the standard deviation included outliers, which in a commercial situation might not be harvested, 10^{th} to 90^{th} percentile distribution in maturity dates might be more applicable for this analysis. With the outliers excluded from the analysis, the 10^{th} to 90^{th} percentile distribution detected more variability among cultivars than standard deviation, hence was used in most of the subsequent analyses.

There was no significant difference in the 10^{th} to 90^{th} percentile distribution of duration (days) from the emergence of the first shoot to harvest maturity *(th)* between 'Spotlight' and 'Diva', nor between 'Diva' and 'Starlet', but the 10^{th} to 90^{th} percentile distribution for 'Starlet' was greater than that for 'Spotlight' (Table 2.1). When the 10^{th} to 90^{th} percentile distribution was based on GDD, the duration from emergence of the first shoot to harvest maturity *(teh)* was similar for each cultivar (Table 2.2; Figure 2.3). In contrast, the 10^{th} to 90^{th} percentile distribution for GDD from shoot emergence to harvest *(teh)* varied, with 'Diva' having a wider spread than 'Spotlight' and, based on days, the 10^{th} to 90^{th} percentile distribution observed in 'Diva' was greater than both 'Spotlight' and 'Starlet' (Table 2.2).

Table 2.1 Duration to harvest maturity, duration of shoot emergence, duration from shoot emergence to harvest and variation in distribution in time for these parameters[z] between three gentian cultivars in calendar days.

Cultivar	*th* (days)***	*te* (days)	*teh* (days)***	SD of *th* (days) $^{NS.}$	SD of *te* (days) **	SD of *teh* (days) **	10th to 90th percentile distribution of *th* (days)**	10th to 90th percentile distribution of *teh* (days)*
'Spotlight'	192^{c}	34^{a} ***	157^{c}	13^{a}	18^{b}	14^{b}	29^{b}	36^{b}
'Diva'	204^{b}	23^{b}	183^{b}	13^{a}	27^{a}	21^{a}	35^{ab}	60^{a}
'Starlet'	215^{a}	21^{b}	193^{a}	14^{a}	19^{b}	15^{b}	41^{a}	43^{b}

[z] Abbreviations as per Figure 2.2.

Within the same column means followed by different letters were significantly different. Mean separation by Tukey's test at; ^{NS} non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2.2 Duration to harvest maturity, duration of shoot emergence , duration from shoot emergence to harvest and variation in distribution in time for these parameters[z] between three gentian cultivars in growing degree-days (GDD).

Cultivar	*th* (GDD)***	*te* (GDD)***	*teh* (GDD)***	SD of *th* (GDD) ^{NS}	SD of *te* (GDD)*	SD of time *teh* (GDD)***	10^{th} to 90^{th} percentile distribution of *th* (GDD)* ^{NS}	10^{th} to 90^{th} percentile distribution of *teh* (GDD)*
'Spotlight'	2959^{c}	436^{a}	2523^{c}	211^{a}	229^{b}	183^{b}	464^{a}	445^{b}
'Diva'	3152^{b}	272^{b}	2880^{b}	202^{a}	339^{a}	266^{a}	526^{a}	754^{a}
'Starlet'	3321^{a}	297^{b}	3024^{a}	206^{a}	237^{b}	198^{b}	596^{a}	578^{ab}

[z] Abbreviations as per Figure 2.2.

Within the same column means followed by different letters were significantly different. Mean separation by Tukey's test at; ^{NS} non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2.3 Average duration to harvest, duration of shoot emergence and duration from shoot emergence to harvest[z] in calendar days for individual plants within a cultivar of gentians.

Plant identity	*th* (days)			*teh* (days)			*te* (days)		
	'Spotlight' **	'Diva' *	'Starlet' ***	'Spotlight' ***	'Diva' $^{NS.}$	'Starlet' $^{NS.}$	'Spotlight' ^{NS}	'Diva' $^{NS.}$	'Starlet' ***
1	188^{b}	219^{a}	218^{ab}	148^{b}	196	195	40^{a}	23^{a}	22^{ab}
2	188^{b}	206^{bc}	211^{bc}	153^{b}	181	198	35^{a}	25^{a}	13^{b}
3	191^{b}	199^{cd}	205^{c}	156^{b}	185	196	27^{a}	15^{a}	9^{b}
4	191^{b}	194^{d}	226^{a}	165^{a}	178	186	34^{a}	18^{a}	40^{a}
5	199^{a}	210^{b}	212^{bc}	165^{a}	183	191	35^{a}	26^{a}	21^{b}

[z] Abbreviations as per Figure 2.2.

Within the same column, means followed by different letters are significantly different. Mean separation by Tukey's test at; ^{NS}non-significant $^{**}P < 0.01$, $^{***}P < 0.001$.

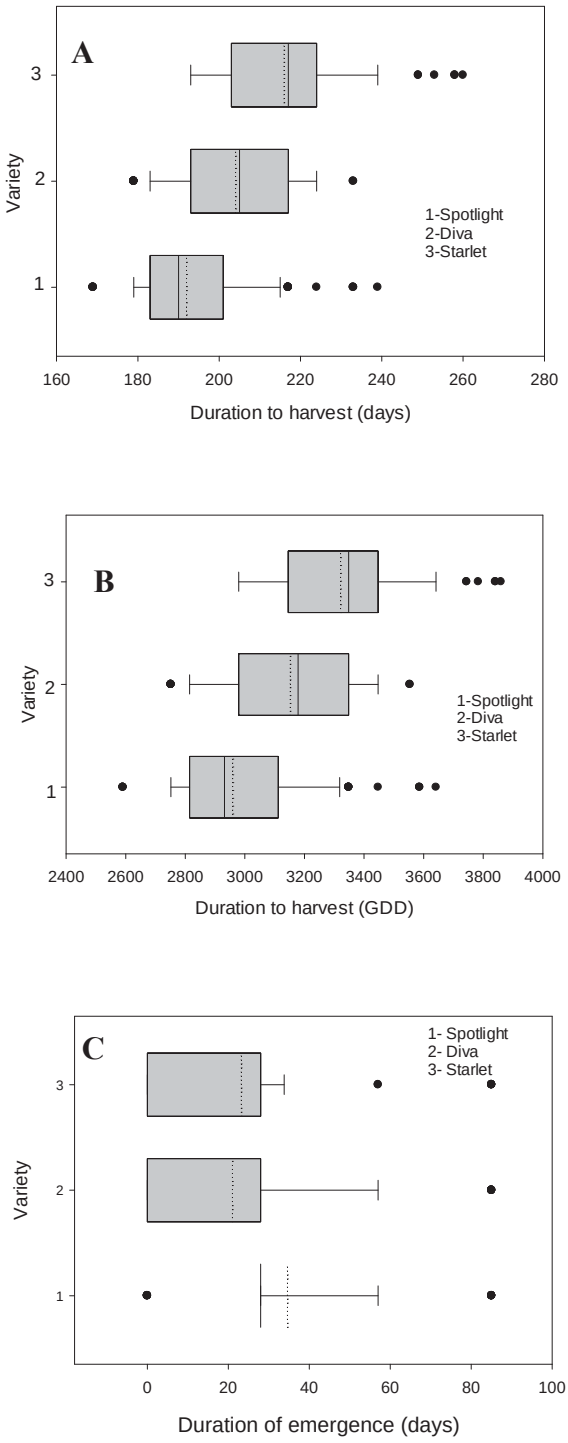


Figure 2.3 Duration to harvest maturity among gentian cultivars ('Spotlight' (*n* = 123), 'Diva' (*n* = 97) and 'Starlet' (*n* = 97)) in either (A), calendar days; or (B), growing degree-days (GDD); (C), duration to shoot emergence in calendar days. Solid and dashed vertical lines in the centre of each box indicate the median and mean, respectively. Boundaries of box indicate 25th and 75th percentiles, whiskers indicate 10th and 90th percentiles and, solid dots indicate one or more individuals as outliers beyond these percentile limits.

Peak harvest maturity (i.e. median harvest date recorded as calendar days from the emergence of first shoot) was 184 days (mid-March) for 'Spotlight'; 204 days (early April) for 'Diva'; and 216 days (mid-April) for 'Starlet' (Figure 2.3A). For both 'Spotlight' and 'Starlet', the spread in time to harvest maturity *(th)* was skewed towards the latter part of the harvest period (Figure 2.3A). For 'Spotlight', a 49-day spread from the median to the date of last harvest was apparent; 'Starlet' had a 44-day spread. By contrast, it was only 25 and 22 days from the first harvest date to the median date for 'Starlet' and 'Spotlight', respectively. The distribution of dates of harvest maturity for 'Diva' was not skewed, with 31 days from the first harvest date to the median, and 26 days from the median to the last harvest date (Figure 2.3A).

Axillary shoots arose from nodes close to the proximal end of flowering shoots, giving the visual appearance of shoots arising separately from the crown. This was more prominent in 'Diva' than the other two cultivars. These axillary shoots were typically weak and short (48 cm) in 'Spotlight', but of commercial quality in 'Diva' (84 cm) and 'Starlet' (60 cm). Most of these axillary shoots flowered at the same time as the shoot to which they were attached and, therefore, did not contribute a separate source of variation in timing of flowering shoots.

2.4.1.2 Duration to shoot emergence

Shoot emergence in 'Spotlight' was significantly later than 'Diva' or 'Starlet', whether expressed as days (Table 2.1) or GDD (Table 2.2). The standard deviation of duration of emergence *(te)* was greater for 'Diva' than 'Starlet' or 'Spotlight' (Table 2.1 and Table 2.2). Similarly, the 10^{th} to 90^{th} percentile distribution of GDD to shoot emergence was greatest for 'Diva', followed by 'Starlet' and 'Spotlight' (Table 2.2; Figure 2.3C).

2.4.1.3 Correlations between key variables

There was a stronger correlation between duration to harvest maturity *(th)* and duration of emergence *(te)* for 'Starlet' (R^2 = 0.51), than 'Diva' (R^2 = 0.33) or 'Spotlight' (R^2 = 0.35) (Figure 2.4A). The correlation was even stronger for 'Starlet' when determined using GDD (R^2 = 0.7; Figure 2.4B). In contrast to the duration to harvest *(th)*, the duration from emergence to harvest *(teh)* was negatively correlated with the date of emergence; later emerging shoots reached harvest maturity more quickly than earlier emerging shoots (Figure 2.4C). This correlation was greatest in 'Diva' (R^2 = 0.7). When the model was fitted using GDD, the negative relationship remained (Figure 2.4C),

although it was weaker, especially for 'Diva' (R^2 = 0.54) and 'Spotlight' (R^2 = 0.25). There were no correlations found between date of emergence and final shoot length ('Diva' R^2 = 0.01; 'Spotlight' R^2 = 0.12; 'Starlet' R^2 = 0.05), or the date of harvest maturity and shoot length ('Diva' R^2 = 0.01; 'Spotlight' R^2 = 0.08; 'Starlet' R^2 = 0.07). Thus for 'Diva', although later emerging shoots reach anthesis more quickly, this did not influence shoot length.

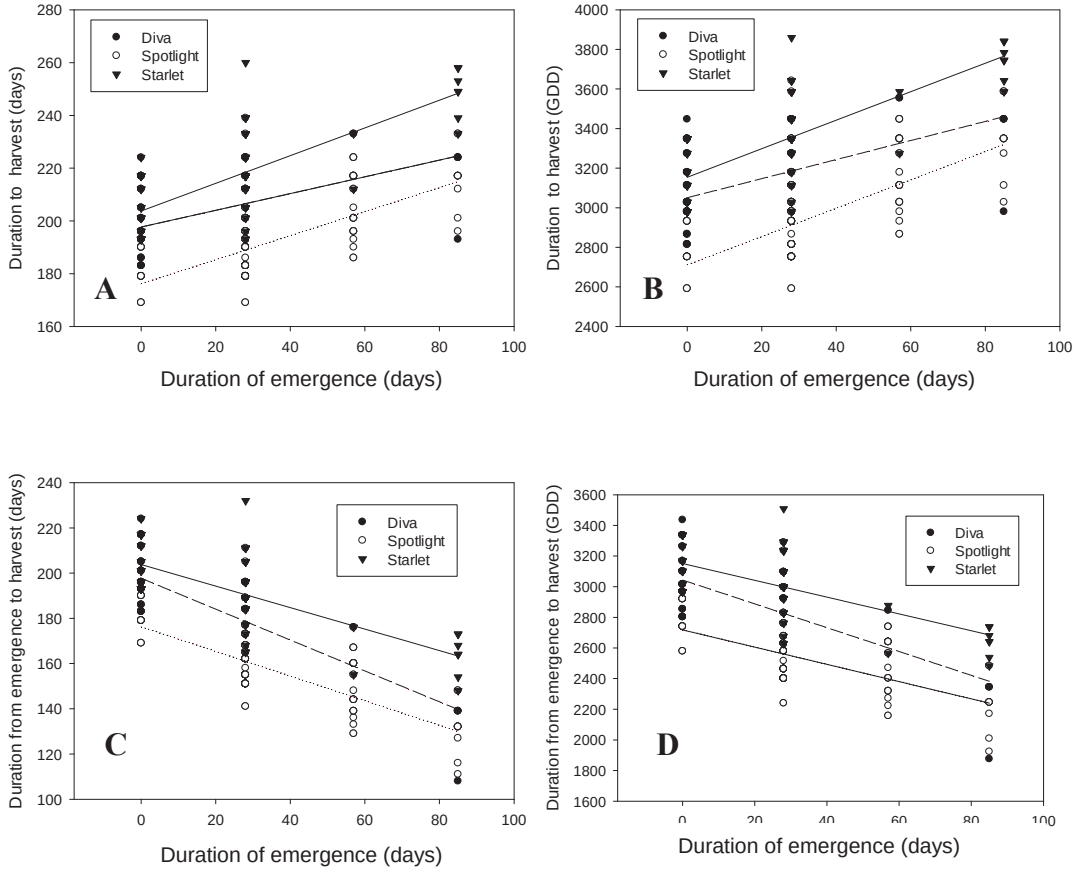


Figure 2.4 (A), Correlation between duration to shoot emergence and duration to harvest ('Diva' (---); R^2 = 0.33, 'Spotlight'(…); R^2 = 0.35, 'Starlet' (—); R^2 = 0.51); (B), GDD to shoot emergence and duration to harvest ('Diva'; R^2 = 0.33; 'Spotlight'; R^2 = 0.35, 'Starlet'; R^2 = 0.70); (C), Duration to shoot emergence and duration to harvest from shoot emergence (Diva; R^2 = 0.70, 'Spotlight'; R^2 = 0.43, 'Starlet'; R^2 = 0.45); (D), GDD to shoot emergence and from shoot emergence to harvest ('Diva'; R^2 = 0.54, 'Spotlight'; R^2 = 0.25, 'Starlet'; R^2 = 0.35). Regression correlations were significant at $P < 0.0001$.

2.4.1.4 Outliers in duration to harvest maturity

In both 'Starlet' and 'Spotlight', there were outliers in duration to harvest maturity beyond the 90th percentile occurring late in the season, but in 'Diva' most outliers were early and occurred before the 10th percentile (Figure 2.3A). For 'Diva', 77% of outliers

(7 out of 9) reached harvest maturity at the beginning of the season, and 23% late in the growth cycle (after the 90th percentile). The outliers of 'Diva' that matured early developed from the first shoots to emerge, and the outliers that matured late all emerged after 57 days. For 'Spotlight', only 20% of shoots classified as outliers flowered early with the remaining 80% (12 out of 15 shoots) emerging after 61 days. In 'Starlet' all outliers (6) were late flowering, with an average duration to shoot emergence of 75 days. When compared with the average emergence time of all shoots, these extremely late flowering outliers emerged later in the season. The average length of these late flowering shoots was 82 cm in 'Diva', 76 cm in 'Spotlight' and 79 cm in 'Starlet'; these values were within the bounds of variation of the average shoot length for 'Diva' at 93 ± 20 cm, 'Spotlight' at 84 ± 9 cm, and 'Starlet' at 75 ± 10 cm. Thus, shoot length did not appear to be influenced by date of shoot emergence.

2.4.2 Spread in time to harvest maturity within cultivars

Individual plants varied in their average duration to harvest *(th)*, with 'Spotlight' spread over 11 days, 'Diva' 25 days and 'Starlet' 21 days (Table 2.3). There was more variation in duration of emergence *(te)* of 'Starlet' plants (31 days) than 'Diva' (12 days) or 'Spotlight' (13 days). By contrast, there was more variation in duration from emergence to harvest *(teh)* in individual 'Spotlight' plants (17 days), than 'Starlet' or 'Diva' (Table 2.3). Similar patterns were apparent when GDD were used instead of calendar days (data not presented).

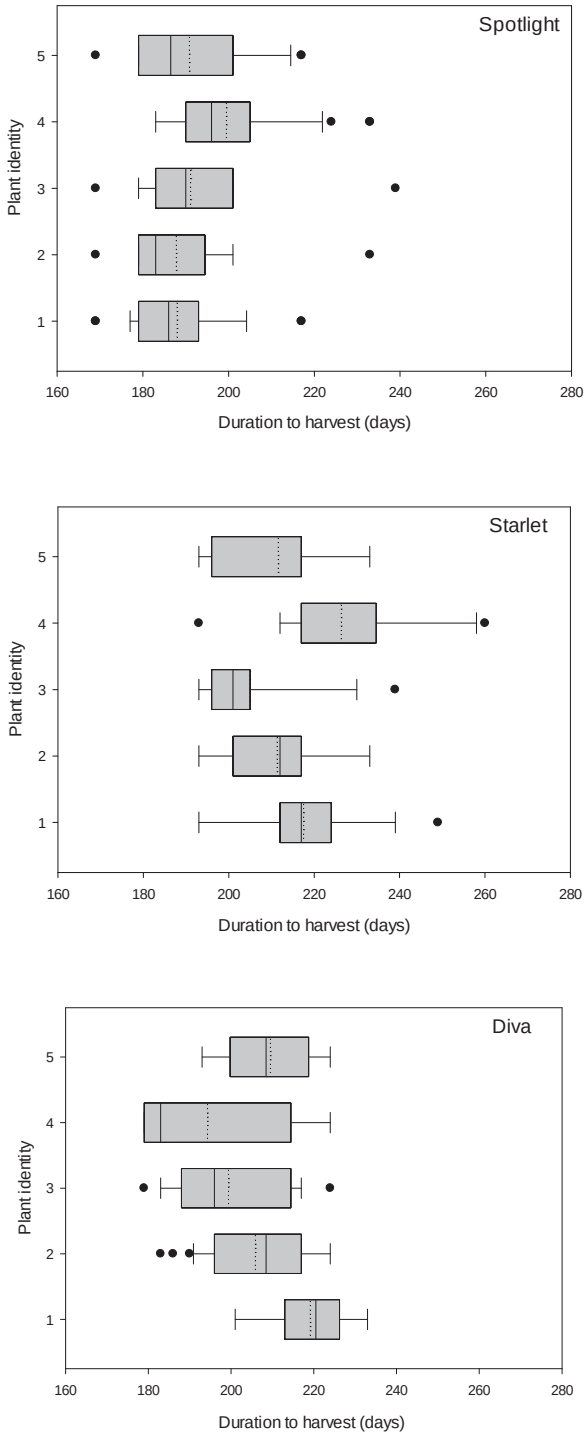


Figure 2.5 Distribution of time to harvest maturity for floral shoots in individual plants of each gentian cultivar. Solid and dashed vertical lines in the centre of each box indicate the median and mean, respectively. Boundaries of box indicate 25th and 75th percentiles, whiskers indicate 10th and 90th percentiles and, solid dots indicate one or more individual shoots as outliers beyond these percentile limits.

There was considerable variation between plants in their 10th to 90th percentile distribution of time to harvest maturity *(th)*. In 'Spotlight', plant 3 took 16 days longer than plant 1 (Figure 2.5). In 'Diva', the maximum difference between individual plants

was 14 days and, in 'Starlet', 9 days. 'Spotlight' had the highest number of outliers (15 shoots), and all replicates of 'Spotlight' had late maturing outliers, though four out of five plants also had early maturing outliers. Only two of the replicates of 'Diva' had outliers, but there was still up to 25 days variation in average date of harvest maturity *(th)* among individual plants. When compared with the other replicates, 'Starlet' plant 4 was late maturing. This was primarily due to outliers taking up to 260 days to mature, compared with an average of 215 days for the five replicate plants. Three of the replicate plants had outliers, the majority late flowering, which indicated greater among-plant variability in 'Starlet' than the other cultivars.

2.5 Discussion

Irrespective of whether expressed as calendar days or GDD, the three cultivars differed significantly in duration to harvest (Tables 1 and 2). Correlations were evident between duration to shoot emergence *(te)* and the duration to reach harvest maturity *(th)*, and between duration to shoot emergence *(te)* and the duration from emergence to harvest maturity *(teh)*, in all cultivars (Figure 2.4). There is little previous research into correlations between duration to shoot emergence and duration to harvest. In results similar to the current study, a positive correlation was reported between duration to shoot emergence and flowering in *Lathyrus vernus* L. (Sola and Ehrlen, 2006). The negative relationship observed between duration to shoot emergence and the duration from emergence to harvest maturity *(teh)* for the three cultivars was unexpected. The finding that late-emerging shoots reach harvest maturity earlier is at odds with the more commonly reported positive relationship observed in *S. multiflorus* (Funnell, 2008). This may be partly explained by acknowledging that the influence of duration to shoot emergence on duration to harvest depended on cultivar, and its effect seemed greater in the latest flowering cultivar, 'Starlet'. In 'Starlet', shoot maturity was likely further delayed by cooler temperatures later in the season. 'Diva', although later flowering than 'Spotlight', had more rapid shoot development, resulting in a shorter duration to harvest in late-emerged shoots. 'Diva' is, therefore, hypothesised to be less affected by cooler temperatures in terms of time to reach harvest maturity.

Under the temperature range experienced during the experiment (Figure 2.1), GDD models did not improve the strength of the correlation in determining duration to harvest for all the cultivars. For this study a base temperature of 0 °C was used. As reported for a range of species, e.g. *Triticum aestivum* L. (Angus et al., 1981), *S.*

multiflorus (Funnell, 2008), and *Arachis hypogaea* L. (Bell et al., 1991), base temperatures can vary according to developmental stage and cultivar. A differential response to temperature in development to harvest maturity has been reported in gentians, in which early cultivars flowered earlier at higher temperatures (20 to 25 °C) than late cultivars, in which flowering was delayed at this same temperature range (Ohkawa, 1983). Thus in the current study, the lack of specific information regarding base and optimum temperatures for growth and development for the cultivars used may have limited the information derived using GDD. For more accurate application of GDD in future work, studies will be required to determine the temperature response curves for cultivars being tested. Additionally, there is some evidence that duration to harvest in late flowering cultivars is influenced by short days (Ohkawa, 1983). Hence, an influence of declining day length may be embedded in the temperature response of duration to harvest.

'Spotlight' had a narrower spread in the duration from emergence to harvest *(teh)* than the two later maturing cultivars (Table 2.1 and Table 2.2). Duration of emergence was also more even than the other cultivars, i.e. a smaller spread. In 'Spotlight', variation among plants had the most influence after emergence. As indicated by the presence of many outliers, variability in maturation of shoots within individual plants was greater in 'Spotlight' than the other cultivars (Figure 2.5). Hence, if in the future narrowing down of the spread in harvest maturity is required in 'Spotlight', it will be important to control the occurrence of such influential outliers.

'Starlet' showed the widest 10^{th} to 90^{th} percentile distribution (Table 2.1), and also the greatest plant to plant variability in duration to harvest (Table 2.3). Since shoot emergence was compact in 'Starlet' it is suggested that declining temperatures in autumn (Figure 2.1) can at least partially account for the spread. Additionally, however, the spread and variation among plants was not due to the variability in duration from shoot emergence to harvest *(teh)*, but due to variation in duration to shoot emergence (*te*; Table 2.3). Hence in 'Starlet', it is hypothesised that, in order to manipulate the spread, the determinants of these two aspects need to be understood in more detail. Physiological or environmental stimuli involved in the induction or breaking of bud dormancy have not been documented for gentians, but chilling to break endodormancy has been inferred (Ohkawa, 1983). Once the requirement for chilling has been met, uniform shoot emergence is enabled (Lang et al., 1987), but paradormancy and

ecodormancy could also influence the development of buds simultaneously or separately (Horvath et al., 2003). Future investigations with 'Starlet' would, therefore, benefit from investigating the influence paradormancy, endodormancy and/or ecodormancy has on duration to shoot emergence, as well as the influence of temperature and day length during shoot development.

'Diva' was relatively late flowering and had a wider spread, in both duration to shoot emergence *(te)* and duration from emergence to harvest (*teh*; Table 2.1). As evident by the failure of GDD to alter the correlations between the variables that determined the duration to harvest (Figure 2.3), no response was detected in extending the duration to harvest maturity *(th)* with respect to the declining temperatures later in the growth cycle (Figure 2.1). Spread in time to harvest maturity *(th)* was similar in 'Diva' and 'Starlet', but the spread in time to harvest from emergence *(teh)*, as well as duration of emergence *(te)*, was widest in 'Diva' (Table 2.1 and Figure 2.3). The observed wide spread in time to harvest from emergence *(teh)* was due to the shoots taking varying times to reach harvest maturity, based on date of emergence (Figure 2.4). Less variability in harvesting dates may, therefore, be due to a greater ability of shoots within 'Diva' to reach harvest maturity at a comparatively uniform time, irrespective of the date of shoot emergence (Figure 2.4). Hence, as observed in populations of *Ulex europaeus* L. (Tarayre et al., 2007) and *Eriogonum abertianum* Torr. (Fox, 1990), 'Diva' appears to display greater phenotypic plasticity in reaching harvest maturity than the other cultivars. With 'Diva', it was hypothesised that the primary source of spread arises from changes in the duration of emergence *(te)* of individual shoots. Once emerged, shoots reach harvest maturity relatively uniformly.

Axillary shoots, which appear to arise as individual shoots, flowered at the same time as the main shoot to which they were attached. Thus in a commercial context, these axillary shoots are unlikely to be a source of variation in time to harvest maturity *(th)*.

Shoots which matured early, and were categorized as outliers, were prominent in 'Diva', and matured late in 'Starlet' and 'Spotlight'. All these shoots were of marketable quality. If the average frequency of late outliers for 'Spotlight' (i.e. 0.096 shoots per plant per day) and assuming a between plant spacing of 20 cm × 20 cm (Anonymous, 1994), to collect 100 floral shoots the harvester would have to walk 208 m of bed. In contrast, during the peak of the flowering season (i.e. between the 25th and 75th percentiles), 100 shoots can be picked within 31 m of bed. This knowledge allows growers to now

consider potential differences in economic returns between these two harvesting periods. While it is desirable to manage the earliness or lateness of outliers, the causes of the observed variation in flowering time of individual shoots, outliers or otherwise, on an individual plant, require further investigation (Chapter 3).

2.6 Conclusion

Gentians show different degrees of variability in time to harvest maturity among cultivars, among plants of the same cultivar, and among shoots within a plant. The spread in time to harvest maturity, as well as the factors contributing to the spread in harvest maturity dates of these clonally propagated plants, appears specific to individual gentian cultivars. For commercial cultivars, reasons for the variation in duration to harvest maturity will need to be investigated by exploring how plant and/or environmental factors influence the growth and development of individual buds/shoots within a plant, from bud initiation to harvest maturity.

Chapter 3 Seasonal Changes in Crown Buds and Flowering of Gentian 'Spotlight'

3.1 Abstract

A better understanding of the origin and development of crown buds in gentian may help explain the observed spread in timing of flower harvest maturity. The types and numbers of buds, the time of their macroscopic appearance, emergence and development to flowering were tracked over time, in two growing environments for two growth cycles of the gentian cultivar 'Spotlight'. Although different types of buds were present, only pre-formed primary-crown buds produced flowering shoots. In the first growth cycle only 73% of these pre-formed buds emerged, leaving the rest of the buds to senesce and degenerate. Hence, the observed spread in time to flower harvest maturity was not due to either bud type or buds that appeared during the current season developing into floral shoots. The appearance of new crown buds for the following season's flower production occurred over an 8-month period, from summer through to winter. The date of harvest maturity for a shoot was not strongly correlated with the time of bud appearance or shoot emergence, hence the hypothesis that this was a source of the spread in timing of harvest maturity was not supported. Compared with those grown in the cooler environment outside, cultivation in a protected environment resulted in 50% longer shoots at harvest, but did not influence the time to flower harvest, and increased the rate of appearance of buds by an additional 0.18 per month. With no chilling, however, within the protected environment the crown buds failed to emerge as shoots during the second growing season. In contrast, crown buds on outdoor-grown plants emerged and flowered. The lack of any correlation between the time of appearance of buds and/or their emergence with that of harvest maturity, as well as the chilling requirement for shoot emergence, highlights that factors associated with growth and development of a crown bud after its appearance may have a greater influence on the duration to harvest maturity in 'Spotlight' and, therefore, the spread in timing.

3.2 Introduction

Gentian (*Gentiana*) is a relatively new flower crop in international markets. It is well known in Japan, the world's largest producer and consumer. Growers in New Zealand (NZ) began exporting cut flowers of gentians in the 1990s, and recent years have seen increased interest in the crop from a range of countries. Breeders in NZ have recently introduced new cultivars derived primarily from *Gentiana triflora* 'Pall' and *Gentiana scabra* 'Bunge', which have an extended colour range, to assist further expansion of the market (Eason et al., 2007).

Growers are faced with a wide spread in the time of maturation of individual shoots on a plant or in a clonally propagated cultivar, potentially resulting in inefficient use of labour at harvest (Chapter 1). Although details of breeding (Morgan, 2004) and post-harvest research on this crop (Eason et al., 2004) have been published, peer-reviewed articles related to growth and physiology are limited. One review article (Ohkawa, 1983) gives a brief description of the growth cycle of the crop and cultural information related to temperature and photoperiodic control of growth in Japanese conditions, but only provides summary data pertaining to historical cultivars, with no statistical validation. The current study was therefore designed to identify factors, both endogenous to the plant and environmental, that could influence the duration to harvest maturity of new cultivars such as 'Showtime Spotlight' (Spotlight).

Gentians are herbaceous perennials, over-wintering as a crown comprised of a underground stem, various-sized buds, storage roots and feeder roots (Ohkawa, 1983). Crown buds are dormant in winter until growth recommences in spring, when they emerge to form flowering shoots. As is evident with some herbaceous perennials (DeHertogh and LeNard, 1993), since these buds are preformed they may have leaves, shoots and flowers already initiated at the beginning of the season, or they may comprise vegetative initials only. Flowering shoots could also grow from crown buds produced *de novo* in the current growing season. With buds at a more advanced stage of development likely to reach flowering earlier (Funnell et al., 2003), it was hypothesized that some of the buds initiated *de novo* in the current growth cycle may flower later in the same season, contributing to the observed spread in time to shoot maturity. Another hypothesis was that differences in the duration to shoot emergence during the growth cycle may be a result of a progressive loss of apical dominance as older shoots elongate, leading to later emergence of less dominant but pre-existing crown buds. Duration to

emergence has previously been shown to correlate with time of harvest maturity in some gentian cultivars (Chapter 2). However, any relationship between the timing of appearance of buds on the crown, their subsequent emergence and duration to shoot maturity (flower harvest), has not previously been examined. The appearance of buds on the crown is defined as the time when the (dormant) buds are first visible to the naked eye; bud emergence refers to the extension of the crown bud following removal of dormancy, and shoots are considered mature when they have reached commercial harvest maturity as a cut flower.

Gentians are usually cultivated outdoors, but protected environments have proven beneficial for other ornamental crops resulting in extended production times, higher yields and improved quality. The growth response of the newer cultivars of gentians to protected environments in relation to cut flower productivity and timing of flowering has not been described. While some gentian cultivars have previously shown a reduction in the rate of development to flower harvest with declining seasonal temperatures (Chapter 2), the response to increased temperatures afforded by controlled environments has not been reported.

In herbaceous perennial plants, new meristems produced in leaf axils can be either reproductive or vegetative, and also active or inactive, i.e. dormant (Bonser and Aarssen, 1996). In a crown, such as occurs in gentians, the origin of buds can be difficult to determine. Anecdotal evidence suggests that shoots of gentians may be produced from both axillary and adventitious buds. As shoots arising from adventitious buds are likely to be more juvenile than axillary shoots, the shoots of adventitious origin are unlikely to develop to flowering at the same rate, even if these two types of shoots emerge at the same time (Del Tredici, 2001). Therefore, the origin and development of crown buds in gentians needed to be determined. Based on the previous observations, the cultivar ‘Spotlight’ displayed a comparatively narrower spread in timing of flower harvest than other cultivars and was not influenced by declining temperatures in autumn, but showed relatively greater variability within a plant (Chapter 2). It was hypothesised that the source of the observed variability in ‘Spotlight’ was mainly due to differences among individual shoots within the plant. ‘Spotlight’ thus was considered a suitable cultivar for determining the types of buds present in a plant, and the influence of growing environment temperature on bud development and growth from their appearance on the crown to flower harvest.

3.3 Materials and methods

3.3.1 General materials and methods

Plants of 'Spotlight' were propagated by tissue culture at The New Zealand Institute for Plant & Food Research Limited, Palmerston North. Plants were deflasked during December 2005 to January 2006 and grown in an unheated greenhouse with natural lighting for 18 months at Palmerston North, NZ (40°37'S 175°60'E). In June 2007, they were placed in continuous darkness in a cool store at 10 ± 1 °C for 12 weeks. Thus at the start of the experiment, the plants had undergone two seasons of growth, and were in cold storage.

Plants were re-potted into plastic pots (5 L), using a growing medium (CAN fines A grade bark 50%; bark fibre 30%; pumice 7 mm 20%) with 1 kg/m^3 serpentine super, 150 gL^{-1} dolomite, 200 gL^{-1} 8–9 month Osmocote® (16N–3.5P–10K, Grace-Sierra International, The Netherlands), and 100 g L^{-1} 3–4 month Osmocote® (15N–4.8P–10.8K), prior to treatments being applied. Throughout the experiment, irrigation was delivered by microtubes to each pot, on a drained capillary bench, for 10 minutes between three and five times a day, depending upon plant demand.

3.3.2 Procedure for tracking buds

Based on the method used by Danningsh (2004) the growing medium on top of each plant's crown was carefully removed to expose the buds. The crowns of the plants were subsequently covered with non-absorbent cotton wool, thus providing easy access while retaining moisture around the exposed tissue. To reduce light penetration into the exposed crown, the top of the covered crown and pot were covered with black weed-mat.

Data were collected at fortnightly intervals for two growth cycles. To track changes in bud number and development over time, a diagram illustrating the crown region of each plant was made at each date of data collection. When buds emerged as shoots, they were tagged to indicate origin and date of shoot emergence. A bud was defined as having emerged as a shoot when it was more than 2.5 cm in height above the point of attachment at its base. As floral shoots reached commercial maturity, i.e. the top-most flower bud was not open but had developed colour (Eason et al., 2004), they were removed from the plant, leaving the basal 4–6 cm of shoot and foliage. To ensure an adequate supply of storage-carbohydrates for the following season's growth, two or

three entire shoots were left on the plant after reaching maturity. These shoots were removed at the beginning of winter (15th June 2008).

Buds observed in the study were described as follows; "Primary-crown buds" were thick (> 1 mm diameter) and purple-coloured; "Secondary-crown buds" were thin (< 1 mm diameter) and pale-coloured. "Root buds" developed directly from storage roots.

3.3.3 Treatments and experimental design

Commencing 19th September 2007, plants were grown in one of two environments, i.e. either outside under ambient conditions at the Plant Growth Unit, Massey University, Palmerston North (40°20'S), or in a greenhouse heated at 15 °C, vented at 25 °C, with 20% shading (i.e. protected environment) and natural photoperiod. Temperature variations in the two environments were recorded (Figure 3.1). Mean monthly temperature in the outside environment varied between 8 and 18°C during the first growth cycle and between 7 and 17 °C in the second growth cycle. Temperature in the protected environment remained above 16 °C throughout the experiment. In order to assess the impact of regularly exposing the crown for tracking in both environments, plants in the control treatment were grown in the same growing medium that was not disturbed. Hence, there were four treatments (i.e. Tracked in the Protected Environment, Tracked Outside under ambient conditions, Control in the Protected Environment, and Control Outside). The experiment comprised a Completely Randomised Design, utilising five single-plant replicates in each treatment. Three additional plants were grown in each environment for dissections at the end of the first growth cycle.

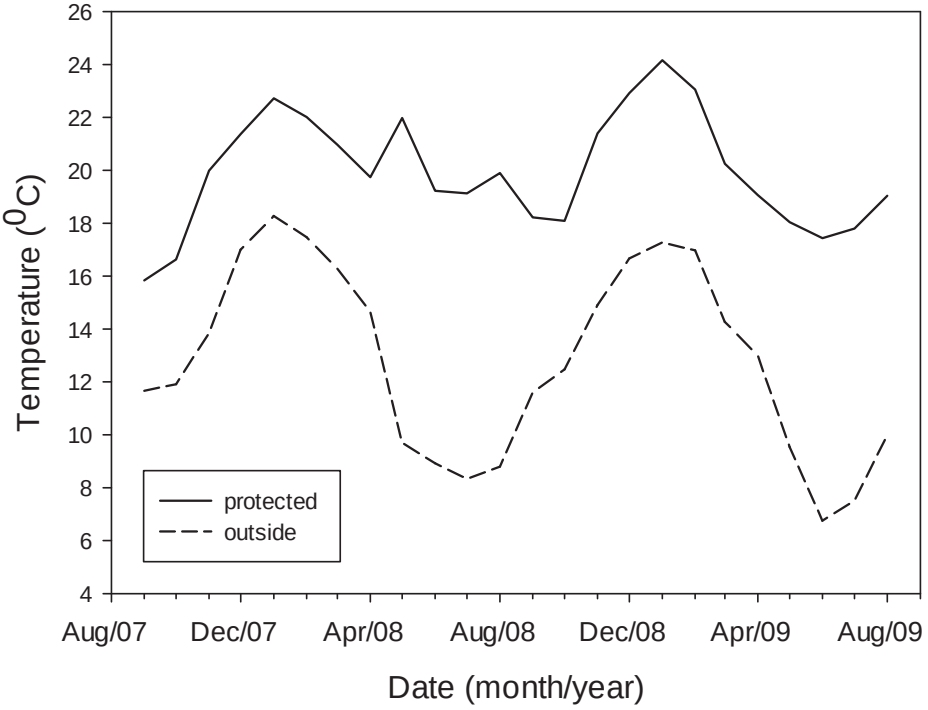


Figure 3.1 Mean monthly temperature encountered in two treatment environments used during the first (September 2007 to August 2008) and second (September 2008 to August 2009) growth cycle.

3.3.4 Variables recorded

For tracked plants, the variables recorded were type of bud (based on origin; crown or root), bud diameter (measured at the base using Vernier callipers on the first day of data collection), status (alive or dead), and date of appearance of new buds. For shoots, the parameters recorded were; position of senesced shoots from the preceding growth cycle, number of visible nodes, shoot length, number of nodes below the lowest flower bud, and date of first visible appearance of floral buds (Appendix I). For plants in the control treatments, the number of shoots was recorded at 2-week intervals. In addition, when the flowering shoots had been removed, the crown was exposed, and the number and types of buds remaining were noted. In all treatments, at flower harvest maturity the following was recorded for each shoot: number of nodes, number of floral nodes, shoot length, and date of harvest. These data were recorded for two growth cycles.

3.3.5 Dissection of crown buds

Primary-crown buds (> 1 mm in diameter) were removed prior to commencement of the second growth cycle (11th September 2008) from the three additional plants grown in

each environment. Because plants had low survival rates in the protected environment (refer to Results; shoot emergence – second growth cycle), only one plant was available for dissection. Buds were dissected using a dissecting microscope. Numbers of scale leaves and primordial structures per bud were recorded.

3.3.6 Data analysis

Data were analysed using the general linear models procedure of Statistical Analysis System (SAS) software version 9.13 (SAS Institute, Cary, N.C., USA). Mean comparisons were conducted using LSD and Tukey's test. Relationships between parameters were evaluated using Proc Reg within SAS. Box and whisker plots (Sigma Plot version 10, Systat Software Inc., San Jose, CA, USA) were used to describe the distribution of bud appearance, shoot emergence and flower harvest with time. The 80% spread was calculated for the time of appearance of buds or harvest maturity of shoots based on the number of days between the 10^{th} and 90^{th} percentiles of the box plot, and the median as the date at which 50% of buds appeared or reached harvest maturity.

3.4 Results

3.4.1 Shoot emergence – first growth cycle

At the beginning of the experiment (September 2007), 94 ± 3% of the buds originated from crown tissue and 6 ± 1 % from storage roots. Primary-crown buds accounted for 84 ± 1% of the crown buds arising from crown tissue, and secondary-crown buds 16 ± 1%. Most shoots had emerged by the end of October (2007), i.e. within 28 days of removal from cold storage (Figure 3.2 and Figure 3.3). With the exception of two shoots arising from root buds in one plant, all shoots emerged from primary-crown buds present at the start of the experiment.

Within the range of the number of buds present on plants at the beginning of the experiment, i.e. between 7 and 42, a higher number of buds was correlated with a higher shoot number (ranging from 5 to 24; $R^2 = 0.94$; $P < 0.0001$; Appendix II). When the total number of shoots that emerged during the growth cycle was compared with the total number of crown buds (i.e. primary and secondary) observed on the first day of the experiment, only 73 ± 7% of crown buds had emerged as shoots. Thus, 27% of primary- and secondary-crown buds did not emerge. At the end of eight months of cultivation in

each environment, from shoot emergence to flower harvest, the majority of these remaining 27% of crown buds had senesced and degenerated (Figure 3.2 E & F).

3.4.2 Shoot growth and development – first growth cycle

Throughout the first growth cycle shoots on plants in the protected environment were longer than those on plants outside, both in tracked and control plants (Figure 3.5A). The difference was most evident ($P < 0.0001$) at harvest maturity where shoots were 50% longer in plants which were tracked and 64% longer in plants from within the Control treatment (Table 3.1). At 0.42 cm/day, the rate of increase in shoot length in the protected environment was almost twice that achieved outside (Figure 3.5A). In contrast, the number of nodes on harvested shoots (20 ± 1), was not different between growing environments ($P > 0.05$). Neither the number of floral nodes per shoot (6 ± 1) nor the shoot number per plant (10 ± 1) differed among treatments ($P > 0.05$). For both growing environments, 98 ± 2% of the shoots which emerged were floral. The few shoots that remained vegetative either developed from root-buds or emerged from the crown late in the growth cycle (i.e. after 17th October 2007).

Table 3.1 Shoot length at harvest, and average duration to harvest (from the date of removal from the cold store) of flowering shoots of both tracked and control plants of 'Spotlight' in either the protected or outside environments during the first growth cycle.

	Shoot length (cm)		Duration to harvest (days)	
Environment	Tracked	Control	Tracked	Control
Protected	66.1 ± 2.5[a]	74.4 ± 2.5 [a]	178 ± 2 [b]	190 ± 2 [a]
Outside	44.2 ± 1.6 [b]	45.4 ± 1.7 [b]	190 ± 3 [a]	184 ± 2 [a]

Means (± standard error) followed by different letters were significantly different at $P \leq 0.05$ for each parameter (Tukey's groupings)

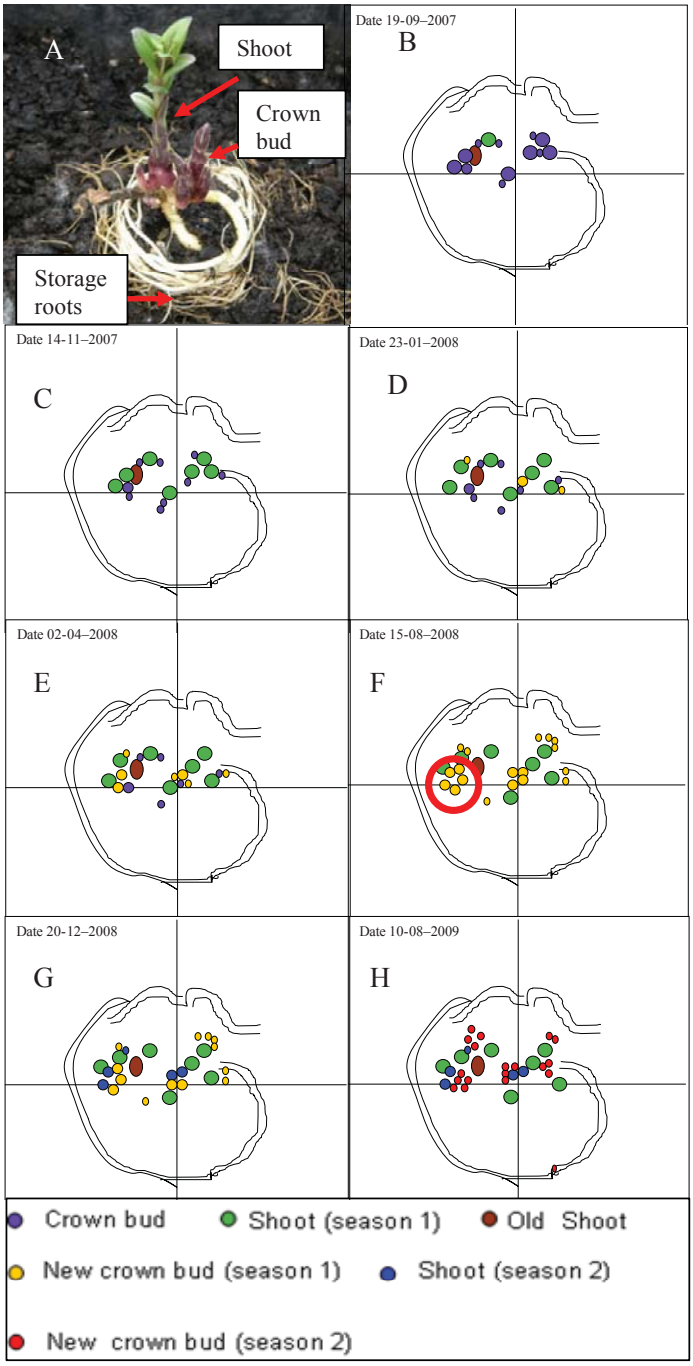


Figure 3.2 (A) and (B), respectively, photograph of a representative plant of 'Spotlight', and diagrammatic illustration of the same, illustrating key organs at the start of experiment, (B-H); Developmental sequence and physical location of buds and shoots on the crown over a period of 2 years, on one representative plant; size of buds ("primary-crown buds" were thick (> 1 mm diameter) and purple-coloured buds; "secondary-crown buds" were thin (< 1 mm diameter) and pale-coloured; "root buds" developed directly from storage roots). Shoots in the diagram are relative to the sizes observed during the experiment, where the smallest coloured dot is representative of less than 1 mm diameter in size; red open circle indicates a single bud cluster.

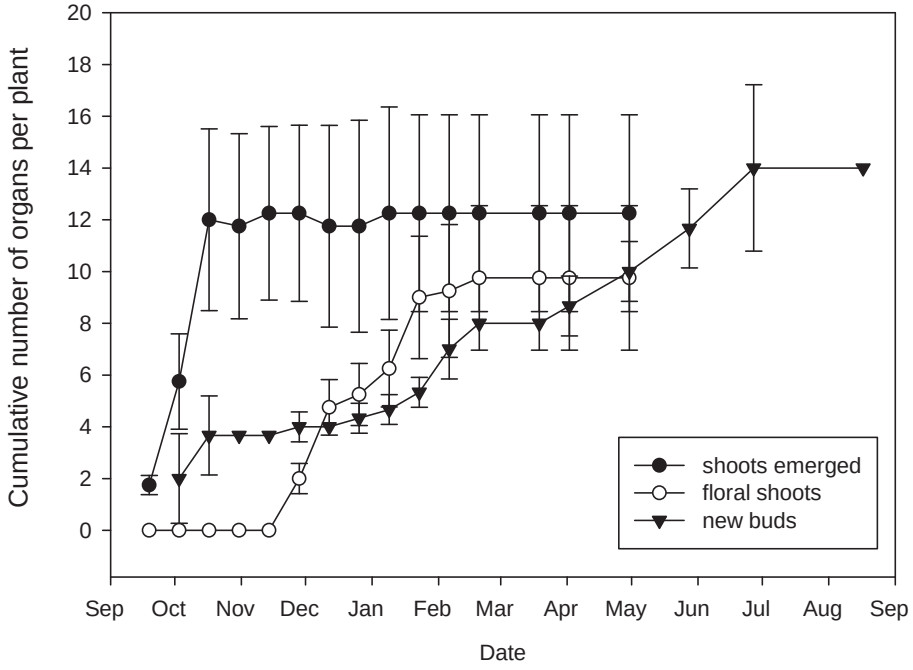


Figure 3.3 Cumulative total of emerged shoots, floral shoots, and new buds per plant for 'Spotlight' grown outside during the first growth cycle (September 2007 to August 2008). The pattern of change within the protected environment (data not presented) was the same as for those grown outside.

3.4.3 Timing of flowering – first growth cycle

For plants which were tracked, floral shoots reached harvest maturity 12 days earlier in the protected than the outside environment ($P < 0.05$; Table 3.1), but this difference was not evident for plants within the Control treatment ($P > 0.05$). In the protected environment, shoots of plants which were tracked reached harvest maturity 12 days earlier than those within the Control treatment ($P < 0.05$). No difference however, was evident between the tracked and control plants growing outside ($P > 0.05$). The 80% spread in time to harvest maturity ranged between 33 and 48 days (Figure 3.4). There was, however, no consistent treatment effect of either growing environment or exposing the crown for tracking, in terms of the date or spread of the timing of shoot harvest maturity.

The duration to shoot emergence and duration to shoot harvest were not correlated in either the protected environment or the outside environment ($R^2 = 0.22$ and $R^2 = 0.1$, respectively). There was no indication of correlation ($R^2 = 0.07$) between the duration to shoot emergence and when floral axillary buds were first observed in a shoot. The date

when floral axillary buds were first observed was 8 days later in the protected (107 ± 3.09 days) than the outside environment (98 ± 3.92 days; $P = 0.05$).

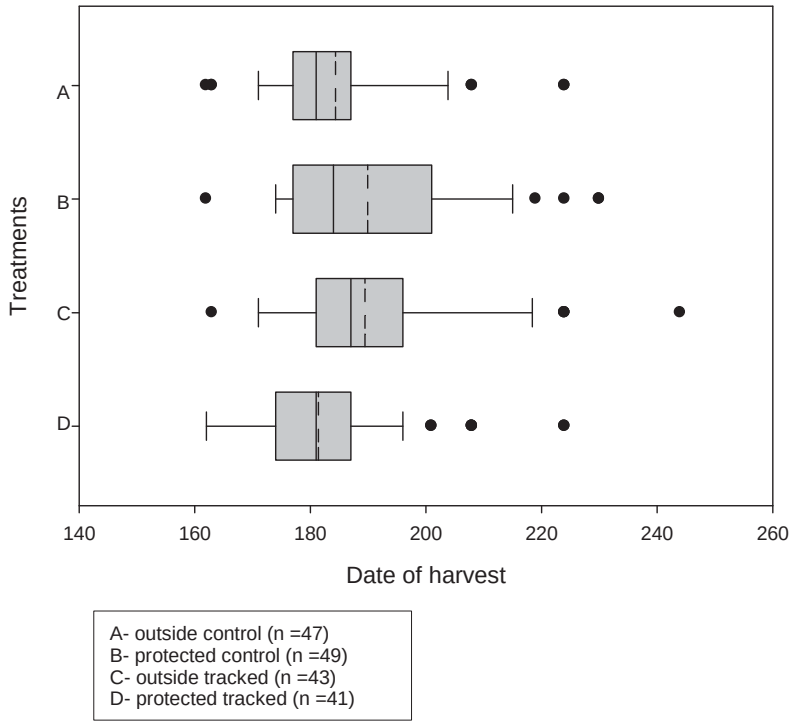


Figure 3.4 Box and whisker plot for number of shoots harvested at each date for tracked or control within two growing environments, during the first (September 2007 to August 2008) growth cycle for 'Spotlight'. Solid and dashed vertical lines in the centre of the box indicates median and mean respectively. Boundaries of box indicates 25th and 75th percentiles, whiskers show 10th and 90th percentiles and solid dots show the outliers these percentile limits. (n = number of shoots harvested from all five replicates).

3.4.4 Timing and quality of crown buds initiated – first growth cycle

In both environments, the rate of appearance of new crown buds was comparatively less in spring (e.g. November), but increased later in the growth cycle (January to June; Figure 3.2 and Figure 3.3). The increase in bud appearance coincided with the date when most shoots could be confirmed as being floral (Figure 3.3). The rate of bud appearance after 98 days (date of observation of floral axillary buds in a shoot) ranged from 0.036 to 0.054 buds per day (i.e. 1.35 per month on average) in the protected environment with those outside achieving 0.028 to 0.050 per day (i.e. 1.17 per month; Figure 3.5B). The cumulative total of newly-appeared buds was initially greater *(*$P <$ 0.05) in the protected environment but, by the beginning of the second growth cycle bud

numbers were not significantly different, with 20 ± 3 new buds in the protected environment and 17 ± 2 outside, for both tracked and control plants. These newly formed buds comprised 19 ± 3 and 14 ± 2 primary-crown buds in the protected and outside environments, respectively. In contrast, the diameter of primary-crown buds was greater ($P < 0.05$) in plants grown in the protected environment (3.7 ± 0.1 mm) than outside (3.0 ± 0.1 mm). Secondary-crown buds were not measured individually as they were less than 1 mm in diameter.

In the first growth cycle, buds with greater diameter on the first day of tracking resulted in longer shoots at harvest maturity, but accounted for less than 50% of the variation in both the protected ($R^2 = 0.45$; $P < 0.0001$) and outside ($R^2 = 0.40$; $P < 0.0001$) growing environments. At the time to harvest maturity, each 1 mm change of diameter in bud size at the start of the experiment resulted in 13 cm of shoot length in the protected environment and 8 cm outside. No correlation was evident between bud diameter and final node number per shoot at harvest maturity.

3.4.5 Sequence of shoot and crown bud development

New crown buds started to appear in October (summer) and continued to appear until July (winter; and Figure 3.3), however primary-crown buds were apparent after December (Figure 3.2). The 27% of crown buds present at the start of the experiment did not emerge and gradually senesced during this period. As they progressively became visible, new crown buds were physically arranged on the rhizome in clusters (Figure 3.2F). Less frequently, clusters of buds also developed at the proximal end of storage roots. New bud clusters that became visible during the second growth cycle were located close to clusters formed during first growth cycle, i.e. at the base of the current season's shoots (Figure 3.2G and H).

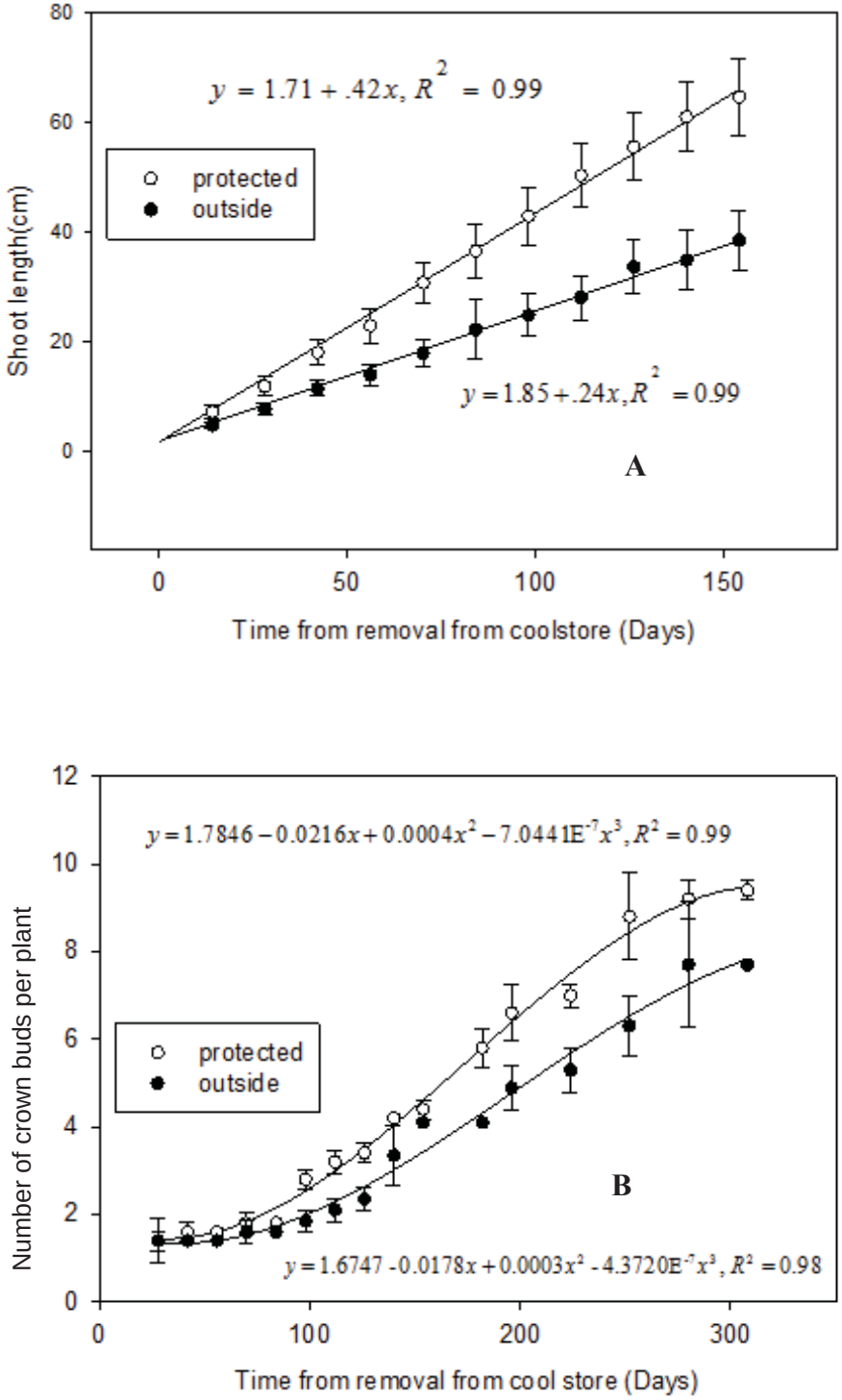


Figure 3.5 Pattern of change in; (A), shoot length until harvesting commenced and; (B), cumulative crown bud number per plant, in two different environments during first growth cycle for 'Spotlight'. Vertical bars = standard error. Regression correlations were significant at $P < 0.0001$.

3.4.6 Crown bud morphology – beginning of growth second growth cycle

When buds were dissected after winter, but before emergence in the second growth cycle, primary-crown buds from all treatments contained a similar number of primordia, i.e. 10 ± 0.71 (including prophylls and primordial leaves). No floral initials were evident in any dissected crown buds.

3.4.7 Shoot emergence – second growth cycle

Although no differences were observed between the outside and protected environments in terms of the number of crown buds initiated in first growth cycle, at the beginning of second growth cycle only 20% (2 out of 10) of the plants in the protected environment remained alive compared with 90% of those growing outside. Shoots that emerged on the surviving plants in the protected environment were not normal in appearance; they remained as a rosette during the second growth cycle, rather than presenting clear internodes. In the second growth cycle, for both tracked and control plants grown in the outside environment 38 ± 8% of all crown buds developed into shoots, with 45 ± 10% of the primary-crown buds developing into shoots. None of the secondary-crown buds emerged.

During the second growth cycle the maximum shoot number was achieved by 21st October 2008, and all shoots became floral. There was no relationship between the time of visible bud appearance and duration to shoot emergence (R^2 = 0.002), shoot length (R^2 = 0.002) or node number (R^2 =0.09) in the tracked plants (three plant replicates). The diameter of primary-crown buds was not correlated with the date of bud appearance (R^2 = 0.002), but marginally correlated with shoot length (R^2 = 0.48) and number of nodes (R^2 = 0.41).

3.4.8 Timing of bud appearance and shoot emergence

In the first growth cycle, 80% of the new crown buds appeared over a period of 256 days in the outside environment, with 50% of the total buds discernible by 18th February 2008 (Figure 3.6). Although crown buds progressively appeared from 19th Sept 2007 to 30th June 2008 (285 days), 80% of the shoots that emerged in the second growth cycle grew from buds which first appeared over a 141 day period in the later part of first growth cycle, between 7th January 2008 and 27th May 2008. The median date of appearance of buds that emerged as shoots was 3rd April 2008 (mid-autumn) in the first growth cycle. The buds which did not emerge in the second growth cycle were evenly

spread throughout the first growth cycle as to when they first appeared. The median date of appearance of new buds was 8th January 2008, indicating that the majority of buds that failed to emerge, first appeared earlier in the growth cycle. The loss of plants during the latter part of the second growth cycle prevented analysis of potential relationships with the date of harvest maturity of shoots.

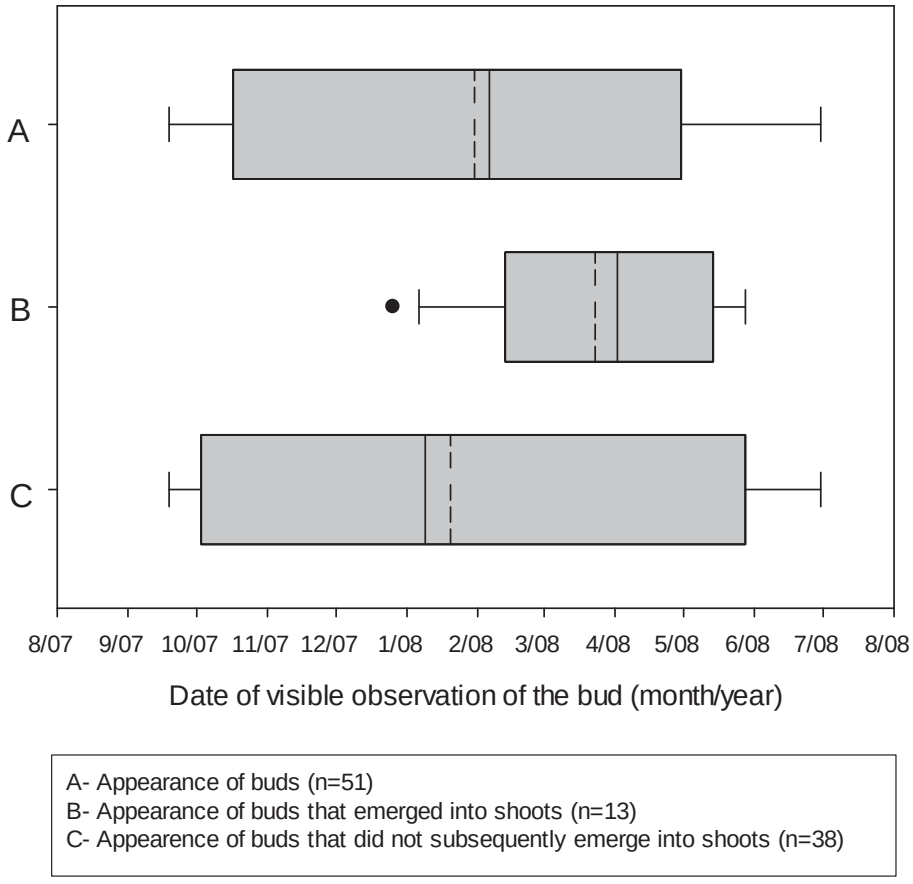


Figure 3.6 During the first growth cycle, distribution of date of appearance of; (A), all buds on the crown; (B), date of appearance of crown of buds that emerged into shoots; (C), the date of appearance of un-emerged buds for 'Spotlight'. Solid and dashed vertical lines in the centre of the box indicates median and mean respectively. Boundaries of box indicates 25th and 75th percentiles, whiskers show 10th and 90th percentiles and solid dots show the outliers.

3.4.9 Influence of exposure of the crown

In both environments the appearance of crown buds and their subsequent growth and development was not significantly influenced by regularly exposing the crown in tracked plants. Neither the number of primary-crown buds nor the total number of

crown buds showed any significant difference between environments. Although not significant, in the protected environment, bud number was higher in control plants than tracked plants (P = 0.09). Tracked and control plants did not show any difference in numbers of shoots nor final shoot length, node number, or floral nodes per shoot however, varied in time to harvest maturity only within the protected environment (Table 3.1) .

3.5 Discussion

3.5.1 Preformed crown buds

Only primary-crown buds that were apparent in the previous growth cycle contributed to the flowering shoots in the current season. In the current study, prior to emergence, the primary-crown buds had 10 ± 0.71 primordia (none floral). In woody perennials most shoot production, both after winter (dormancy) or following disturbance, is from preformed buds (Del Tredici, 2001). In the current experiment no crown buds appeared and developed into flowering shoots in the same growth cycle nor did any root buds from the previous growing growth cycle produce flowers. Therefore, contrary to the stated hypothesis, the observed spread in time to shoot floral maturity was not due to either bud type or buds that appeared during the current growth cycle developing into floral shoots. Hence the plant-related factors that affect the spread of harvest maturity in 'Spotlight' are yet to be identified.

In both seasons, only a proportion of the primary-crown buds present at the beginning of the current growth cycle emerged (Figure 3.2 and Figure 3.6). The remaining primary and secondary buds (27% in first growth cycle and 38% in second growth cycle) had senesced and degenerated by the end of the growing period, so made no contribution to the following season's flowering shoots. In other species, growth and development of one large bud causes smaller buds in the cluster to cease growth and become dormant (Stafstrom, 1995). These dormant buds only grow when the larger bud(s) is removed. Thus, it is hypothesized that in gentians some of the crown buds remained suppressed due to paradormancy of emerging buds. If indeed paradormancy does suppress growth of these un-emerged crown buds, reduction of paradormancy during the current growth cycle by removing existing shoots could be used to increase the total number of shoots emerging and, therefore, the yield of cut flower shoots per plant (Chapter 4).

3.5.2 Appearance of new crown buds

Crown buds were continually produced over an 8-month period (Figure 3.2 and Figure 3.3) spanning summer through winter and, therefore, each sequentially produced bud is likely to have experienced different environmental conditions between appearance and shoot emergence the following season. Hence, it is possible that these different populations of crown buds could emerge and perform differently during their subsequent cycle of growth to harvest. Further to this, in commercial cultivation any horticultural activities occurring from summer through to winter, which disturb the crown, could adversely influence bud initiation. Such environmental or man-made disturbances could alter the dates of bud initiation and development, thereby reducing the number and quality of the buds being produced, e.g. through reduced number of primordia or decreased resource accumulation (Sosnová and Klimesová, 2009). If so, it is hypothesized that the time of bud initiation could lead to differences in yield, quality and time of floral maturity when they develop into shoots. Further investigation based on time of bud initiation and tracking subsequent growth through to shoot harvest maturity is required to test this hypothesis.

In temperate woody perennials, declining temperature and/or photoperiod are associated with the formation of dormant buds (Arora et al., 2003). Compared with woody perennials, the exact environmental cues for initiation of dormancy in herbaceous perennials are not clear. During the current study the formation of new dormant crown buds began in summer when the plants were not experiencing low temperatures or short photoperiods, leading us to question the environmental stimuli that trigger bud initiation. Thus, factors that trigger bud initiation in gentians like ‘Spotlight’ remain to be ascertained (Chapters 6 & 7).

In contrast to plants outside in full sun, increased appearance of buds was observed on plants in the protected environment with 20% shade (Figure 3.5B). While it is hypothesised that temperature differences (Figure 3.1) were primarily influencing these differences in bud appearance, the influence of environmental (temperature and day length) and physiological factors (above-ground and below-ground changes in growth) need to be investigated further as potential factors influencing initiation of new crown buds in ‘Spotlight’ (Chapters 6 & 7).

3.5.3 Timing of bud appearance and shoot emergence

Maximum shoot emergence was achieved by October in both seasons. Although the appearance of new buds was distributed from summer through to winter, only buds that appear in the latter part of the period over which new crown buds became visible (i.e. from October to July) emerged as shoots the following growth cycle (Figure 3.6). There was no relationship between the time of appearance of crown buds in the first growth cycle and duration to shoot emergence ($R^2 = 0.002$; $P < 0.0001$) in the second growth cycle. The loss of plants during the latter part of the second growth cycle prevented analysis of potential relationships with the duration to harvest maturity, but in the first growth cycle no correlation was observed between duration to shoot emergence and either the time of observation of floral axillary buds or duration to shoot harvest maturity. This was consistent with the previous findings for 'Spotlight' (Chapter 2). Thus, the hypothesis that the time of appearance of buds and/or their emergence as shoots was correlated with the time of stem harvest maturity is not supported by the current data set.

New buds were typically apparent as clusters of several buds, located close to the current and previous season's shoots or buds (Figure 3.2). Buds that appeared first in a cluster were not first to emerge, nor the greatest in diameter. However, buds with a greater diameter also produced longer shoots and more nodes in both seasons. Buds produced within the cluster in the latter part of the period over which new crown buds became visible (i.e. from October to July) were more likely to emerge as a shoot than early- and late-formed buds. Since the time of bud appearance was not related to the duration to emergence, it is hypothesized that the factors determining which bud emerges from a cluster as a shoot depend on factors other than the time of appearance of the bud within the cluster. It is possible that correlative inhibition between buds enables only a few buds to grow out of the cluster. Therefore, it appears that the physical arrangement and internal coordination of development between buds within the cluster determines which buds from a cluster develop into shoots. Thus, correlative inhibition between buds of gentians and how it relates to productivity warrants further study (Chapters 4 & 9).

Induction, maintenance and breakage of dormancy could be integrated within the process of bud formation (Horvath et al., 2003). Therefore, it is possible that buds which develop into shoots originate only from buds experiencing similar environmental

conditions over a narrow period (i.e. autumn though to early winter; Figure 3.6). To achieve uniformity in duration from date of emergence and date to harvest maturity, buds may require a common number of chill units to break dormancy prior to shoot emergence. In the current study this was apparently fulfilled during the 12 weeks of cold storage prior to the first growth cycle and the natural cold temperature in the outdoor environment in winter (Figure 2.1) prior to the second growth cycle. For the current experiment, this scenario is considered likely because plants within the heated greenhouse did not receive temperatures below 16 °C (Figure 2.1) and did not develop flowering shoots in the second growth cycle. Therefore, the observed spread in time between bud appearance to shoot emergence and harvest maturity may be relatively narrow compared with the observed spread in bud appearance dates. As a result, provided the chill unit requirement is being saturated, the timing of shoot maturity in 'Spotlight' may not be influenced by either the time of bud appearance or shoot emergence; the poor correlation between duration to shoot emergence and duration to harvest maturity was consistent with the previous observations of 'Spotlight' (Chapter 2). Hence, rather than the time of appearance of crown buds, the factors associated with growth and development of a crown bud after its appearance may have a greater influence on the duration to harvest maturity in 'Spotlight'. In order to identify the factors related to the dates of shoot emergence and harvest maturity in 'Spotlight', the physiological and environmental factors regulating bud development, dormancy and flower initiation need further study (Chapters 8 & 9).

3.5.4 Influence of environment on shoot emergence and development

In the current study, shoot emergence following removal of putative endodormancy by chilling (12 weeks, 10 °C) was faster in plants in the warmer environment than plants growing outside, but neither the duration to shoot harvest maturity (Table 3.1), nor the spread of dates when shoots reached harvest maturity (Figure 3.4), were consistently different between treatments. Environmental conditions, primarily higher temperatures (Figure 3.1), are likely to have stimulated rapid shoot emergence and development in the protected environment (16 °C to 24 °C average monthly temperature) compared with outside (8 to 18 °C). The difference in monthly average temperature between the protected and outside environments varied from 4 °C in summer to 12 °C in winter. Although the final shoot length varied greatly, the final number of nodes per shoot was not different between the two environments. Thus the increased shoot length was due to

increased internodal length in the protected environment. Increased shoot length with increased temperatures up to 24°C and increased flower numbers up to 27 °C were observed in *Sandersonia aurantiaca* Hook (Davies et al., 2002). In other studies, increased temperatures during spring caused greater shoot length extension in *Vitis vinifera* than plants grown in cooler temperatures (Keller and Tarara, 2010), and growing in a heated environment reduced the number of days to flowering in *Scadoxus multiflorus* subsp. *Katherinae* (Funnell, 2008). In the present study, however, temperature in the growing environment had no effect on flowering date, percentage of flowering shoots, or number of floral nodes. This was consistent with the observed time to flower harvest of 'Spotlight' in response to growing temperature in a previous study (Chapter 2).

In both environments, at the beginning of the second growing season, buds had similar numbers of primordial structures (Section 3.4.4). The failure of buds to emerge as shoots in the protected environment in the second growth cycle supports the hypothesis of an obligate requirement for cold (eg, 60 to 90 days at 0 °C; (Ohkawa, 1983)) for shoot emergence in 'Spotlight'. During the current experiment, outside-grown plants experienced a progressive reduction in average monthly temperature from 18 to 8 °C (Figure 3.1). In contrast, plants grown in the protected environment were not exposed to temperatures below 16 °C. In most temperate species, the main factor governing breakage of endodormancy in buds is exposure to low temperature to satisfy chilling requirements (Lang et al., 1987). In the current experiment, the survival rates of plants in the second growth cycle were lower (20%) in the protected environment than outside (90%). Hence in order to achieve any benefit of increased growth for 'Spotlight' from a heated protected environment, prior exposure of plants to chilling is required. Further investigations are required with cultivars like 'Spotlight' to confirm and quantify whether true endodormancy occurs in gentians, and to characterise the possible effect of chilling in stimulating a greater level of shoot emergence (Chapter 8).

As reported for other plant species (Remphrey and Davidson, 1994), in the protected environment plants were larger, more new crown buds appeared, and bud diameter was larger. As observed in the first growth cycle, higher bud numbers resulted in higher shoot numbers (Appendix II) and, in both seasons, larger diameter buds resulted in longer shoots at harvest maturity. Thus it can be hypothesized that in 'Spotlight' if more, large-diameter primary-crown buds are initiated in a given season, higher quality

floral shoots can be expected the following season. Therefore, although there was no difference between the growing environments tested in the dates of flower harvest, if chilling requirements can be satisfied, the advantages of longer shoot length and increased initiation of crown buds may make cultivation of gentians in a protected environment attractive commercially.

3.5.5 Conclusions

The types of buds, dates of bud appearance and shoot emergence, and growing temperature did not influence duration to shoot harvest maturity of 'Spotlight'. As only crown buds contributed to floral shoots, shoot productivity could possibly be enhanced by manipulation of growth and development of these preformed buds. Growing plants in a warmer environment resulted in increased shoot length at harvest and a greater number of new crown buds of a larger size. These results indicate the potential of exploring environmental and/or plant-related factors controlling initiation and dormancy (both para and endo) of primary-crown buds to increase crop yields.

Chapter 4 Crown buds; changes in paradormancy during the growing season

4.1 Abstract

Based on previous studies, not all over-wintering crown buds of the gentian cultivar 'Spotlight' emerged during spring, and some (27%) remained dormant throughout the growing season. It was hypothesized that paradormancy by early emerged shoots prevent emergence of the remaining crown buds. So as to study the emergence of crown buds, changes in the population of crown buds were examined following clipping of the current season's shoots at two separate times in two growing environments. Microscopic analysis of sectioned crowns revealed that the crown included individual crown buds or clusters of crown buds which were not macroscopically visible, prior to summer. Irrespective of the date of clipping or growing environment, clipping allowed emergence of all macroscopically-visible crown buds, plus more that were not visible at the time of clipping. It was concluded that crown buds were, therefore, under paradormancy. The shoots which emerged from crown buds following clipping remained in a rosette. In contrast, the shoots that emerged from axillary buds on shoots which had been clipped were elongated and, developed into floral shoots.

4.2 Introduction

Shoot productivity of gentians is determined by crown buds, as only these preformed buds contribute to floral shoot production (Chapter 3). In a single growing season 27% of the crown buds present at the beginning of the season remained without emerging in the gentian cultivar 'Spotlight'. As reviewed in Chapter 3, this incomplete emergence could be due to paradormancy, as observed in other plants (Hall and Hillman, 1975; Horvath, 1998; Lang et al., 1987). Thus in gentians it was hypothesized that, via paradormancy, rapid emergence of some crown buds and subsequent development of shoots at the beginning of the season, inhibited emergence of these other (27%) crown buds.

There is no published work except for one review article that supports the notion that paradormancy exists in gentians, as additional shoots were stimulated to emerge if existing shoots were removed, i.e. clipped, early in the growing season (Ohkawa, 1983). Identification of the types of buds that developed into shoots, fate of the subsequent

shoot development, varietal information, or statistically validated data supporting this claim, were not presented within Ohkawa's review. In the experiment reported here, it was hypothesized that paradormancy limits the number of crown buds emerging as shoots in the cultivar 'Spotlight', and that clipping of shoots would indicate whether crown buds were under some level of paradormancy.

With *Euphorbia esula* L. the quantity of shoots emerging following clipping varied, dependent on the timing of clipping during the growing season (Horvath et al., 2006). This time dependency in the response to clipping has also been suggested to occur in gentians, although statistically validated data were not presented (Ohkawa, 1983). As also applied in the current research, it is hypothesized that this time-dependent response is due to changes in the existence of different types of dormancy (endo, para, eco) separately or concurrently within the plant (Faust et al., 1997). Clipping the shoots at different times during the growing season, therefore, should indicate the different degrees of paradormancy existing in gentians during a single growing season.

Release from paradormancy and subsequent shoot development can be considered as two separate physiological processes (Cline, 1997). During the previous experiments with the cultivar 'Spotlight', a higher temperature within the growing environment did not alter the timing of phenological events during the growing season, but final shoot length was longer (Chapter 3). Hence, if clipping relieves the remaining crown buds from any influence of paradormancy, as found with late emerging shoots of the cultivar 'Starlet' (Chapter 2), growing environment temperature could extend an additional influence on the subsequent development of these later emerging shoots. During the current study therefore, the effect of differences in growing environment temperature on shoot emergence and development after clipping, was monitored.

Primary-crown buds for the current season's growth start to become macroscopically visible in December (Figure 3.2, Chapter 3) of the previous growth cycle. However, it is possible that crown buds are initiated prior to December (summer), but not macroscopically visible at this time. In order to determine what type of crown buds contribute to the population of shoots after clipping, in addition to determining the presence of non-emerged crown buds which were macroscopically visible, it was considered important to also identify whether new crown buds were being initiated before clipping. During the current experiment this was achieved by microscopic examination of samples of the storage stem/root region of the crown.

Prior to the commencement of this experiment the nature of the parental tissue, i.e. stem or root, from which crown buds were initiated, or whether they were axillary or adventitious in type, was not known. As reviewed in Chapter 3, the juvenility and subsequent emergence and development of a bud as a shoot could potentially vary based on the type of bud, i.e. axillary or adventitious (Del Tredici, 2001). It was expected therefore, that microscopic examination of the tissue from where crown buds typically developed, would potentially provide an indication of both the nature of the tissue from which the buds originate from, and their type.

The objective of current experiment was to determine, for the gentian cultivar 'Spotlight', whether unemerged crown buds are under paradormancy during the growing season and, if so, does this change with the season and growing environment temperature.

4.3 Materials and Methods

4.3.1 Plant materials and production

Plants of 'Spotlight' were propagated by tissue culture at The New Zealand Institute for Plant & Food Research Limited, Palmerston North. Plants were deflasked during December 2005 to January 2006 and grown in an unheated greenhouse with natural lighting for 18 months at Palmerston North, NZ (40°37'S 175°60'E). In June 2007, they were placed in continuous darkness in a cool store at 10 ± 1 °C for 12 weeks. Thus at the start of the experiment, the plants had undergone two seasons of growth, and were in cold storage. Plants were repotted prior to treatments being applied, and managed under similar conditions, as per Section 3.3.1.

4.3.2 Treatments

Commencing 19th September 2007, plants were grown in one of two environments, i.e., either outside under ambient conditions at the Plant Growth Unit, Massey University, Palmerston North (40°20'S), or in a greenhouse heated at 15 °C, vented at 25 °C, with 20% shading from structural components (i.e. protected environment). Plants remained in these two growing environments for two growth cycles. As illustrated in Figure 4.1, in both environments plants were either clipped or not (control) on two dates during the first growing cycle. The first date of clipping (clip 1) was 12th December 2007, when in plants within the control treatment maximum shoot emergence had occurred, and

axillary buds had become visible on some early developing shoots; the latter being indicative that flower initiation had commenced (refer Appendix I). The second date of clipping (clip 2) was the 20th February 2008, when flower buds were visible on shoots. As illustrated in Figure 4.1, both unemerged crown buds and some new crown buds were visible on plants within the control treatment at these two dates of clipping. In plants that were clipped, all visible shoots were cut to the height of the growing medium, which typically left on the plant between 0.5 and 1 cm of shoot, i.e. above the point of connection to its point of origin on the crown. In the control treatment, following commercial practice, when shoots reached commercial maturity they were cut leaving between 4 and 6 cm of shoot and foliage attached to the crown (refer to Chapter 3). Towards the end of the period of harvesting floral shoots, up to three entire shoots were left on plants within the control treatment after reaching harvest maturity, and were subsequently clipped at the beginning of winter (15th June 2008).

4.3.3 Macroscopic examination

Before clipping, the number of visible crown buds was recorded (i.e. after exposing the crown by removing the growing medium on the surface (refer Section 3.3.2). Subsequent to clipping, the number of shoots emerging was monitored at weekly intervals till the end of the first growth cycle in September 2008 (Figure 4.1). One representative plant from each treatment, clipped on 12th December 2007, was uprooted after 10 weeks (Figure 4.1) and the presence and origin of both visible crown buds and shoots determined.

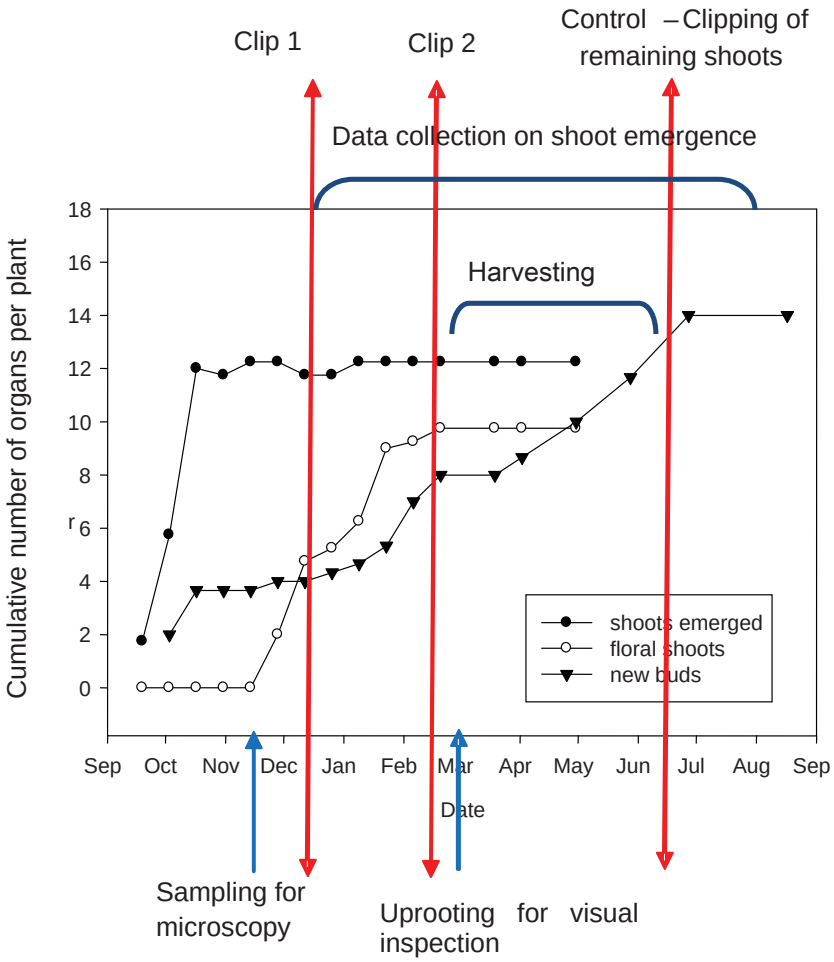


Figure 4.1 Stages of shoot and bud development, and period of harvesting during the first growth cycle of plants of 'Spotlight', with reference to when plants were clipped (Clip 1 - 12th December 2007, Clip 2 - 20th February 2008, or control - 15th June 2008) and samples taken for microscopy and visual inspection. (Adapted from Figure 3.3).

4.3.4 Histological examination

Before the first date of clipping when all shoots had emerged (17th November 2007; Figure 4.1), samples of the stem/root region of the crown which had visible crown buds, were taken for histological examination. samples of fresh tissue were fixed using FAA (formaldehyde: glacial acetic acid: ethanol: distilled water at 2:1:10:7 by volume) overnight at 4°C, and dehydrated using a graded ethanol series (Eaton et al., 2010). Tissues were then prepared for wax infiltration through a graded series of Ethanol: Histoclear™ (National Diagnostics, Atlanta, Georgia, USA). The Histoclear was subsequently gradually replaced with Paraplast X-tra (McCormick Scientific), and embedded in 100% Paraplast X-tra. Depending on the type of tissue, both transverse and longitudinal sections were cut at 8 to 12 µm thickness, using a Leica RM 2145

Rotary Microtome, and mounted onto polysine-coated slides. Transverse and longitudinal sections were taken progressively along the length of crown tissue or bud, so as to identify the vascular connections and buds within crown tissue, and the arrangement of buds within the clusters. Slides were stained with 0.05% toluidine blue for 10 minutes, washed in distilled water, and dehydrated in a graded ethanol series. After the final transfer to 100% fresh ethanol, the Paraplast X-tra was removed from the sample using Histoclear. Slides were mounted with cover slips using Entellen (Merck, Darmstadt, Germany). Morphological features of crown buds and crown tissue were recorded by observing the sections using either a compound light microscope (Zeiss, Germany) or Stereomicroscope (Leica Microsystems, Wetzlar, Germany), with a Leica DFC 320 digital camera attached (Leica Microsystems, Wetzlar, Germany).

4.3.5 Statistical design and data analysis

Each treatment comprised a minimum of three single-plant replicates, with each having, at the time of clipping, between 2 and 4 macroscopically visible buds and between 3 and 12 developed shoots. The experiment was conducted as a completely randomized design (CRD) with a factorial arrangement of treatments, where the main effect variables were growing environment (protected and outside) and clipping date (December, February, control (i.e. non-clipped)). The proportion of shoots emerged were calculated and analysed using the General Linear Model's procedure in the Statistical Analysis System (SAS) software version 9.13 (SAS Institute, Cary, N.C.). Mean comparisons were done using Least Square means (LSMEANS) or Duncan's multiple range test (DNMRT).

4.4 Results

4.4.1 Shoot emergence after clipping

Shoots in control treatments (non-clipped) reached harvest maturity but no new shoots developed during the rest of the growth cycle following the date of first clip (Figure 4.1). By contrast in plants that were clipped, shoots emerged during the following four weeks (Table 4.1 and Figure 4.2A & B). The number of shoots which emerged per plant varied from 3 to 12 in the clipped treatment. Expressed on the basis of the number of crown buds visible at the start of the experiment, the proportion of shoots which emerged following clipping was not significantly different among the two environments, and no interaction between the two main effects was found ($P > 0.05$).

Plants in the clipped and control treatments varied in their percentage shoot emergence ($P < 0.0001$), but not between the two clipping dates ($P = 0.05$). Across both environments, plants clipped in December averaged 222% shoot emergence compared with 187.5% for those clipped in February (Table 4.1).

Table 4.1 Shoot emergence, as a percentage of macroscopically visible buds[Z], from plants of 'Spotlight' grown in two environments 4 weeks following three separate dates of clipping.

	Shoot emergence (%)[Y]	
Date of clipping	Greenhouse	Outside
12/12/07	244 ± 80	200 ± 0
20/02/08	200 ± 100	175 ± 14
Non-clip control	0	0

[Z]note; all visible buds emerged

[Y] expressed as a percentage of the number of crown buds macroscopically visible at the time of clipping. Values are means ± standard error

When uprooted 10 weeks after clipping, irrespective of clipping date and growing environment, no un-emerged crown buds were macroscopically visible in clipped plants, and it was confirmed that all shoots had arisen from the storage stem/root region (Figure 4.2C & D).

Crown buds were present at the start of the experiment, and more progressively appeared during the current growing season in plants from the control treatment. At the time of natural senescence of shoots in early winter, this amounted to 4 ± 0.5 buds per plant in the protected environment and 4 ± 0 outside. These new crown buds remained without emerging however, as additional shoot emergence was not observed during the period of the experiment (Figure 4.1; Table 4.1).

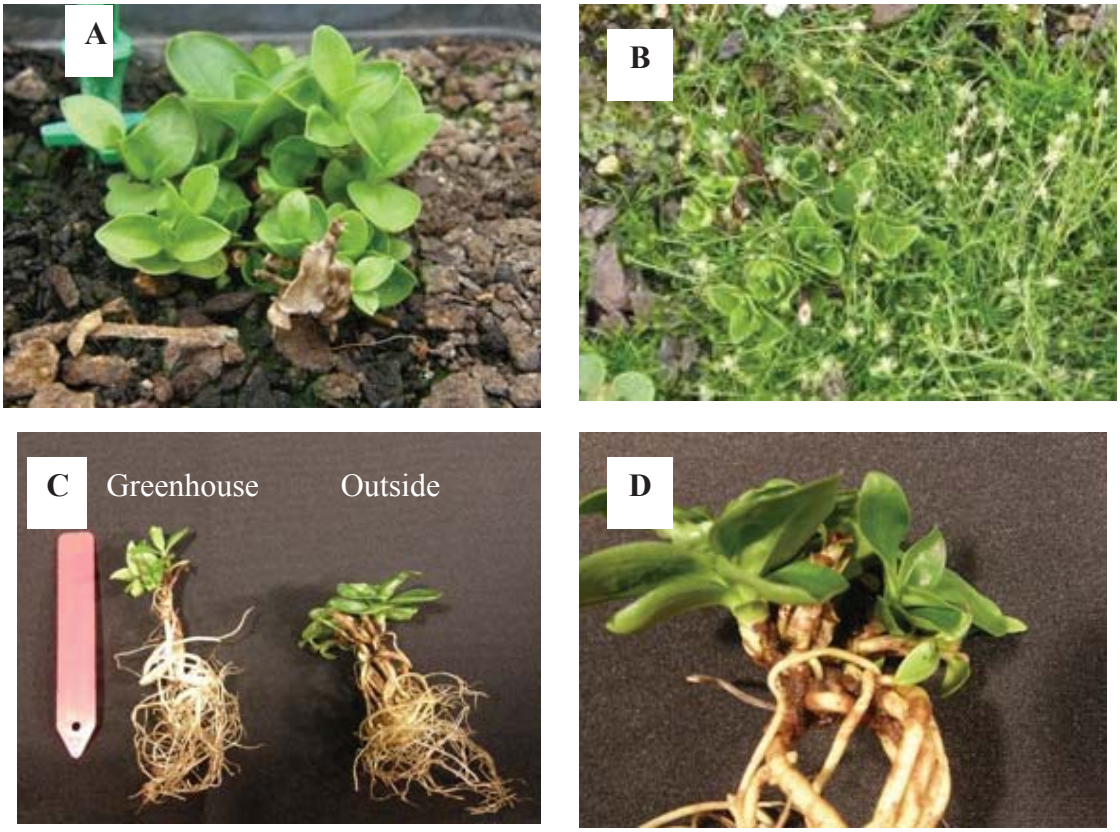


Figure 4.2 Emerged Shoots of plants of 'Spotlight' clipped on 12th December 2007, as observed on 9th January 2008; (A) greenhouse and, (B) outside. (C) Shoot emergence from the crown area of clipped plants from the outside and greenhouse environments. (D) Exposed crown showing no remaining crown buds on an outside grown plant.

Figure 4.3 Two types of shoots which emerged following clipping of plants of 'Spotlight'; (a) axillary shoot from current season's axils and, (b) rosette from crown bud.

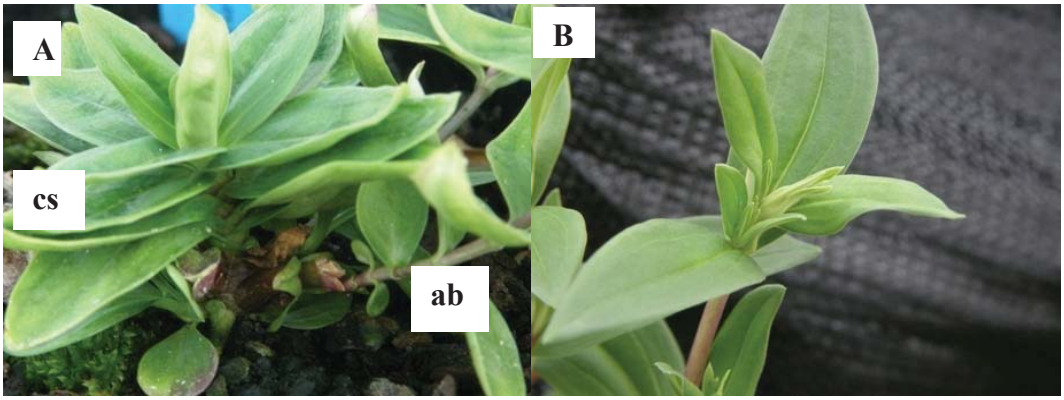


Figure 4.4 Shoot emergence following clipping in plants of 'Spotlight'; (A) shoots directly from the crown as a rosette and as elongated shoots from the axillary buds of current season's floral shoot, (B) flower buds in shoots which emerged from an axillary bud (flower buds visible in March). cs - crown shoot, ab - axillary shoot.

Plants clipped in December (clip 1) also developed axillary shoots from leaf axils at the bases of the stems that were clipped (Figure 4.3). In comparison to the shoots produced directly from the crown after clipping, these axillary shoots showed greater internode elongation (Figure 4.3), and flowered within the current season's growth cycle (June, 2008; Figure 4.4B). In contrast, those shoots which emerged directly from the crown following clipping, remained as a rosette, with no visible internodes, and did not flower during the current season's growth cycle in either growing environment (Figure 4.4A).

When shoots of plants in the control treatment were clipped in winter (15th June 2008), following natural end-of-season senescence of shoots, the crown buds did not emerge as shoots during the current season's growth cycle in either the protected environment or outside. For plants which remained in the outside growing environment over winter, crown buds within the control treatment emerged in the following spring and progressed to flower during this second growth cycle. In contrast however, crown buds on plants which remained in the protected environment, did not flower in the next growth cycle, and remained as rosettes.

4.4.2 Histology of crown region with buds

While when sampled prior to clipping, crown buds were visible macroscopically on sections of the storage stem/root region, microscopic examination allowed numerous additional clusters of buds to be seen, i.e. crown bud clusters (Figure 4.6A). While each individual cluster of buds arose from the storage stem/root tissue without any apparent phyllotaxy, within a cluster more organisation was apparent. Each individual crown bud was enveloped by scale leaves and, similarly, clusters of crown buds were enveloped by

additional scale leaves (Figure 4.5B-C & Figure 4.6B). In some instances vascular connections between the crown bud and the centre of the crown tissue, from where it arose, could be determined (Figure 4.5C). The exact identity of the type of tissue that bud clusters were derived from, i.e. stem or root, was not clear from this series of sections, since the vascular tissues could not be located (Figure 4.6C & D).

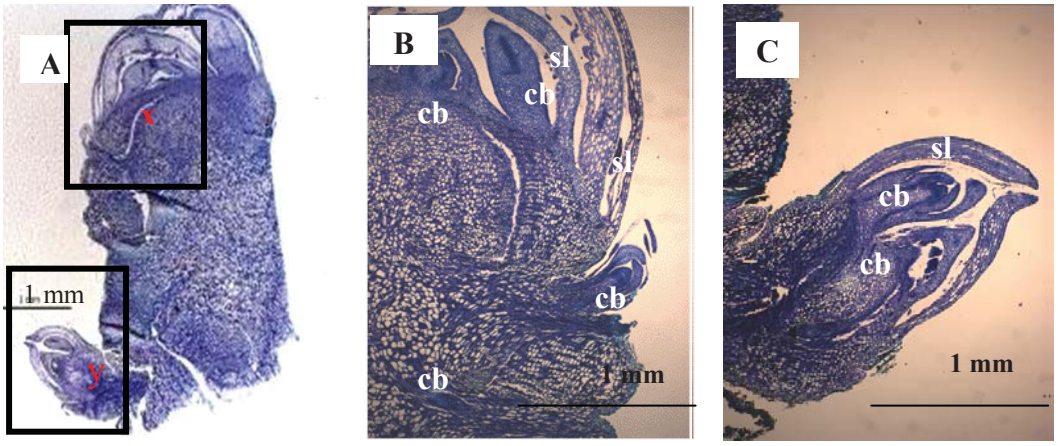


Figure 4.5 Longitudinal sections of the crown of 'Spotlight' prior to applying clipping treatments; (A) Emergence of buds from different positions ('x' and 'y') on a segment of crown; (B) Region 'x' of image (A) at higher magnification, presenting three crown buds, with two covered with a single scale leaf; (C) Region 'y' at higher magnification, presenting buds arising as a cluster. cb - crown bud, sl - scale leaf. Scale bar = 1 mm.

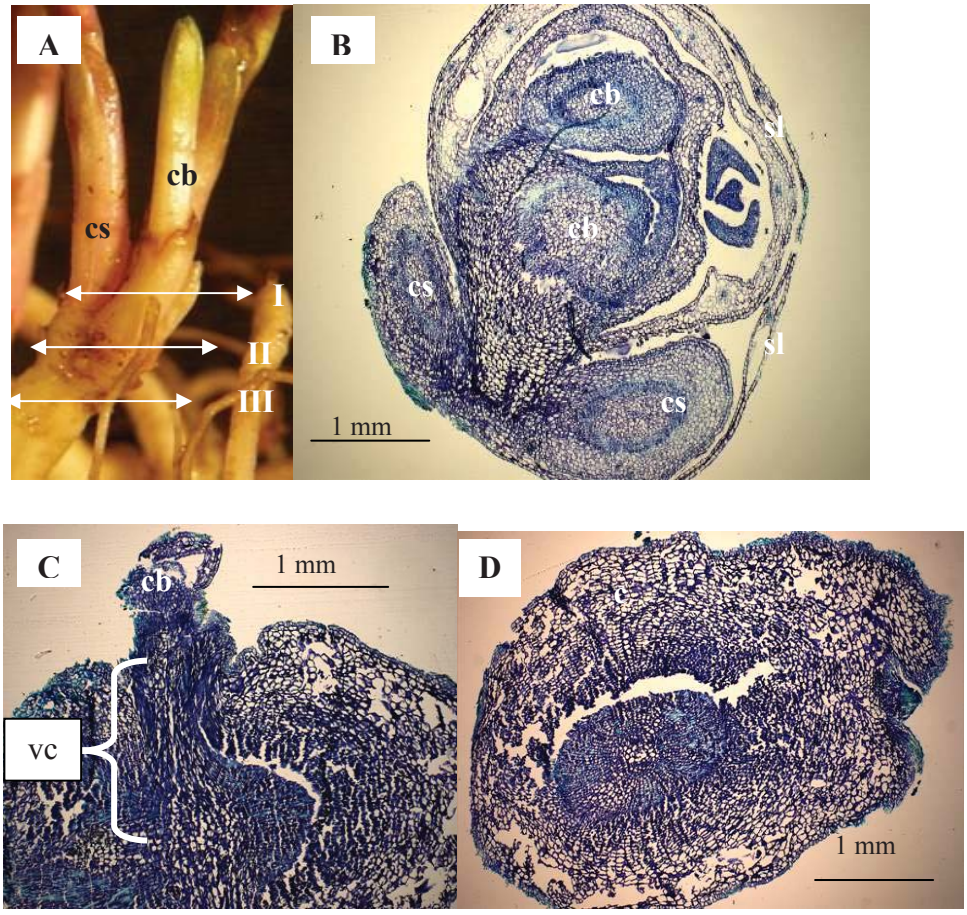


Figure 4.6 (A) Macroscopic appearance of crown region of 'Spotlight' prior to applying clipping treatments; (B) transverse section at position I in image (A), immediately above the visible crown bud, (C) transverse section at position II, at the level of the bud where attachment to the main stem is visible, (D) transverse section at position III, below the crown bud. c - cortex, cb - crown bud, cs - shoot developed from the crown, sl - scale leaf, vc - vascular connection. Scale bars = 1 mm.

4.5 Discussion

Similar to previous observations reported in a review article (Ohkawa, 1983), clipping of shoots facilitated emergence of crown buds. In contrast, plants within the control treatment comprised the normal season's flowering stems and crown buds, but did not comprise any new shoots. It can therefore be concluded that the crown buds which remained without emerging, before clipping, were under paradormancy imposed by the earlier emerging shoots.

The number of shoots that emerged following clipping was double the number of unemerged crown buds observed prior to clipping (Table 4.1). Histological examinations illustrated that crown buds were present in clusters, with scale leaves enveloping several buds (Figure 4.5A-C, Figure 4.6 B), while being visible as a single

bud macroscopically (Figure 4.6A). Hence at any point in time of observation, a single visible crown bud may subsequently give rise to several buds/shoots. This indicates that at the time of clipping the crown carried approximately 27% unemerged crown buds (refer Chapter 3), with the potential to emerge as shoots within the current season, and new crown buds initiated for the next season (i.e. new buds macroscopically visible before clipping and microscopically visible new individual buds or clusters). It is hypothesised therefore, that the shoots that emerged after clipping were derived from previously unemerged buds as well as both macroscopically and microscopically visible new buds destined to emerge in the next season. Clipping is, therefore, an effective technique to release crown buds from paradormancy.

The shoots which emerged from crown buds following clipping remained in a rosette (Figure 4.4A). In contrast, shoots which emerged from axillary buds on shoots which had been clipped were elongated and, developed into floral shoots (Figure 4.4B). It is hypothesized therefore, that the shoots which developed from crown buds are juvenile compared to axillary buds on shoots. Alternatively, floral shoot elongation and development may require flower induction to occur first, as with *Arabidopsis* (Weigel and Nilsson, 1995). If so, it is concluded that the signal (e.g. low temperature or day length) for flower induction (Bernier, 1988) was either not received or the meristem of the crown buds which emerged after clipping were unable to respond to the floral inductive signal. In order to determine the environmental requirements for shoot elongation and floral induction, and to identify any differential response based on growth stage of the plant, further investigations would be required with plants comprising buds at different developmental stages under varying environmental regimes (refer Chapters 8 & 9).

In the present experiment, clipping resulted in emergence of crown buds, when it was conducted in summer. When all shoots of plants within the control treatment were clipped in winter, after the natural end-of-season senescence of shoots, crown buds did not emerge in either the protected environment or outside. As reported previously, only plants within the control treatment grown outside developed into floral shoots during spring of the next season of growth, the few shoots that emerged on plants grown in the greenhouse remained as a rosette, while the majority of the buds remained unemerged (Chapter 3). This rosette formation and non-emergence of crown buds in the absence of exposure to temperatures below 15 $^{\circ}$C, is indicative of the fact that true endodormancy

and/or vernalization does occur in the cultivar 'Spotlight'. In *E. esula,* shoots clipped later in the season (presumably autumn or winter), did not develop any floral shoots, but flowered when endodormancy was broken by cold (Horvath et al., 2006). Similarly in the current experiment, development of floral shoots was observed in plants exposed to cold temperature outside, indicating the importance of prior chilling for shoot emergence and development in gentians. Although not significant ($P > 0.05$), the proportion of shoots emerging reduced when plants were clipped two months later, and no shoots emerged after all shoots were finally removed in control treatments in winter, in either environment. (Ohkawa, 1983) also reported that when shoots were clipped in gentians before flower initiation 100% shoot emergence occurred (presumably in summer), but few shoots emerged when clipped after flower initiation. Similar reports with other species describing changes in shoot emergence following clipping at different times are evident, i.e. greater shoot emergence when clipped in summer as compared with spring or autumn/winter, such as in *Pityopsis graminifolia* (Michx.) Nutt (Brewer and Platt, 1994), *Gentianella campestris* (L) Borner (Lennartsson et al., 1998), and *Rorippa palustris* Greene (Sosnová and Klimesová, 2009). Studies applying successive dates of clipping to plants of *E. esula*, lead to the conclusion that a progressive reduction in the number of shoots emerging later in the season was due to the development of endodormancy (Anderson et al., 2005). As evident in other species, both forms of dormancy (para and endo) can co-exist during some periods of the growth cycle (Faust et al., 1997). Similarly therefore, it is hypothesized that crown buds in gentians are under paradormancy after initiation, but develop endodormancy later in the season.

Although with 'Spotlight' the majority of new primary-crown buds became macroscopically visible on the crown from December, and continued to increase in number over a 8-month period (Figure 4.1 and Chapter 3), histological data from the present study showed bud clusters were being initiated earlier in the growing season (i.e. November). The physical sites of initiation of crown buds did not show any organised phyllotaxy relative to the storage stem/root tissue, which supports the hypothesis that these crown buds are adventitious. In contrast however, the presence of many buds covered with a single scale leaf, indicates that these buds developed as axillary buds, i.e. collectively forming a crown bud cluster.

Analysis of longitudinal and transverse sections of crowns in the current experiment, did not enable determination of the nature of the tissue that bud clusters were derived from (i.e. stem, root, or transitional between these two). So as to better determine the origin and type of crown buds, future investigations need to assess the arrangement of buds within the cluster, and their progressive development. To achieve this in future experiments, crown bud clusters and the connective tissues could be sectioned at different stages of development (refer Chapter 5).

Clipping has the potential to promote emergence of all crown buds in a given season, by eliminating the paradormancy imposed by early emerged shoots. During the previous study (Chapter 3) it was hypothesised that any cultural practises occurring from summer through to winter, that disturb the crown, could adversely influence bud initiation. Histological examination of storage stem/root tissue during the current study however, confirmed that the initiation of new crown buds commenced earlier in the season (i.e. prior to December; summer), while not being visible macroscopically. Any disturbance to the shoots or crown therefore, could influence the initiation of buds for next season even prior to summer (i.e. spring). As evident with other species, this could potentially arise due to reduced resources for bud initiation. In *Acomastylis rossii* Greene, carbohydrate allocation from mature leaves to preformed buds, and the rhizome, occurred starting from summer of the previous growth cycle (Meloche and Diggle, 2003). Reduction of reserves in the lignotuber (storage organ) that supports regrowth, and reduced plant survival, was observed in *Arbutus unedo* L., *Erica arborea* L. (Canadell and Lopez-Soria, 1998), and in *Macadamia integrifolia* Maiden & Betche (McFadyen et al., 2011) following clipping. Within the current experiment, clipping during the current growth season therefore, could also deplete the resource availability for development of buds, and subsequent shoot growth for the next growth season. As a further consequence of the loss of paradormancy, the activation of growth of preformed crown buds which have not yet developed endodormancy, could expose these shoots to adverse conditions in winter. The consequences of shoot removal during the season, on quality of the floral shoots produced, and next season's growth and productivity, therefore remain to be investigated.

4.5.1 Conclusion

New crown buds were initiated as clusters on the crown during spring, and became visible from summer. By releasing buds from paradormancy imposed by growing shoots, clipping facilitated emergence of both crown buds which had not emerged during the current season, and newly initiated buds destined for the next growing season. The number of shoots emerging following clipping was not affected by the growing environment, but was influenced by the time of shoot removal. This change was presumed to be due to the type (endo or para) and/or degree of dormancy existing in the plant.

Chapter 5 Anatomy, ontogeny and origin of crown bud clusters of gentians

5.1 Abstract

Shoot productivity and overwintering survival of gentians, is determined by growth and development arising from crown buds. During vegetative propagation some cultivated selections fail to produce an adequate number of crown buds following planting, and do not survive through to the next growth cycle. Understanding of morphological features of crown buds and the associated development in gentians is required if manipulation of bud initiation, emergence and development is to be achieved. Whether bud clusters originate from axillary or adventitious meristems has not previously been published. In the current study, therefore, histological features of the shoot/stem, storage root, transition zone between shoot and root, axillary buds, and crown bud clusters, were examined in both seedlings and vegetatively propagated plants. Typical histological features of shoot, root and axillary buds were identified and described using both light and confocal microscopy. The transition zone was the predominant area for initiation of crown buds, which was functionally similar but morphologically different within seedlings and plants propagated by tissue culture. The initial of a bud cluster presented characteristics typical of adventitious buds, in terms of their origin and presence of external vascular connection to the parental tissue. In contrast, crown buds forming subsequently within the cluster developed as axillary buds at the base of the initial adventitious bud, collectively forming on a compact stem. Stem elongation within the cluster following application of gibberellic acid (GA_3) enabled identification of a hierarchical arrangement of buds within the cluster, with one bud at each node. It also enabled confirmation that with the exception of the initial bud, all crown buds within the cluster were axillary in type.

5.2 Introduction

Productivity of gentians is associated with the growth and development of crown buds (Chapter 3), hence strategies to manipulate initiation, emergence and subsequent development of these buds are of importance to the industry. Further to that, the timely initiation of an adequate number of crown buds on plants is perceived as a limiting factor during vegetative propagation in commercial nurseries (Ed Morgan, pers. com;

Takashi Hikage, pers com.). This factor has led to delays in starting commercial production and the inability of plants to survive into the next growth cycle. Crown buds arise on tissue within the storage stem/root region of the crown as separate units comprising several associated buds, with one such unit comprising two or more buds referred to as a bud cluster (Chapter 3 and 4). An understanding of morphological features associated with the origins of crown buds and bud clusters, and plant ontogeny, is required to develop techniques for manipulation of bud initiation, emergence and development. Prior to the current research, there have been no previous reports on these matters in gentians.

In other perennial plant species emergence of new shoots requires three conditions; a perennial root system, carbohydrates/nutrients for new growth, and surviving meristem(s) or bud(s) (Vesk and Westoby, 2004). In gentians, the presence of a perennial crown appears to fulfil the first two conditions and, the third condition requires understanding of the initiation and subsequent ontogeny of the crown buds. Other than the pre-existing growth arising from above-ground shoots, studies related to woody-plants of temperate origin have categorised subsequent shoot emergence based on their bud origin as: stem-collar, underground stem (storage), root or shoot sprouts (Del Tredici, 2001). Based on preliminary investigations with gentians, bud clusters were produced from different positions on the crown (Chapter 4), but whether the tissue was specifically shoot/stem, root or the transitional zone between these two (transition zone), was not determined.

In terms of types of buds, axillary buds are typically of exogenous origin, whereas adventitious buds develop from endogenous (tissues deep within the parental axis) or exogenous tissue (relatively superficial tissues) of any plant organ (Evert, 2006). As adventitious shoots are more juvenile compared to axillary shoots (Del Tredici, 2001; Vesk and Westoby, 2004), even if shoots of these two types emerged at the same time, they are likely to develop differently, leading to variation in times at which shoots reach anthesis. Whether crown buds in gentians are axillary or adventitious in type has not been reported, but following the removal of correlative inhibition via clipping, shoots that emerged from crown buds were juvenile compared to those from axillary buds of floral shoots (Chapter 4). In order to control initiation of crown buds and investigate their subsequent timing of emergence and development as shoots, determination of whether crown buds are axillary or adventitious is first required.

Crown buds of 'Spotlight' developed as multiple buds in a cluster (Chapter 4), as also found in other species such as in the *Myrtaceae* (Burrows et al., 2008) and *Cucurbitaceae* (Gerrath et al., 2008). In gentians 27% of crown buds did not emerge within a growth cycle (Chapter 3), and were found to be under correlative inhibition (Chapter 4). It was reasoned that developing an understanding of the arrangement order of buds within the cluster might provide some insight into why some buds develop into shoots, while others stay dormant within the cluster during the growth cycle. Buds within the cluster could be axillary or adventitious and, if axillary, paradormancy could exist within the buds of the cluster. In order to identify the growth potential of crown buds within the cluster therefore, a prior understanding of whether buds within the cluster are adventitious or axillary was required.

Across the three experiments presented within this chapter, the aim was to identify the anatomy and morphology of both bud clusters and the buds within these clusters. Specific objectives were to determine for gentians the;

1. basic anatomical features of shoot/stem, root and transition zone,
2. origin, initiation and development of crown bud clusters,
3. origin, arrangement, initiation and development of crown buds within the cluster.

In commercial cultivation, gentians are frequently propagated vegetatively via tissue culture. Given the comparatively artificial nature of these propagules, it was recognised that increased understanding of the above aim and objectives might be achieved by comparing the origin of crown buds within seedling material with that from tissue culture. Histological studies have identified the origin and development of buds on seedlings of herbaceous perennials like *Euphorbia esula* L. (Myers et al., 1964), *Cardopatum corymbosum* L. (Chiatante et al., 2008) and also in many woody perennials like *Quercus* species (Pascual et al., 2002), and species within the *Myrtaceae* (Burrows, 2000). By tracing the vascular connection of the buds to the tissue of origin, and the morphological identity of this tissue (i.e. shoot/stem, root or transition zone), in these studies on other perennials an understanding was achieved of the type of buds present. Given the aim of the current chapter, a similar histological approach seemed worthy of application to gentians. To provide histological data for subsequent comparison to that focussed on crown buds, Experiment One detailed the anatomy of the shoot and associated axillary buds, of floral shoots and roots of both mature plants and seedlings.

Hence, specific objectives for Experiment One were to describe for gentians, the histological features of;

1. shoot/stem, root and axillary buds in mature plants,
2. the ontogeny of development of the transition zone in seedlings,
3. subsequent crown bud formation in relation to parental tissue in seedlings.

In Experiment Two, by exclusively utilising newly established plants derived from tissue culture, the specific objectives were to;

1. identify the origin of crown bud clusters, i.e. whether from the root, shoot, or transition zone,
2. identify whether bud clusters are axillary or adventitious in origin,
3. describe the ontogeny of development of the transition zone (i.e. site for bud initiation),
4. identify whether buds within crown bud clusters are axillary and/or adventitious.

In Experiment Two the physical compactness of crown bud clusters made it difficult to identify the type of buds within a cluster, and their hierarchical arrangement. So as to resolve these areas of uncertainty, in Experiment Three plants with elongated crown tissue, following application of GA_3, were utilised. It was expected that GA_3 would enable internode elongation (Atwell et al., 1999) to produce an elongated stem of the cluster, which would be compressed under natural conditions. The specific objectives of Experiment Three were to;

1. confirm whether buds within the clusters were axillary or adventitious and,
2. identify the hierarchical arrangement within the cluster.

5.3 Materials and methods

5.3.1 Management of planting material and sampling

5.3.1.1 Experiment 1; Basic anatomical features of shoot/stem, root and transition zone

Plants of the gentian cultivar 'Spotlight' were propagated vegetatively as described in Section 3.3.1. At the start of the experiment, these plants had undergone two growth seasons and, therefore, were considered to be mature. Seedlings of both *Gentiana lutea* L. and *Gentiana straminea* 'Maxim' grown in an unheated greenhouse at The New Zealand Institute for Plant & Food Research (Palmerston North) were supplied at the end of their first growth cycle (11th June 2008). Seedlings of *G. triflora* and *G. scabra*, were grown in an unheated greenhouse at the Hachimantai City Floricultural Research Station (Hachimantai, Japan 39°92´N) and were assessed in their first and second growth cycles.

The mature plants of 'Spotlight' were potted into plastic pots (5 L) and the seedlings into black polythene bags or pots (1.7 L), using the growing medium described in Section 3.3.1. Plants were grown in a heated greenhouse (heated at 15 ºC, ventilated at 20 ºC) and irrigation was delivered to the mature plants by microtubes to each pot, on a drained capillary bench, for 10 minutes between three and five times a day, depending upon plant demand. Seedling *G. lutea* and *G. straminea* were grown under a capillary irrigation system using drippers which supplied between 50 and 60 ml of water per plant per day supplemented with one overhead watering per week. Seedlings of *G. triflora* and *G. scabra* in Japan were grown in black polythene bags (1.7 L) using a standard potting media, with irrigation supplied via overhead watering, at a frequency depending upon plant demand.

Three representative samples were collected for macroscopic observation or histology of each plant source at different stages of growth. For the mature plants of 'Spotlight', samples were collected in late spring (November 2007) for histological examination (refer Section 5.3.2) of floral shoots and storage roots (primary and secondary). For the seedlings of *G. lutea* and *G. straminea*, whole plants were sampled for macroscopic observation at three month intervals from acquisition until the end of the third growth cycle. For microscopy, leaves were removed and crown tissue between the shoots and roots of seedlings of *G. lutea* and *G. straminea* sampled in late autumn (May 2009).

Whole plant samples from seedlings of *G. triflora* and *G. scabra*, which were either in their first or second growth cycle, were lifted and used for macroscopic observation at the end of summer (August 2010).

5.3.1.2 Experiment 2; Crown bud clusters - Origin and ontogeny

Plants of the gentian cultivar 'Diva' were propagated by tissue culture and grown as described in Section 3.3.1, with plants potted into black polythene bags (1.7 L) on 21st May 2008, using the growing medium and irrigation schedule as described in Section 5.3.1.1.

Over a period of three growth cycles, three representative samples of crown tissue and bud clusters were taken at different developmental stages based on duration after deflasking and size of the bud clusters. Sampling for microscopy occurred from the time of deflasking, through to when bud clusters were visible on the plant. For direct macroscopic observation, plants were sampled at a time interval of three months, until the third growth cycle.

5.3.1.3 Experiment 3; Hierarchical arrangement of crown buds

Plants of genotype '03/04-114' were propagated by tissue culture at The New Zealand Institute for Plant & Food Research (Palmerston North), deflasked and grown as described in Section 5.3.1.2, and subsequently grown outside for the first growth cycle. Plants with non-emerged crown buds were selected and re-potted (13th October 2008) into black polythene bags (3.4 L), using the growing medium described in Section 5.3.1.1. For their second growth cycle, plants were grown on a drained capillary bench in a heated greenhouse (heated to 15 ºC, vented at 20 ºC). During this second growth cycle, when new shoots emerged following removal of the existing shoots (i.e. clipping; 19th December 2008), either 100 mg l^{-1} gibberellic acid (GA_3, OlChemIm Ltd., Czech Republic) or water was applied onto the remaining crown buds, shoots and exposed crown, as a spray to run-off (≈ 50 ml per plant; 21st January 2009). By the end of this second growth cycle (July, 2009), plants treated with GA_3 developed elongated stems within the clusters, resulting in a physical separation of individual buds.

There were eight replicate plants per treatment, each producing between 1 and 5 bud clusters per plant. Five individual replicate samples each of the elongated stem with crown buds from GA_3-treated plants, or non-elongated stem with bud clusters from

control-treated plants, were taken for dissection and confocal microscopy in October 2009. Samples of floral shoots and storage root were also collected.

5.3.2 Histology - Light Microscope

Within Experiment One and Two, the samples of fresh tissue were fixed using FAA (formaldehyde: glacial acetic acid: ethanol: distilled water at 2:1:10:7 by volume) overnight at 4°C, and dehydrated using a graded ethanol series (Eaton et al., 2010). Tissues were then prepared for wax infiltration through a graded series of Ethanol: Histoclear™ (National Diagnostics, Atlanta, Georgia, USA). The Histoclear was subsequently gradually replaced with Paraplast X-tra (McCormick Scientific), and embedded in 100% Paraplast X-tra. Depending on the type of tissue, both transverse and longitudinal sections were cut at 8 –12 μm thickness, using a Leica RM 2145 Rotary Microtome, and mounted onto polysine-coated slides. Transverse and longitudinal sections were taken progressively along the length of crown tissue or buds, so as to identify the vascular connections and buds within crown tissue, and the arrangement of buds within the clusters. Slides were stained with 0.05% toluidine blue for 10 minutes, washed in distilled water, and dehydrated in a graded ethanol series. After the final transfer to 100% fresh ethanol, the Paraplast X-tra was removed from the sample using Histoclear. Slides were mounted with cover slips using Entellen (Merck, Darmstadt, Germany).

Morphological features of crown buds and crown tissue were recorded by observing mounted sections using either a compound light microscope (Zeiss, Germany) or Stereo-microscope (Leica Microsystems, Wetzlar, Germany), with a Leica DFC 320 digital camera attached (Leica Microsystems, Wetzlar, Germany). Based on observations made, the developmental sequence of crown tissue and bud clusters was identified. The numerical value for the number of individual buds present, within a single crown bud cluster, was used to identify the progressive stages of the development of buds within the cluster (i.e. Stages 1 to 4).

5.3.3 Histology - Confocal Microscope

Fresh samples were either sectioned by hand or using a Leica Jung CM 1800 Cryotome (Leica Microsystems, Wetzlar, Germany), to 20-30 μm thickness. In Experiment Three, for plants treated with GA_3, sectioning was undertaken at two positions along the elongated stem, i.e. distal and proximal end of the tissue carrying bud clusters (i.e.

cluster stem). In control treatments, sections were taken at the proximal end of the cluster stem. Sections sampled from the proximal end of floral shoots and roots were also taken from plants within the control treatment.

Fresh tissue sections were washed with PBS buffer and 2% Tween 20 for 10 min, stained with 0.01% Acridine orange (AO) for 1 h at room temperature, and washed with PBS (Yang et al., 2007). Slides were observed under the confocal microscope (Leica Microsystems, Wetzlar, Germany), with lignified cell walls giving autofluorescence and non-lignified walls giving fluorescence stained with AO.

5.3.4 Macroscopic observations

In Experiment One, the presence of leaves or leaf traces within the transition zone, and buds or shoots, were observed on the seedlings *G. lutea*, *G. straminea*, *G. triflora* and *G. scabra*. Based on their location of development, buds and shoots were further classified as crown buds arising from the transition zone or axillary buds from axils of leaves.

In Experiment Two, the presence of buds, shoots and bud clusters were observed relative to their location of development on plants of 'Diva'. Bud clusters were dissected by first removing the largest bud, and progressively the successive scale leaves and adjacent buds, so as to reveal the buds present in the center of the cluster. Based on the location of crown buds and scale leaves on the cluster stem, the order of arrangement/formation of buds within the clusters was determined. Buds were numbered incrementally, based on the relative size and distance from the apex of the cluster stem.

In Experiment Three, in order to identify the hierarchy of development of crown buds, the position of each bud within an elongated bud cluster was mapped in plants remaining in the greenhouse. As used in Experiment Two, this methodology utilized the location of crown buds and scale leaves on the shoot. Once emerged, shoots were numbered and marked based on the earliest emerged to the latest, as at 14/12/09 (date that maximum shoot emergence was observed). To determine differences in the rate of development of emerged shoots within the cluster, the length of individual shoots at each position was recorded four weeks after the date of final shoot emergence.

5.4 Results and discussion

5.4.1 Experiment 1; Basic anatomical and morphological features of shoot, root, axillary buds and transition zone

5.4.1.1 Histology of floral shoots and storage roots

Transverse sections of floral shoots of 'Spotlight' clearly showed the vascular tissue arranged in a circular ring with pith at the centre (Figure 5.1A & B), as also evident in shoots/stems of other dicotyledonous plants (Bowes, 1996; Evert, 2006). Based on the light-blue staining and the shape of these cells, xylem vessels were identified within the ring, with the tissue external to the xylem vessels presumed to be cambium and phloem (Figure 5.1C). The presence of the cortex and epidermis was visible external to the vascular ring (Figure 5.1A & B). In contrast, within transverse sections of both primary and secondary storage roots, vascular tissue was not evident as a ring, but fully occupied the centre of the root as a circle (Figure 5.1D & E), as reported in other dicotyledonous plants (Clegg and Cox, 1978; Evert, 2006). In cross sectional view, the primary storage roots had well-defined xylem rays, compared to secondary storage roots (Figure 5.1F & G), and was similar to that reported as occurring in roots of both *Arabidopsis* and woody perennial species (Chaffey et al., 2002). Hence floral shoots and storage roots of gentians can readily be distinguished by typical features revealed by histological examination.

5.4.1.2 Histology of axillary buds on floral shoots

In the transverse sections through the node and axillary buds on floral shoots (Figure 5.2A-G), vascular bundles were arranged as a ring on the shoot/stem (Figure 5.2B) and, with sequential acropetal sections, the vascular bundles associated with the buds progressively became evident as being separate, and external to the central stele (Figure 5.2C-G). Vascular tissue for the leaves and buds was directly connected to the vascular ring of the shoot, causing a distortion on the vascular ring at the node (Figure 5.2C-E) as described in other species (Evert, 2006). As evident by the presence of sepal and petal primordia (Figure 5.2H-I), at the time of sampling floral initiation had commenced within the axillary buds of the shoot.

In the absence of any previously published histological studies on the morphology of gentians, the present study (Experiment One) enabled establishment of a baseline for histological features of a typical shoot/stem, root, and axillary buds. These typical

features can, therefore, now be used for contrasts in subsequent discussion involving determining the origin and development of both crown bud clusters and buds within clusters.

5.4.1.3 Transition zone/crown tissue in seedlings

5.4.1.3.1 Macroscopic features of transition zone

At the time of first sampling, the above-ground component of seedlings of *G. straminea* consisted of a rosette of leaves (Figure 5.3A). Based on the fact that none of the seedlings had leaf scars or intact leaves on the stem-like structure below the rosette, this compressed and thickened structure in-between the root and the rosette (Figure 5.3A, B & D) was tentatively identified as the transition zone, i.e. region between the true stem and root (refer Section 5.4.1.3.2 for histological confirmation). Towards the end of the second and third growth cycle, many crown buds were visible in the transition zone, as both individual buds and clusters (Figure 5.3B, C, E & F). During the course of this study, some of these crown buds developed into shoots (Figure 5.3E). Although crown buds arising from the transition zone were the primary source of buds evident, axillary buds were visible occasionally within the axils of the earliest formed leaves, and were different to the crown buds arising from the transition zone in both size and shape (Figure 5.3C).

Longitudinal sections along the shoot/stem axis of intact seedlings of *G. straminea*, permitted the rosette, transition zone, and primary root, to be visible (Figure 5.3G & H) macroscopically. As described in the following section, the region indicated by an 'x' within Figure 5.3H was used for further microscopy. In terms of the visual appearance of macroscopic features, such as the transition zone and presence of crown buds, seedlings of *G. lutea*, *G. triflora* and *G. scabra* (Appendix III) were similar to that noted for the seedlings of *G. straminea* (Figure 5.3A-G). One additional feature noted with plants of *G. scabra* and *G. triflora* however, was that they also developed crown buds on the proximal region of storage roots (Appendix III).

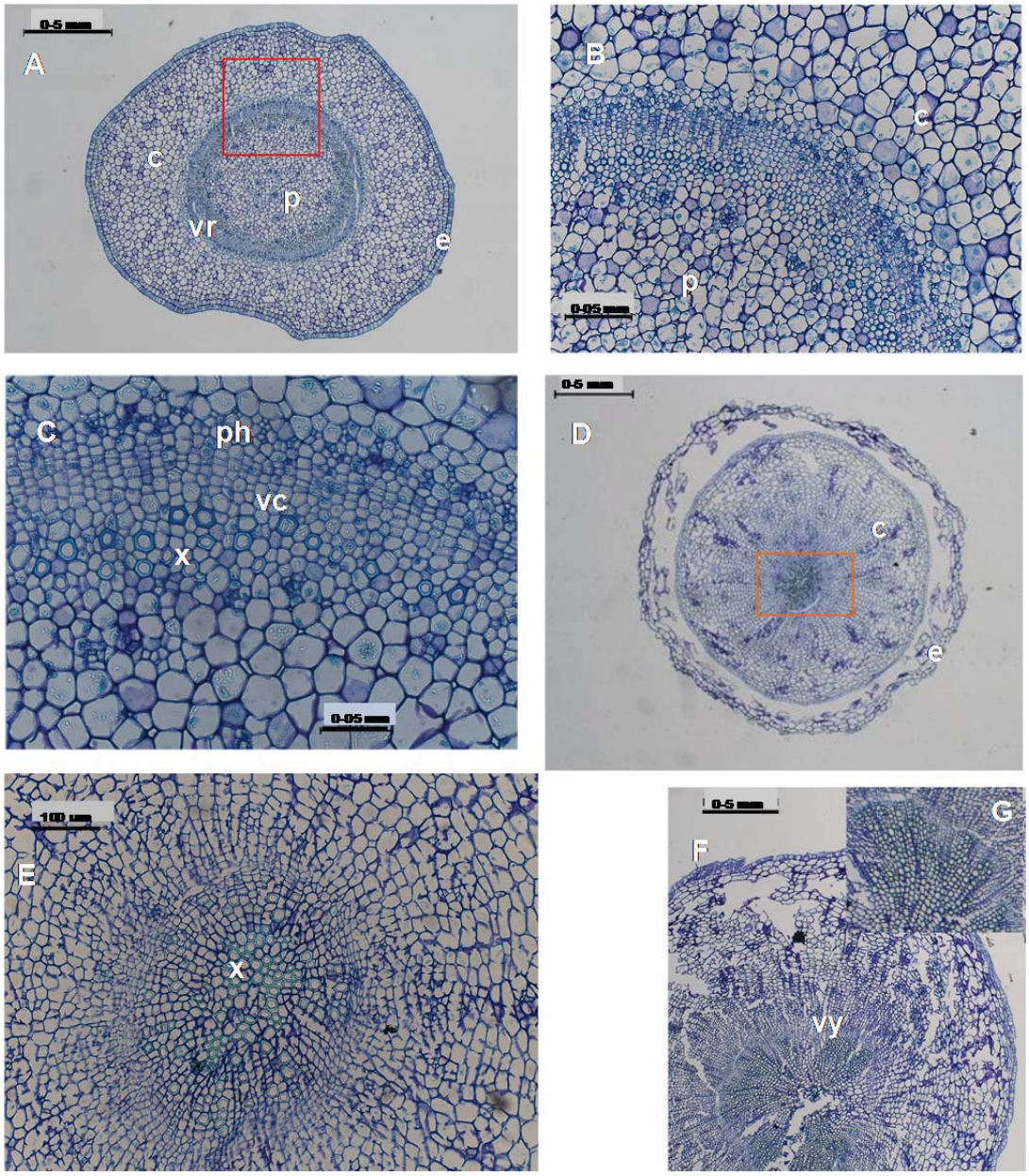


Figure 5.1 Transverse section of (A-C) floral shoot and (D-F) storage root of 'Spotlight' at increasing magnification; (A) full section indicating vascular ring, (B) magnified view of the vascular ring (red coloured square in Figure 5.1A), (C) arrangement of xylem vessels and phloem fibers, (D) full section of secondary storage root, (E) magnified view of secondary storage root indicating xylem of vascular tissues in the center, (F) full section of a primary storage root. (G) Radial arrangement of xylem vessels. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels: c – cortex, e – epidermis, p – pith, ph – phloem, vc - vascular cambium, vr - vascular ring, vy – vascular rays, x – xylem.

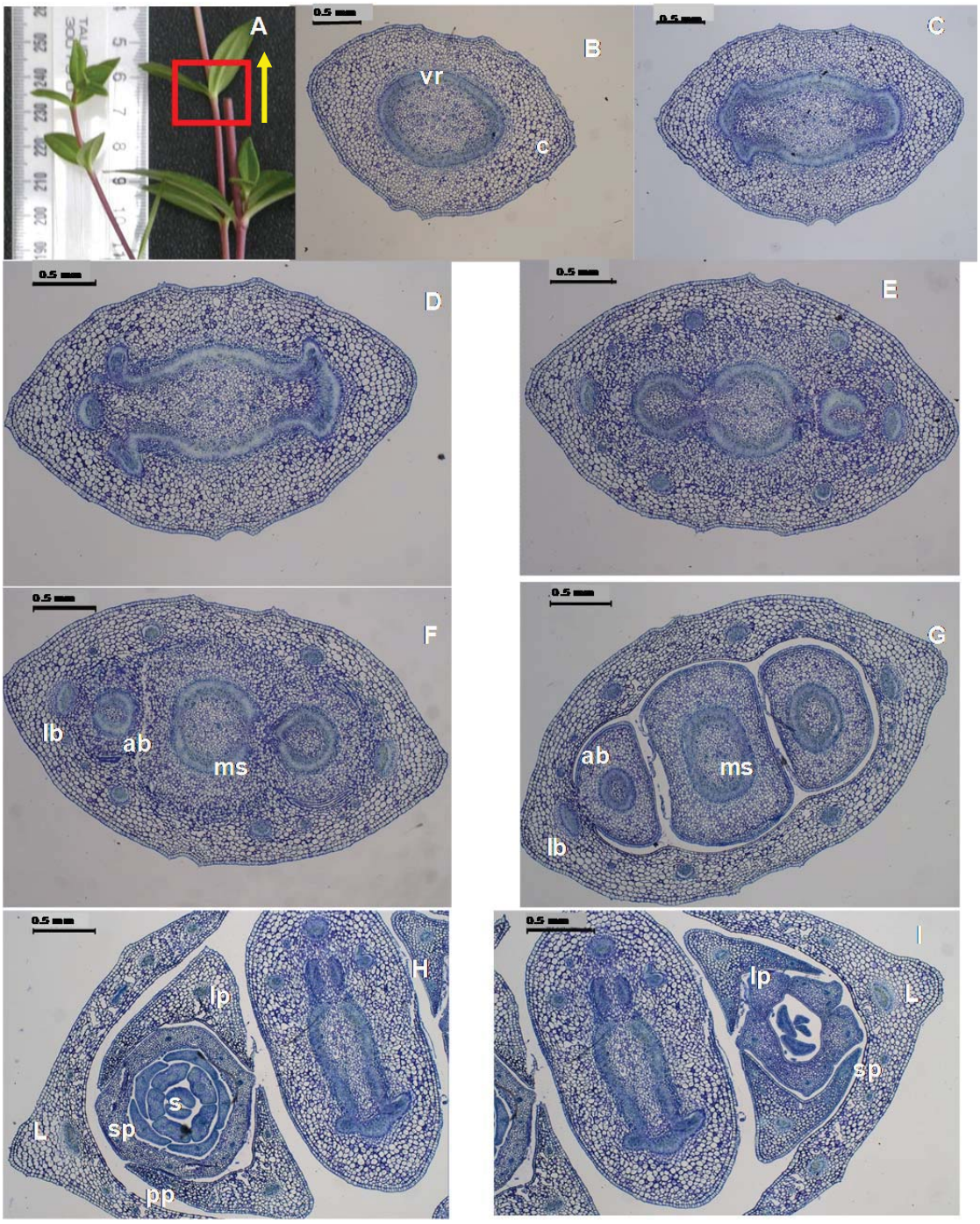


Figure 5.2 Floral shoot of 'Spotlight' sampled in Nov 2007 (late spring); (A) the region highlighted by red coloured square from which (B - I) present an acropetal sequence of transverse sections, including the node and associated axillary buds, indicating; (B-E) changes in vasculature of the main shoot and, (F-G) development of leaf base and bud primordia. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels: ab - vascular tissue of axillary bud, c – cortex, L – leaf, lb – vascular tissue of leaf base, lp - leaf primordia, ms - vascular tissue of main shoot , p - petal primordia, sp - sepal primordia, v - vascular ring.

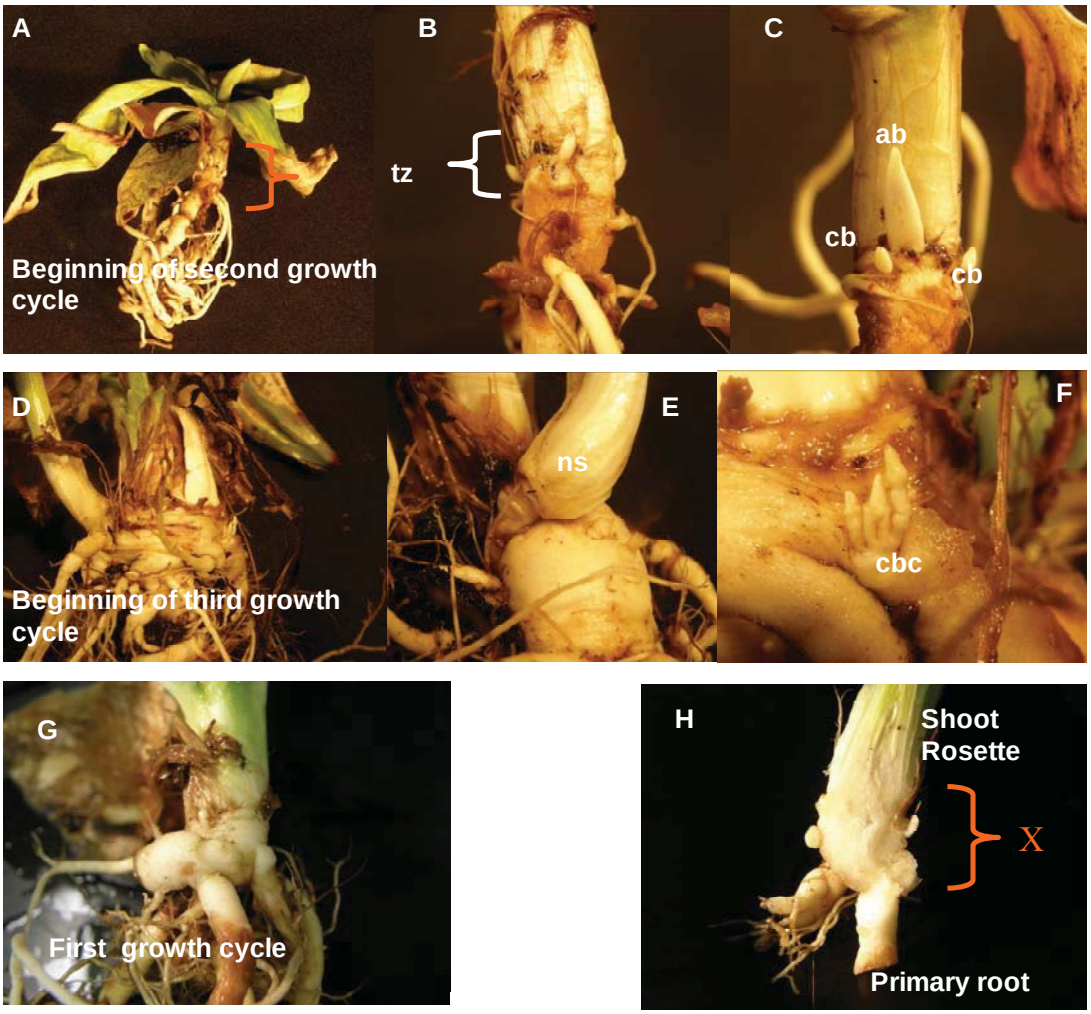


Figure 5.3 Seedlings of *Gentiana straminea*; (A-C) in the beginning of the second growth cycle from germination, (D-F) in the beginning of the third growth cycle, and (G and H) in their first growth cycle; (A) whole plant with region highlighted by orange coloured parenthesis magnified in subsequent images, (B) magnified view of transition zone with buds, (C) buds within leaf axils and transition zone, (D) whole crown region with an axillary bud above transition zone, and magnified in (E) showing the base of an emerging new shoot from transition zone, and (F) a crown bud cluster arising from the transition zone. (G) intact and, (H) longitudinal, hand-cut sections through the stem axis of juvenile seedlings in their first growth cycle indicating, area taken for microscopic sections presented in Figure 5.4, Figure 5.5, Figure 5.6 denoted by 'X'. ab – axillary bud, cb – crown buds, cbc - bud cluster, ns - new shoot from transition zone, tz - transition zone.

5.4.1.3.2 Histological features of transition zone

As evident in other plants, the transition zone of gentians has vascular characteristics between those observed in a shoot/stem and root, in that vascular bundles gave the appearance of gradually diverging from the centre with progressive sampling positions of transverse sections from the root towards the shoot (Clegg and Cox, 1978; Roland and Roland, 1980). In seedlings of *G. straminea*, at the root end vascular tissue were

centrally located (Figure 5.4B-D), with scattered xylem vessels occupying the central stele (Figure 5.4D). For sections of the transition zone from the shoot-end, the vascular tissue was visible surrounding the pith (Figure 5.4E, F, & K). Based on the details of histological features presented below, it is proposed that the vascular tissues were beginning to diverge from the centre with progression towards the stem (Clegg and Cox, 1978). Compared to transverse sections from the shoot of 'Spotlight' (e.g. Figure 5.1A), and as further evidence of morphological differences of the transition zone, the vascular bundles within this region of the transition zone were not arranged as a ring, but more in the shape of a square (Figure 5.4F). Arrangement of xylem vessels as four separate groups was visible in the central column of vascular bundles (Figure 5.4E & K). There were outward extensions of xylem vessels from the four corners of these groups of xylem tissue (indicated by 'X' in Figure 5.4E & K), each terminating in clusters of unidentified cells of greater density close to the epidermis (Figure 5.4E-J). These results were therefore similar to those reported within the transition zone for *Helianthus* sp. where six vascular groups were observed (Clegg and Cox, 1978) and, in *Arabidopsis*, two groups (Busse and Evert, 1999). In *G. straminea*, bud initiation was visible arising from groups of cells in each of the four corners of the square-shaped vascular cambium (Figure 5.4K-R and Figure 5.5B-D). In some cases, several bud initials were being initiated from a single corner (Figure 5.5B). Within the transition zone, where these buds/bud initials were present, vascular traces arising from the primordia or scales were not visible however. At any one time, in transverse sections of the transition zone, while not usually macroscopically visible, buds were found to be at differing degrees of development, from bud initials through to buds with well-developed primordia (Figure 5.4K-R and Figure 5.5B-D). As evident from acropetal sectioning of one bud which was also visible macroscopically, bud clusters were clearly attached as an external outgrowth to the transition zone (Figure 5.4L - Q). Near the rosette of leaves in the transition zone of *G. straminea*, the vascular bundles were spread among the parenchyma cells of the shoot (Figure 5.4S - U) as observed in *Arabidopsis* (Busse and Evert, 1999), and are likely to be the traces of leaves that form the rosette (Busse and Evert, 1999). Based on the histological observations therefore, the region below the leaf whorl in juvenile seedlings of gentians can be considered to be the transition zone.

Within longitudinal sections of young seedlings of *G. lutea* and *G. straminea* the undulations visible on the external surface of the transition zone (Figure 5.6A) were

visible as protrusions from the outer, corky periderm (Figure 5.6B). Buds with leaf primordia (Figure 5.6F, H and Figure 5.5F), and undifferentiated cell masses (Figure 5.6C-E, G & J), were present along the periderm of the transition zone. On the assumption that dividing cells are smaller in diameter (Chiatante et al., 2008), it is hypothesised that the undifferentiated cell masses observed within the current study are potential sites for initiation of crown buds. In longitudinal sections, these putative meristematic zones could be identified as regions of high cell density, arranged on the periderm of the transition zone, each at different stages of development (Figure 5.6C-J). Some bud initials were embedded within the periderm (Figure 6E, G & J), while others protruded (Figure 5.6C-D, F & H). Within microtome and hand-cut sections, vascular traces could be seen extending from well-developed buds to the central vascular tissue (Figure 5.3H & Figure 5.5F). In some plants flattened axillary buds were sometimes visible at the basal node of the shoot, whereas crown buds were located just below, originating within the transition zone (Figure 5.3C).

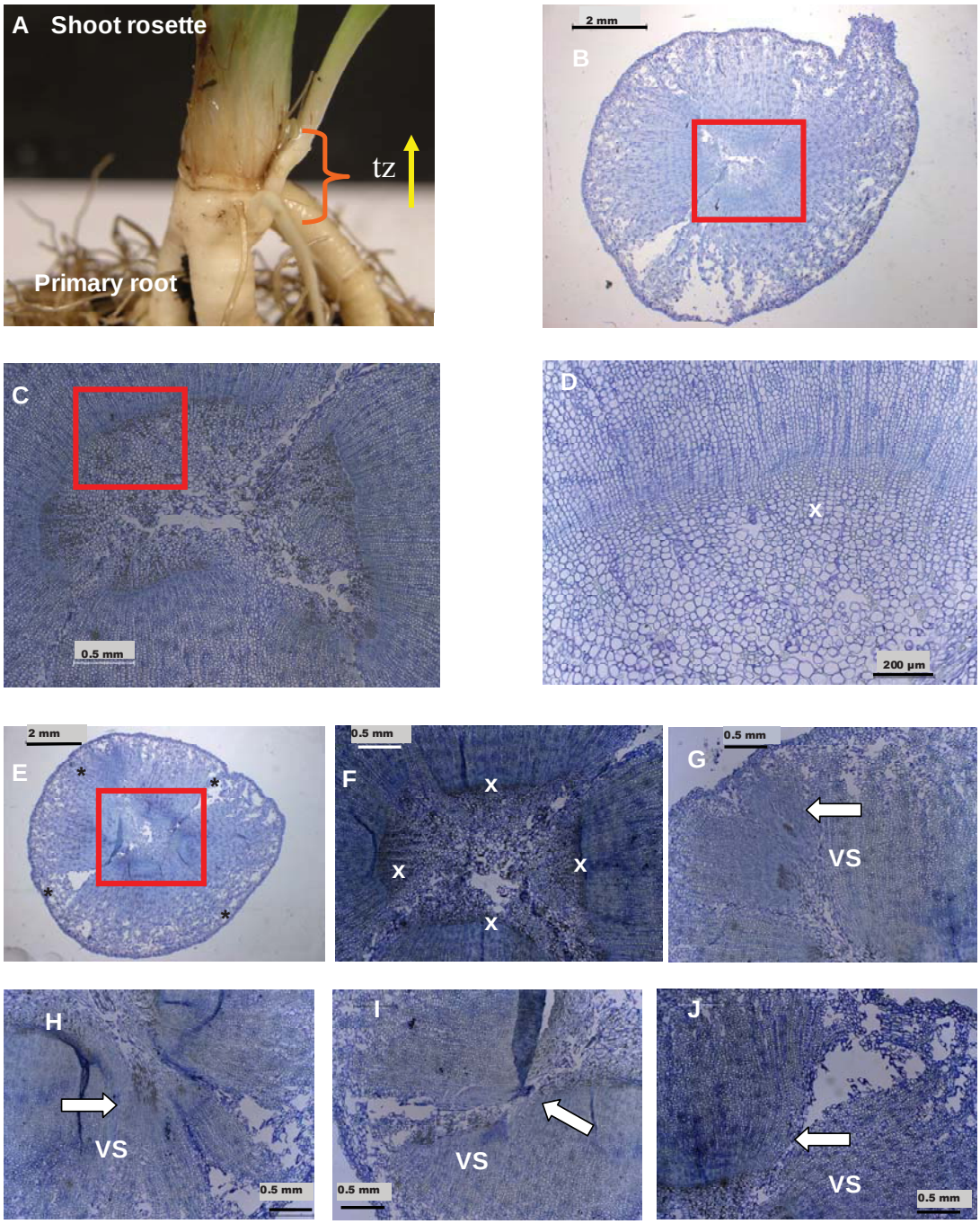
The confirmation of the presence of the transition zone and the understanding of the histological features present and associated with bud initials, can now be used for contrasts between true seedlings and gentian plants propagated from tissue culture, in subsequent discussions involving the origin and development of crown bud clusters.

5.4.1.4 Development of crown buds in the transition zone of seedlings

Based on the macroscopic and microscopic studies presented above, the transition zone was regarded as the primary site of initiation of crown buds in seedlings. During the first growth cycle following germination, numerous crown buds and bud initials were visible in the transition zone in all three species investigated. Crown buds developed from all four corners of the vascular column, albeit in different planes (Figure 5.4, Figure 5.5 & Figure 5.6). All buds in gentian seedlings were not equally developed, and ranged from being bud initials through to fully differentiated buds with prophylls (Figure 5.4, Figure 5.5 & Figure 5.6). Crown buds consisted of individual buds in the first growth cycle, but could later develop into bud clusters (Figure 5.3 & Appendix III). The vascular connections between the shoot apical meristem and adaxial leaf cells are considered important for axillary bud formation (McConnell and Barton, 1998), and for other plant species the associated visible leaf traces within the shoot has been used to confirm their morphological status as being axillary (Meloche and Diggle, 2001). In contrast to axillary buds (Figure 5.2), buds originating within the transition zone of

seedlings of gentians developed externally from the corners of vascular groups (Figure 5.4), showed no vascular traces as seen for leaves, and the buds themselves were less elongated than typical axillary buds (Figure 5.3, Figure 5.4, Figure 5.5 & Figure 5.6). The first initials of crown buds on seedlings of gentians therefore, can be considered to be adventitious.

The initiation of adventitious buds and bud clusters at the hypocotyl (equivalent to the transition zone in the current study) was evident in other herbaceous perennials like *Cardopatum corymbosum* L. (Chiatante et al., 2008), *Diodia Virginia* L. (Baird et al., 1992), and *E. esula,* exogenously (Raju, 1975) or endogenously (Myers et al., 1964). In some woody perennials, initiation of adventitious buds was observed within the collar, which is also derived from the hypocotyl (Del Tredici, 2001; Klimešová and Martínková, 2004; Pascual et al., 2002). As evident in the current study, both gaps and the ends of vascular strands located at the four corners of the vascular groups, potentially provide the sites of connection for the vascular traces of the adventitious bud initials, that originated in the periderm (Figure 5.4 & Figure 5.5). With tissue within the transition zone in other gentian spp. being capable of greater bud initiation *in vitro* than the shoot (Miku a et al., 2002; Miku a et al., 2005), this region of the seedling appears to offer ideal conditions for initiation of adventitious buds. Hence, based on the morphology of the different species of gentian seedlings investigated during the current study and, as evident in other herbaceous and woody perennials, it is concluded that in gentians the transition zone is the site for formation of bud clusters under natural conditions. In the absence of previous histological studies on development of crown buds in gentians under natural conditions, this study has enabled identification of features that can be considered to occur naturally and, therefore, be used to determine what if any differences may be evident in plants from tissue culture (as discussed in subsequent sections).



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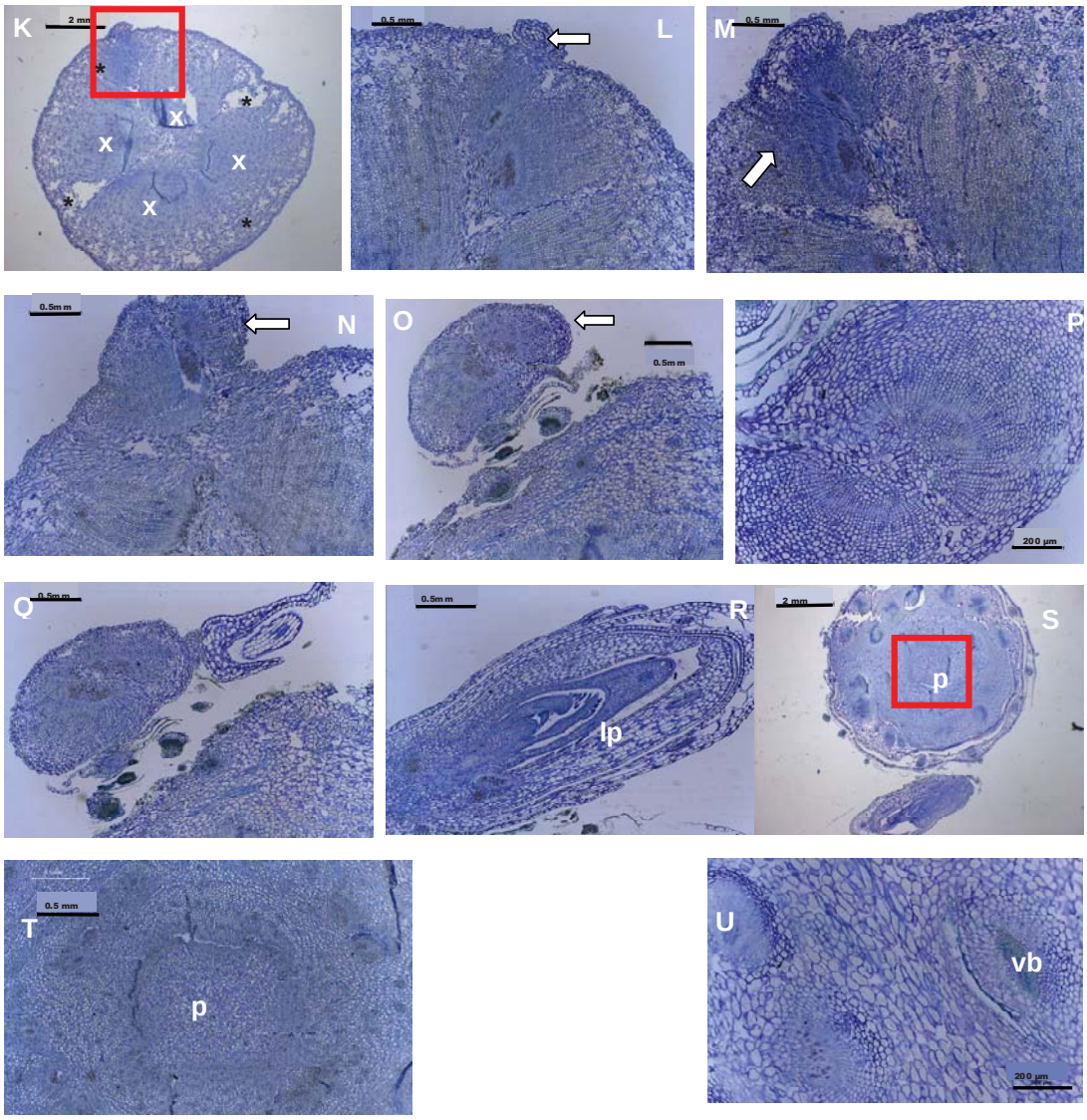

Figure 5.4 Acropetal sequence of transverse sections within the transition zone of a seedling of *Gentiana straminea* in its first growth cycle from germination; (A) intact plant illustrating transition zone, (B) full section closer to root with vascular bundles concentrated towards the centre, red square indicating position of magnified image of (C) and, within (C) indicating with the red square the magnified position shown in (D) illustrating xylem vessels. (E) Full section with vascular bundles diverging to form separate groups with red square magnified in (F), and four gaps indicated by "*" with extensions of vascular strands magnified in (G), (H), (I) and (J) as indicated by white arrows. (K) Development of a bud from one gap indicated by red box (L - Q) sectioned acropetally indicating base of the bud (white arrows) and, (R) primordia. (S) Shoot closer to rosette showing, (T) pith in the centre and, (U) separation of vascular bundles. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels: lp - leaf primordia, p - pith, vb - vascular bundles, vs - vascular strands, x - xylem.

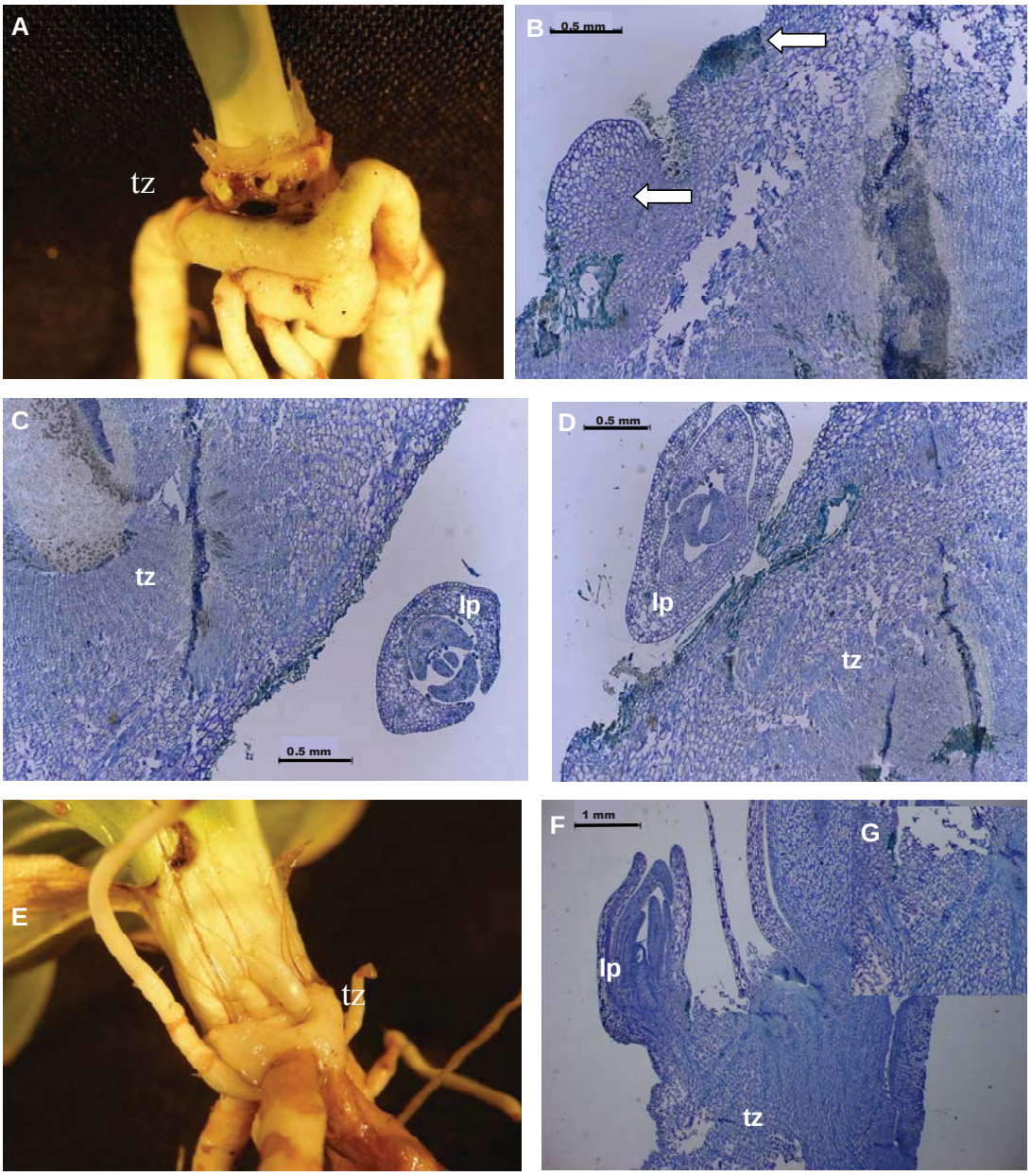

Figure 5.5 Transverse sections of transition zone of a seedling of *Gentiana straminea* in first growth cycle following germination; (A) intact plant illustrating transition zone, (B) section indicating development of two bud initials (white arrows) from one corner of the gaps of vascular grouping within the transition zone, (C, D) two well-developed buds with primordia at another corner of the transition zone. (E) Intact plant of a seedling of *Gentiana straminea* illustrating transition zone and crown buds, (F) longitudinal section through transitional zone illustrated in (E), indicating bud with primordia and, (G) vascular traces connecting bud and transition zone: lp - leaf primordia, tz -transition zone.

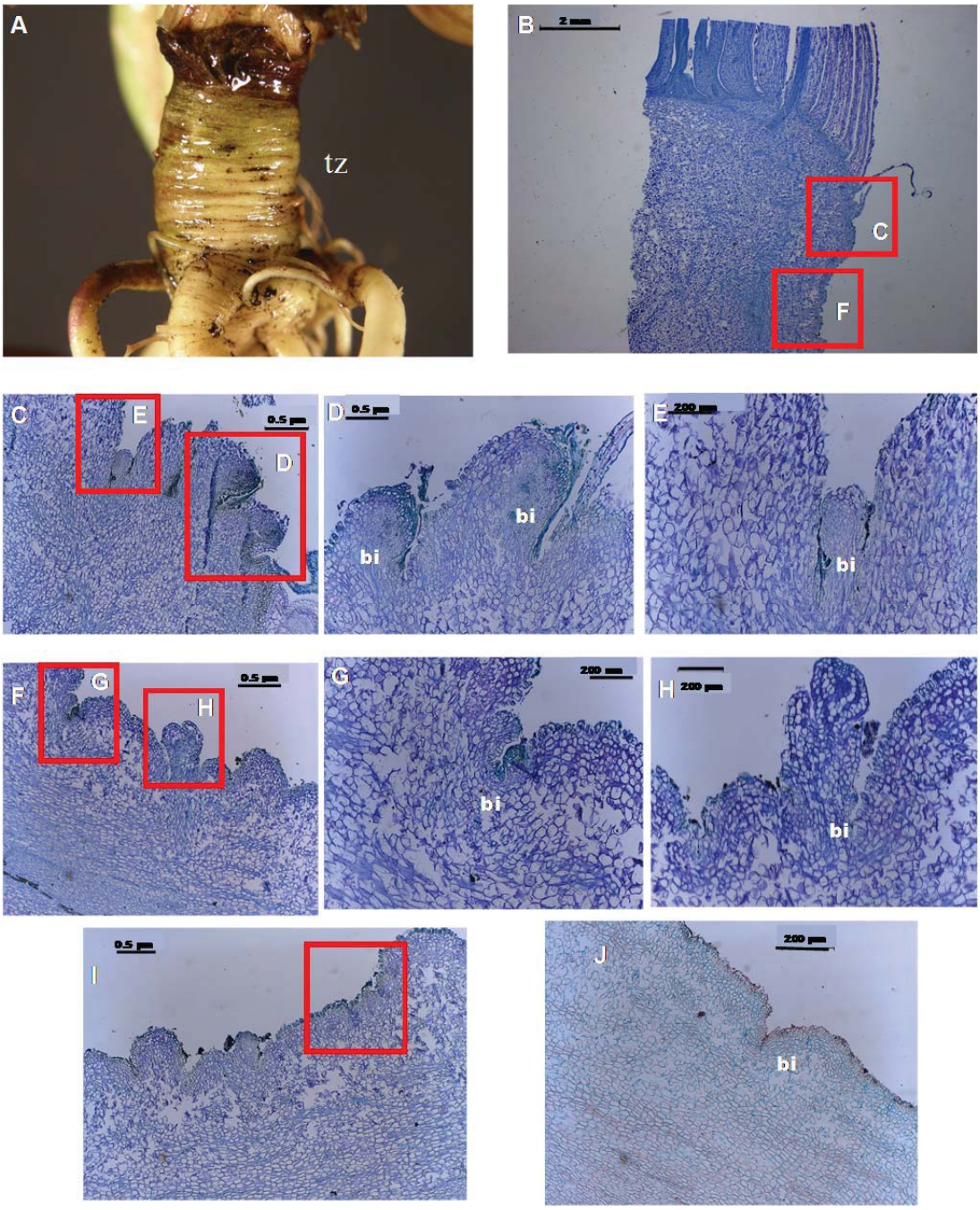

Figure 5.6 Longitudinal sections of transitional zone of a seedling of *G. Lutea* in first growth cycle following germination; (A) intact plant illustrating transition zone, (B) full section at transition zone indicating positions where magnified images of (C) and (F) were taken. (C) Positions of bud initials further magnified in (D) and (E), indicating bud initials at different stages of development. Positions indicated in (F) further magnified in (G), indicating bud initials and (H), buds with primordia. (I) Section taken on a different plane of axis, indicating the position magnified in (J), a potential site for bud initiation. bi - bud initial, tz - transition zone.

5.4.2 Experiment 2; Crown bud clusters - Origin and ontogeny

5.4.2.1 Macroscopic features of the transition zone in plants from tissue culture

Crown bud clusters developed from multiple positions in tissue cultured plants of 'Diva'. During the first season of growth following deflasking, bud clusters were most commonly initiated at the base of the primary shoot where the shoot and storage roots joined (Figure 5.7C & E), others from the lower nodes on the primary shoot (Figure 5.7B & E) and, less frequently, from the proximal end of the storage roots (Figure 5.7E). In plants with bud clusters at the lowest node, swelling of the shoot, in the area below the lowest node, was clearly visible as the season progressed (Figure 5.7B-D). As buds elongated into shoots in these clusters, a storage root system usually developed in close association with the base of the cluster (Figure 5.7F). Irrespective of where the crown bud clusters formed, in line with the natural seasonal growth cycle, the primary shoot above the crown bud cluster senesced during subsequent months.

In cases where the storage roots did not develop in association with the bud cluster, the region of shoot below the bud cluster became thickened (Figure 5.7C & D). On the thickened area of the primary shoot below the cluster, small buds formed on the surface (Figure 5.7C & D), and some eventually developed into crown bud clusters. Hence, as was apparent in more mature plants of 'Diva' derived from tissue culture, the area below the lowest node on the shoot became the crown tissue, i.e. transition zone (Figure 5.7D).

5.4.2.2 Histological features of transition zone in plants from tissue culture

5.4.2.2.1 Ontogeny of transition zone

In contrast to the situation evident in seedlings (Figure 5.4B, C, & K), soon after being deflasked, the sections of the shoot where the transition zone would subsequently form (refer Section 5.4.2.1) comprised tissues which were not organised as being typical of either shoot, root or transition zone (Figure 5.8A-E). Only root initials were identifiable in plants at this stage in the pre-transition zone (Figure 5.8D). Compared to the transition zone in true seedlings (Figure 5.4), even when well established (i.e. six months following deflasking), plants derived from tissue culture appeared rather disorganised at the proximal end of the transition zone (Figure 5.9B). In serial sections progressing acropetally, xylem within this disorganised tissue became evident as several separate groups (Figure 5.9C & D), with these groups forming a ring at the base of the

shoot (Figure 5.9E & F). As illustrated by layers of xylem vessels within the vascular ring, shoots presented evidence of secondary thickening (Figure 5.9F). As expected within a shoot, pith was visible in the centre, and was comparatively smaller in width compared to the external cortex (Figure 5.9F).

When the bud clusters first became large enough to be visible under microscopic examination, crown buds, storage roots, and root initials with vascular bundles in the centre, were visible at the root end of the eventual transition zone (Figure 5.10A). Progressing acropetally up the shoot, the vascular tissue became organized into separate units (Figure 5.10B) and formed a ring (Figure 5.10C & D). This ring of vascular tissue contained secondary thickening. Even when the bud clusters were macroscopically visible, a similar histological arrangement was evident within the transition zone, but secondary thickening was further increased at the basal internode of the shoot, which also developed a thicker cortex (Figure 5.11B & Figure 5.12B-E). Based on these progressive sections with time, in contrast to that evident in seedlings, it appears that in a plant propagated vegetatively by tissue culture, development of the transition zone may take a longer time. This was evident by the formation of crown buds from multiple locations including roots and shoots, in plants from tissue culture as compared to seedlings where crown buds were formed on the typical transition zone between shoot and root during the first growth cycle. It is possible therefore that the inability to develop an adequate number of crown buds following propagation can be attributed to the transition zone still developing within the plants.

5.4.2.2.2 Initiation of bud clusters on transition zone of plants from tissue culture

Although not visible macroscopically, within either longitudinal or transverse sections viewed under the microscope, it was possible to identify different positions of initiation of crown bud clusters. Most plants had crown bud clusters with primordia originating from the base of the primary shoot but, with vascular connections to the cells located external to the vascular ring (i.e. no gaps were formed in the vascular ring; Figure 5.13D). In contrast however, some of the plants had buds developing on the root-end of the transition zone, which was in close association to the proximal end of storage roots (Figure 5.10A, Figure 5.13A & B). As a further example of the divergence in origins from which crown bud clusters could arise, in one instance one shoot had already emerged from a crown bud cluster developed at the base of the primary shoot (Figure 5.13E), and its base remained enveloped with the same scale leaf as the other buds in

that cluster (Figure 5.13F). Bud clusters with external vascular attachment to the primary shoot, as well as initiating at a distance from the primary shoot (presumably from the proximal end of the storage root) were visible on one plant (Figure 5.13G). Although attached externally to the base of the primary shoot, xylem strands were visible within the vascular connection (Figure 5.13H). Some bud clusters were initiated on the eventual transition zone in-between two shoots (Figure 5.13D). In addition to these examples, there were crown bud clusters attached at the lowest node (Figure 5.10 & Figure 5.12). Hence bud clusters were able to form at multiple positions, even on a single plant and display different degrees of development in their vascular connections.

As noted above, in young plants from tissue culture, in the transition zone, at the junction of the storage root and shoot the cellular tissue was not well organised. As a result, bud clusters initiated at the lowest node were the most reliable source for histological tracking of the progressive development of vascular connections of the crown bud cluster. As presented in the following sections (a-c), the stages of development of the first bud initial for the cluster are described, based on bud cluster size, i.e. whether visible microscopically or not.

a. ***Earliest microscopically visible stage of bud cluster development;*** When sectioned acropetally, crown bud formation was visible close to the proximal end of both the storage roots and on the primary shoot (Figure 5.10A-E). The bud formed on the shoot was a crown bud which would eventually form a cluster (Figure 5.10J), and at this stage the presence of this bud did not cause major changes to the vascular ring of the shoot (Figure 5.10C & D). The bud cluster developed external to the vascular ring (Figure 5.10C, F & G), on one side of the primary shoot (Figure 5.10C-E). The base of this bud cluster was visible as a single mass of cells (Figure 5.10H & I), which appeared rather disorganised compared to that evident in a typical flowering shoot, root or transition zone (Sections 5.4.1.1 & 5.4.1.3). Hence at this stage of development the type of tissue was not distinguishable (Figure 5.10F-I).
b. ***Bud clusters become slightly visible macroscopically;*** On examining sections of a crown bud cluster developed on the shoot, gaps in the vascular ring were visible at the node when only one bud had become visible macroscopically (Figure 5.11C - F). In contrast to what occurs with axillary buds on the floral shoot (Figure 5.2) the base of the bud cluster formed externally on the shoot (Figure 5.11D-F). The base of the bud cluster was initiated from a mass of cells

similar to that evident at stage '*a*', but the base was apparent as a stem at this stage (Figure 5.11F-H).

c. ***Bud clusters clearly visible macroscopically;*** When bud clusters were clearly visible macroscopically (Figure 5.12A), as described below, the vascular ring had become modified on both sides of the primary shoot below the cluster (Figure 5.12E & F). On the side of the eventual transition zone where the bud cluster developed, a major change was visible on the primary shoot as an outgrowth of the cortex (Figure 5.12E & F). Within this the crown bud cluster developed a vascular strand connecting the bud directly to the stele, without formation of a gap in the vascular ring. This was also as occurred in the formation of an axillary bud on a flowering shoot (Figure 5.2C-E). As the bud was apparent at a node on the primary shoot, the gap created in the vascular ring on the opposite side of the cluster, was possibly the position of the original axillary bud which had senesced (Figure 5.12A). Although there were two axillary buds present at a node of a floral shoot (Figure 5.2), when bud clusters were initiated at the lowest node, only one bud cluster was developed (Figure 5.10, Figure 5.11 & Figure 5.12).

5.4.2.3 Origin for bud clusters; adventitious or axillary?

Based upon the macroscopic and microscopic features of clonally propagated and seedling gentians, crown buds developed on;

- the transition zone, which could include axils of lower nodes (Figure 5.3, Figure 5.4, Figure 5.5, Figure 5.10, Figure 5.11 & Figure 5.12),
- thickened shoot-tissue below the first node (Figure 5.7C-D),
- the base of the shoot (Figure 5.7 E-D & Figure 5.13) and,
- the proximal end of storage roots (Figure 5.7 E).

In contrast, on floral shoots axillary buds were developed from, and associated with, a node within the axils of a leaf base. In the cultivar 'Spotlight', formation of crown buds was also observed at the distal end of storage roots (Chapter 3), but at a low frequency. The first bud initial of the crown bud cluster was, therefore, initiated without the presence of a node and a leaf base, which is regarded as a prerequisite for axillary bud formation (Cutler et al., 2008; Evert, 2006; McConnell and Barton, 1998) and, therefore, supports the conclusion that the origins of a crown bud cluster is not via an axillary bud. Further evidence supporting this conclusion included that crown bud clusters were developed at different origins/locations, and present without any particular

order (Chapter 4). As noted in other plant species, axillary buds differ from adventitious buds by adventitious buds not being related to a leaf axil (Cutler et al., 2008), and can develop from root, hypocotyls, leaf or the shoot (Evert, 2006). Hence, based on the location of their development, and their ability to develop without a leaf axil, the first bud initials from which crown bud clusters develop must be considered to be adventitious.

A typical transition zone was not present in vegetatively propagated plants at the start of their first growth cycle (Figure 5.11 & Figure 5.12) and, therefore, raises the question whether adventitious buds could be formed within axils of a shoot. As adventitious buds can originate from parenchyma or cambial cells on any plant organ (Evert, 2006; Kerstetter and Hake, 1997), it was hypothesized that nodes could be a site for adventitious bud formation, prior to becoming part of the transition zone. The presence of an axillary bud at a node created gaps in the vascular cambium of the main shoot (Figure 5.2) (Cutler et al., 2008), whereas adventitious buds could arise endogenously from the periphery of the vascular cylinder cambium or parenchyma (Bowes, 1996; Evert, 2006), or exogenously from more superficial tissue like the epidermis (Evert, 2006) of any plant organ (Kerstetter & Hake 1997). As evident by microscopic examination of crown bud clusters at the proximal end of the shoot (no visible node), there were no gaps in the vascular ring, rather buds initiated externally outside the central vascular cylinder (Figure 5.13). This evidence therefore, supports the notion that crown bud clusters originate adventitiously in these vegetatively propagated plants. In seedlings, buds formed from the vascular strands arising from the vascular groupings within the transition zone (Figure 5.4). Even when bud clusters developed on one side of the node on a shoot, the bud clusters were attached to the vascular cambium externally, and no disruption to the central vascular cylinder was observed (Figure 5.10, Figure 5.11 & Figure 5.12). In such occasions, a gap in the vascular ring was observed only on the opposite side of the cluster, which could be due to the original leaf/scale, leaf axil or the true axillary bud on the node. Hence based on the preceding evidence, the vascular connection of the bud initial for the crown bud cluster, was different from that seen with typical axillary buds, and could be considered adventitious in origin. Thus the initiation of a crown bud cluster in gentians presents characteristics typical of an adventitious bud, in terms of their origin and vascular connection to the parental tissue.

As evident during the current study, the initial crown bud that forms, subsequently develops into multiple buds, i.e. a crown bud cluster. The current section of this chapter has focussed on the connection between the base of the crown bud cluster, i.e. first bud within the cluster, and the transition zone; the vascular connections and progressive development of buds within the cluster itself, are discussed in a subsequent section (refer Section 5.4.3).

5.4.2.4 Contrast between a seedling and a plant from tissue culture, in development of the transition zone and bud initials

In seedlings the transition zone was established early in the first growth cycle, prior to initiation of crown buds (Figure 5.3 & Figure 5.4). In plants derived from tissue culture however, the tissue on which crown buds and clusters initiate and develop, i.e. around and below the lower node or at the base of the shoot, develops after deflasking (Figure 5.7 to Figure 5.12). The typical histological characteristics of the transition zone (Section 5.4.1.3) were not evident within plants from tissue culture, except for the vascular grouping at the base of the shoot (Figure 5.9 & Figure 5.12). As evident by the arrangement of vascular strands, this grouping had no connection with initiation of crown buds. Although histologically different from the transition zone of seedlings, in plants from tissue culture the swelling of the shoot below the lower nodes and the base of the shoot, was the site for formation of adventitious buds, and was similar in function to the transition zone in a seedling. The transition zone was therefore, the main area for initiation of crown buds in seedlings as well as plants from tissue culture. Following deflasking however, plants from tissue culture may require additional time to develop the transition zone before formation of crown buds can commence.

Although crown bud formation was more prominent at the base of the shoot, during the first growth cycle in plants derived from tissue culture, some crown bud clusters developed around the lower node, and were externally visible as true axillary buds Figure 5.11 & Figure 5.12). If a crown bud starts to emerge as a shoot from a cluster on the lowest node, or from the base of the primary shoot, that bud typically becomes the dominant shoot within that growth cycle, sharing the same transition zone and root system with the primary shoot of the deflasked propagule. In such cases the primary shoot above the point of development of the new shoot naturally senesces. Thus the shoot-tissue, below the axil where the cluster is formed, becomes the transition zone. Such bud clusters on nodes were not observed during the second growth cycle of plants

derived from tissue culture, i.e. when a transition zone had already been established. Further to that, in some plants crown bud clusters were observed on the proximal end of storage roots. Hence although the transition zone is primarily the site for bud initiation under natural conditions, it is possible that bud clusters could also be formed at the lower nodes, base of the shoots, and roots. Bud formation from multiple organs of the plant was similarly observed in *E. esula* (Myers et al., 1964), in which adventitious buds formed exogenously or endogenously (Raju, 1975), and hence may be a common occurrence in some herbaceous perennials.

As found in other plant species, the formation of the transition zone may be associated with both the development of secondary thickening and the storage of carbohydrates in this area (Baird et al., 1992; Bellingham and Sparrow, 2000; Bowes, 1996; Del Tredici, 2001). Following completion of one growth cycle from deflasking, crown buds frequently appeared in the area of the lowest internode, which by this time had become an established transition zone (Figure 5.7) with an enlarged cortex and secondary thickening (Figure 5.9 to Figure 5.12). Since parenchyma cells within the cortex have a storage function (Bowes, 1996), a larger cortex at the lowest internode, could be an indication of the storage (carbohydrates) function of this tissue. It is therefore hypothesised that stimulation of development of the transition zone (i.e. crown tissue) may be associated with both early and high quality crown bud initiation in plant material vegetatively propagated by tissue culture. This hypothesis was further explored in both Chapters 6 and 7.

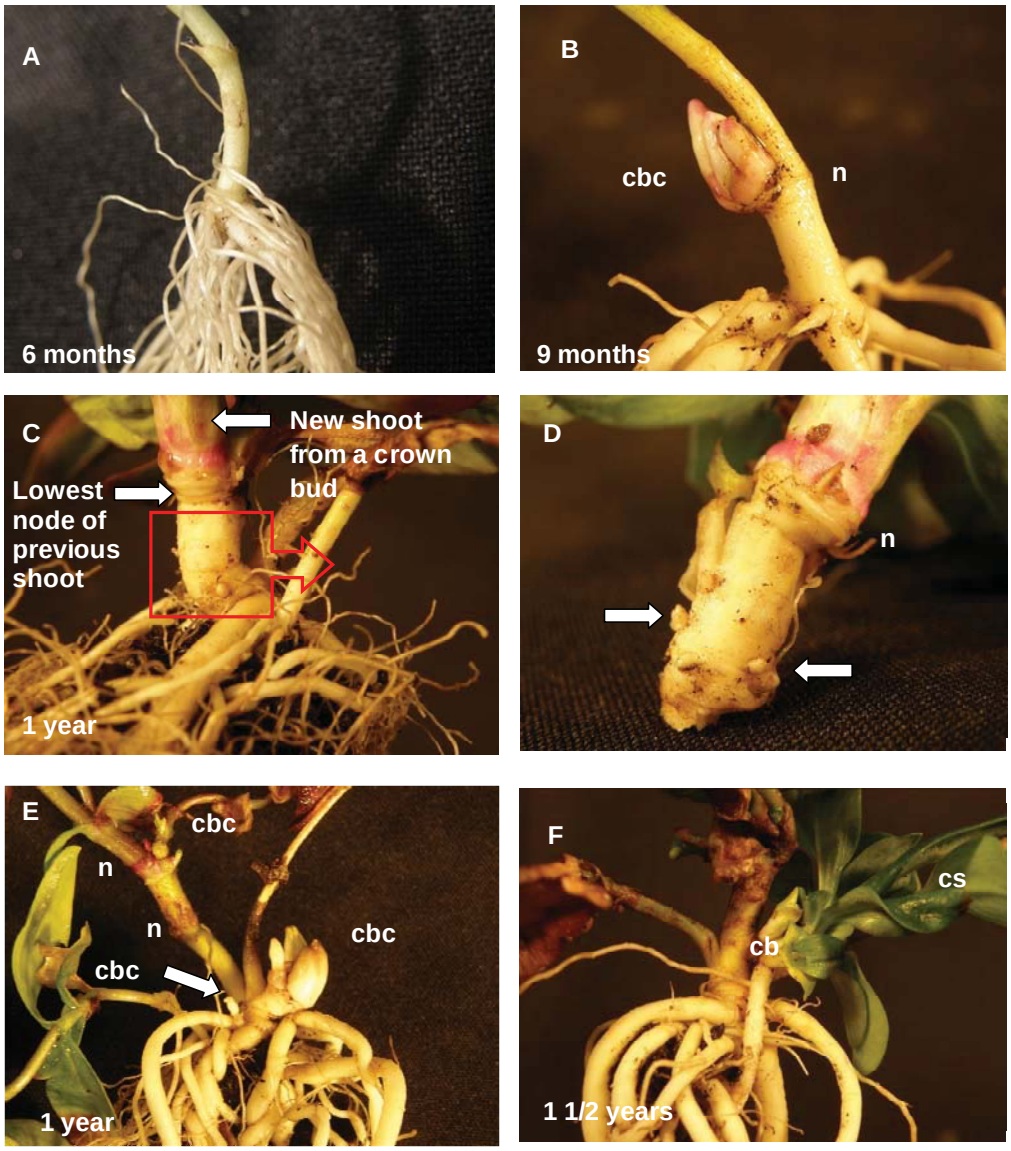

Figure 5.7 Development of the transition zone and bud clusters from different positions on plants of 'Diva' produced from tissue culture, at increasing periods following deflasking; (A) transition zone of a plant before visible evidence of a bud cluster after 6 months, (B) bud cluster developed at a node after 9 months, (C) thickening of the region below the lowest node after one year, (D) magnified view within red coloured square of (C), illustrating crown buds (white arrows). (E) Bud development at different positions on a single plant after one year (June 2009; white arrow indicates a cluster arising from proximal end of storage roots), and (F) subsequent shoot emergence from the bud cluster on the transition zone of the same plant after a further 6 months. cbc - crown bud cluster, cb – crown bud, cs - crown shoot, n – node.

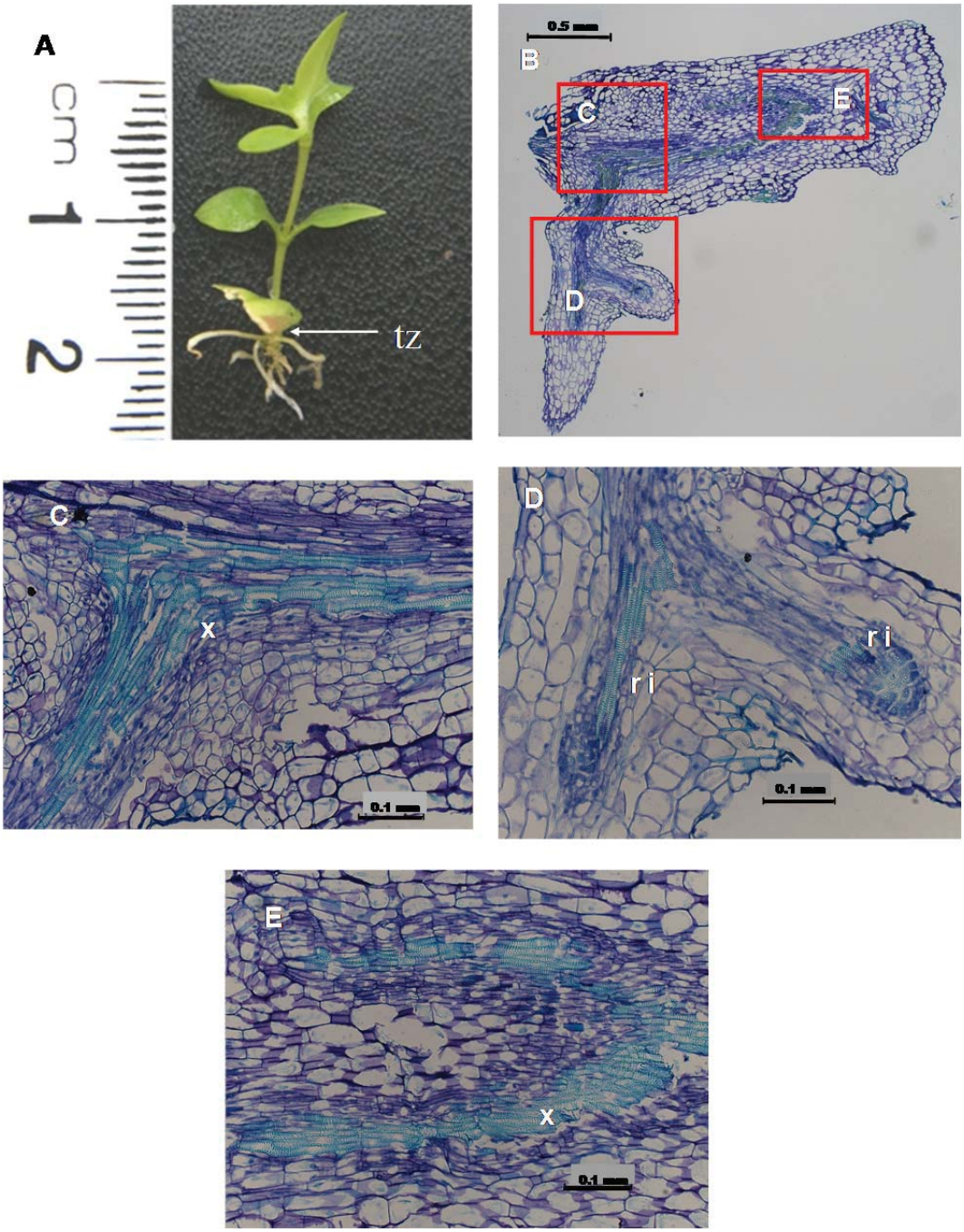

Figure 5.8 Transverse sections of an eventual transition zone of a plant of 'Diva' derived from tissue culture following deflasking; (A) intact plant indicating transition zone, (B) full section at the base of the shoot indicating positions of magnified images of where (C), (D), and (E) were taken, illustrating vascular tissues and root initials. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels: ri - root initials, tz - transition zone, x - xylem.

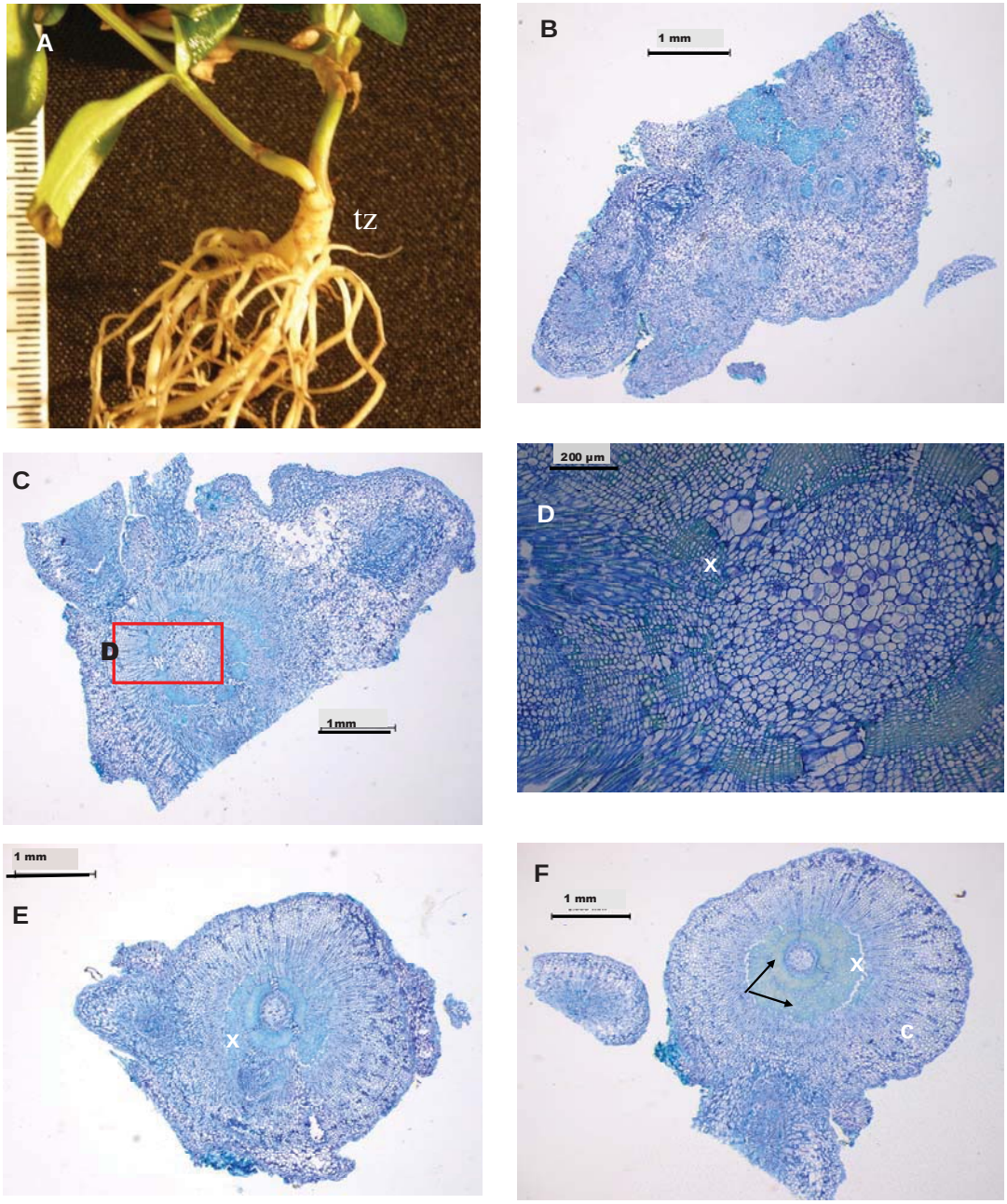

Figure 5.9 Acropetal sequence of transverse sections of the eventual transition zone of a plant of 'Diva' derived from tissue culture six months following deflasking; (A) intact plant indicating transition zone, (B) partial section at the root end indicating unorganized tissue, (C) partial section indicating position from which magnified image of (D) was taken, indicating the vascular groupings at the base of the shoot. (E-F) progressive development of vascular ring and presence of xylem as two layers (black arrows). As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels: c – cortex, tz -transition zone, x – xylem.

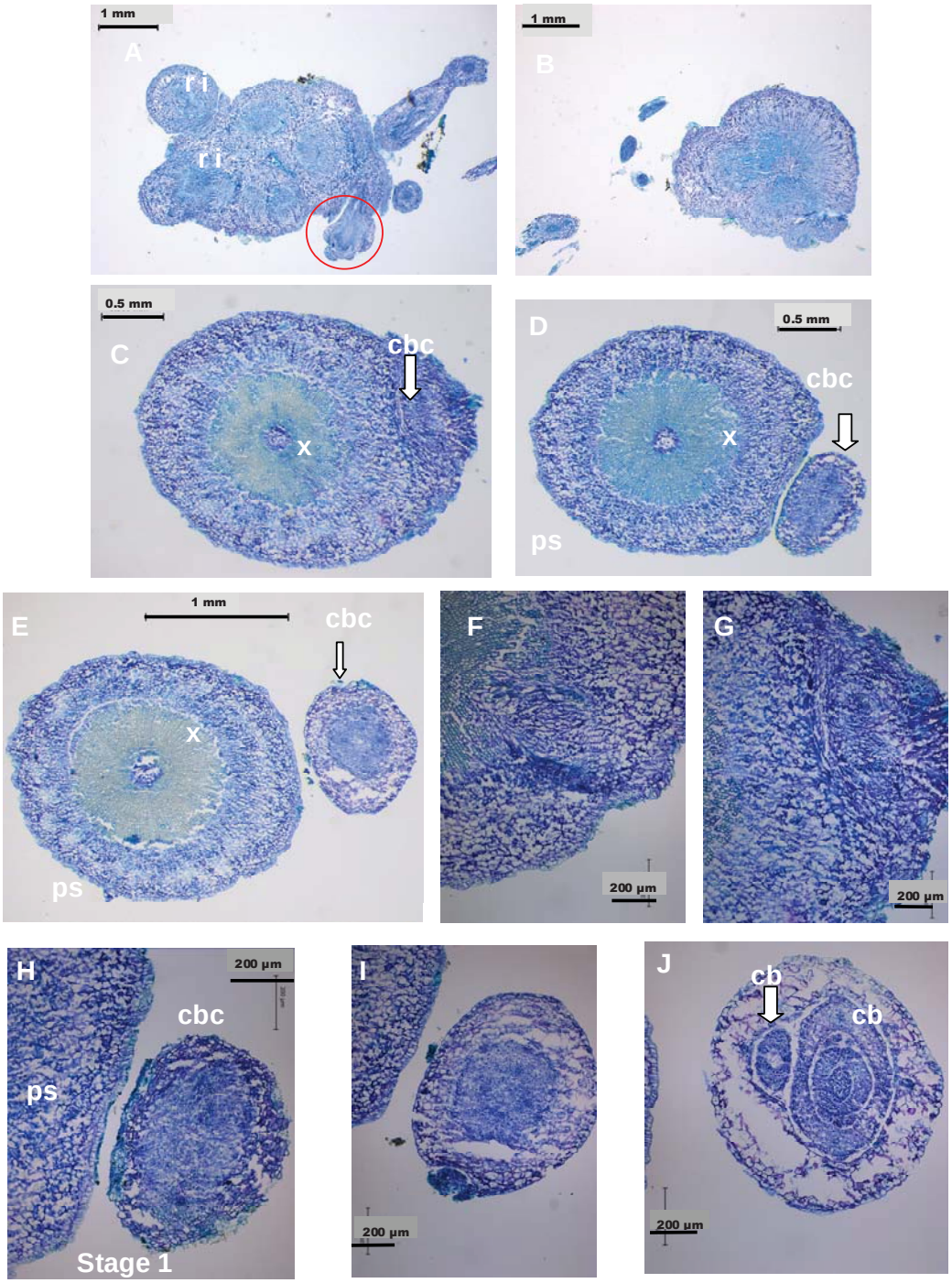

Figure 5.10 Acropetal sequence of transverse sections through the eventual transition zone (i.e. shoot) of a plant of 'Diva' derived from tissue culture at six months from deflasking; (A) a crown bud (red circle) and root initials at root end and, (B) vascular tissues converging at base of the shoot with (C-E), a bud cluster at stage 1, and the eventual transition zone with secondary thickening. (F-J) magnified images of acropetal sequence of transverse sections below, and through, the bud cluster indicating; (F-H) non-differentiated tissue at attachment to main shoot and, (I-J) association between buds within the cluster. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels; cb - crown bud, cbc - crown bud cluster, ps - primary shoot, x - xylem vessels.

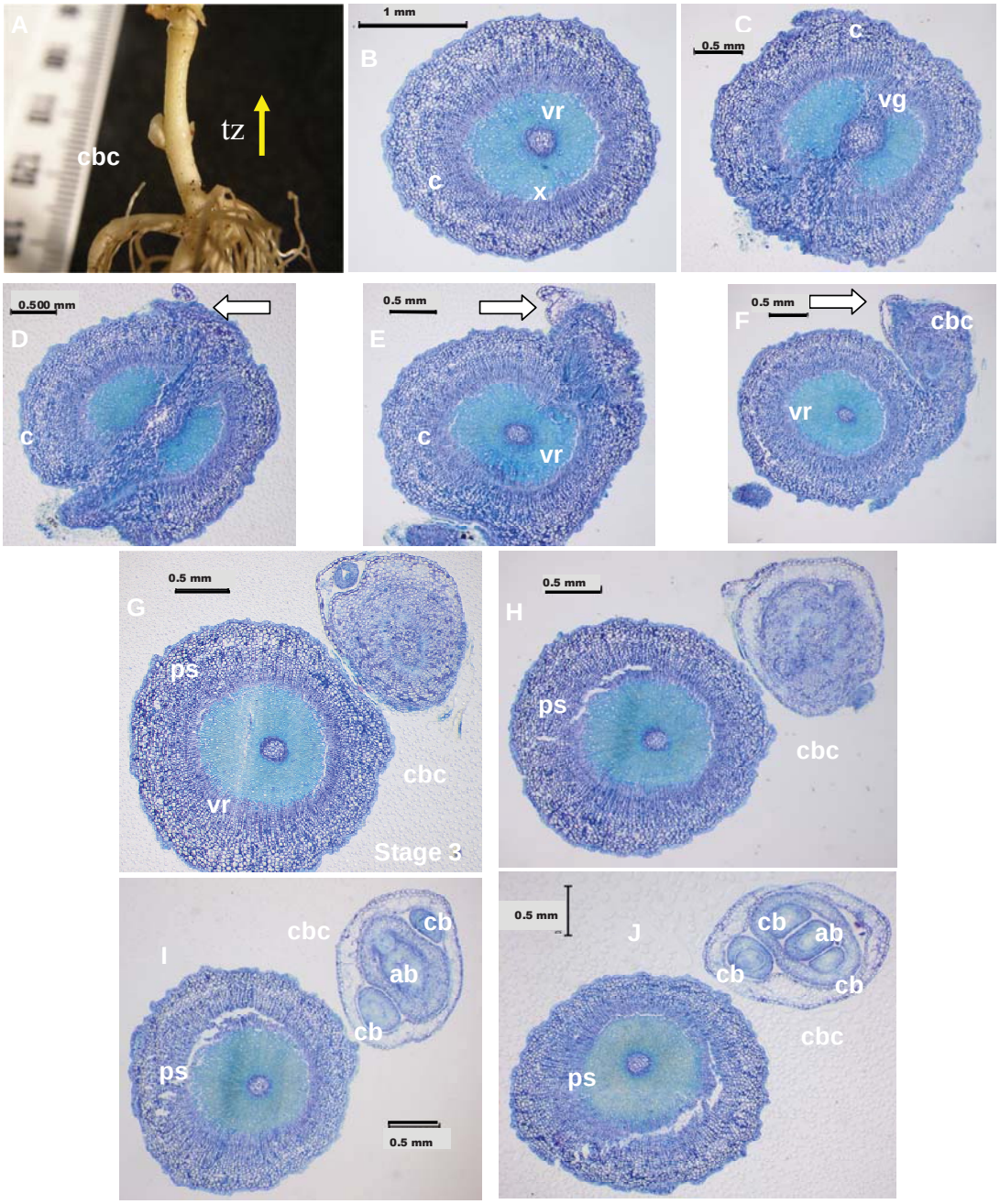

Figure 5.11 Acropetal sequence of transverse sections through the eventual transition zone (i.e. shoot) of a plant of 'Diva' derived from tissue culture at six months from deflasking; (A) intact plant indicating transition zone and crown bud cluster, (B-F) full sections indicating changes in vascular ring at the lowest node and the initiation of a crown bud cluster (white arrow) at the node, (G-J) full sections below and through the bud cluster indicating changes along the bud cluster at Stage 3, (G-H) at the base of the cluster and, (I-J) within the buds of the cluster. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels. ab – apical bud, c - cortex, cb – crown bud, cbc - crown bud cluster, ps - primary shoot, vg - gap in vascular ring, vr - vascular ring, x - xylem vessels.

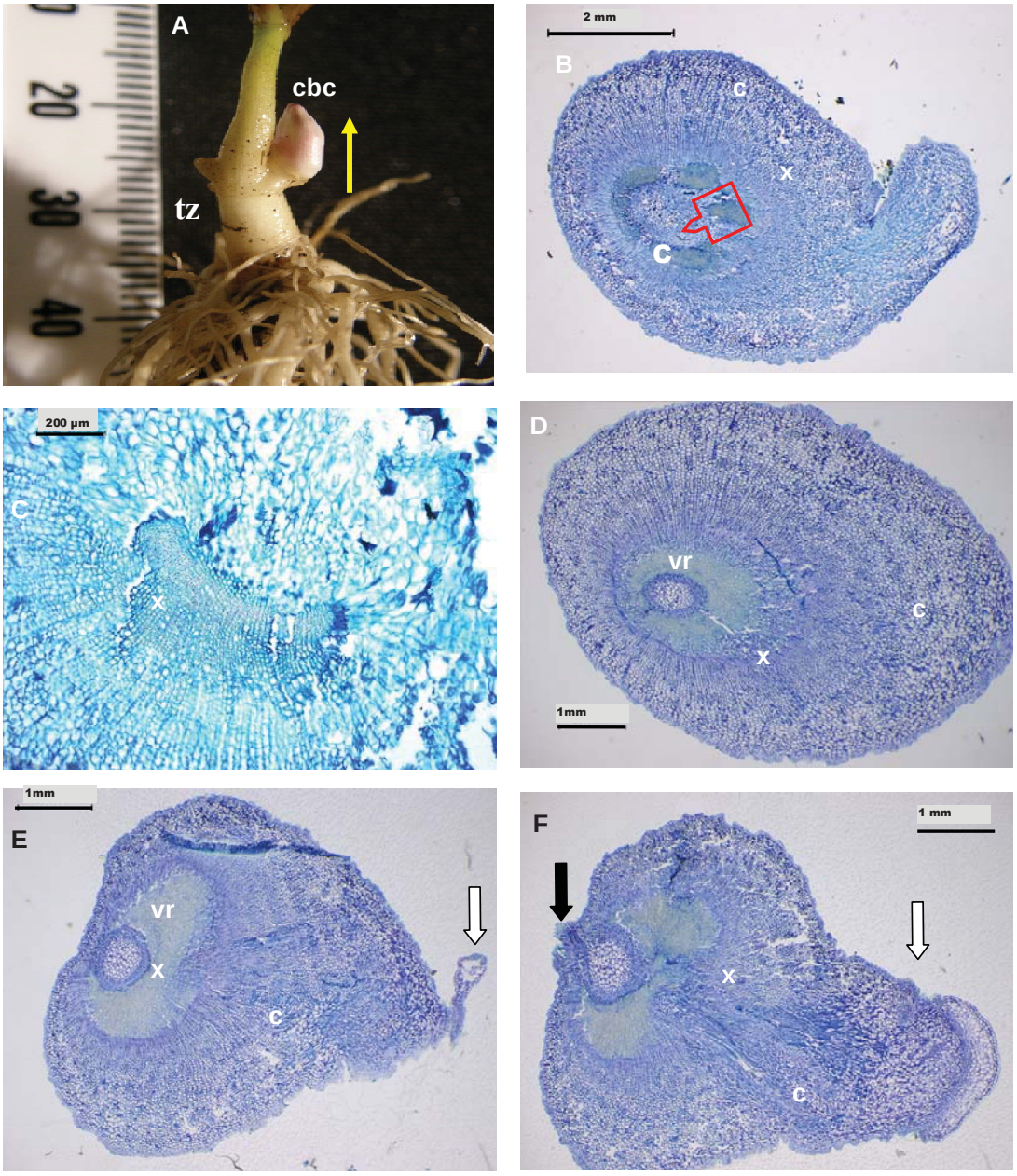

Figure 5.12 Acropetal sequence of transverse sections through the eventual transition zone (i.e. shoot) of a plant of 'Diva' derived from tissue culture at eight months from deflasking; (A) intact plant indicating transition zone and crown bud cluster, (B) red object indicating segment magnified in (C) illustrating vascular grouping at the root end. (D) full section at the base of shoot illustrating enlarged cortex and, (E, F) development of the bud cluster on the shoot (white arrow), and the gap in the vascular ring on the other side (black arrow). As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels. c - cortex, cbc - crown bud cluster, tz -transition zone, x - xylem vessels, vr - vascular ring.

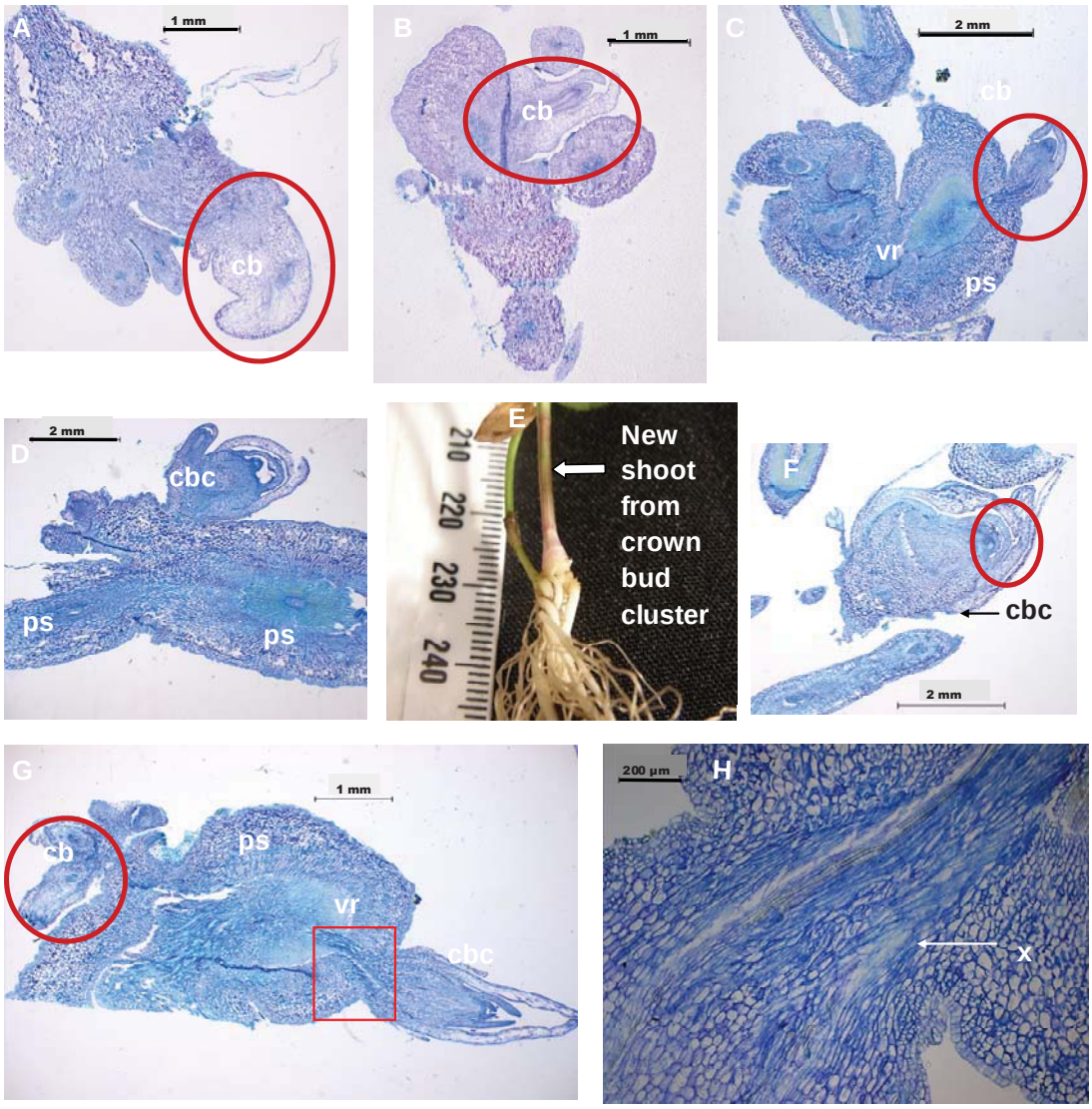

Figure 5.13 Sections through crown bud clusters initiated at different positions on plants of 'Diva' during the first growth cycle from deflasking; (A, B) transverse sections indicating crown buds at proximal end of roots (red circle), (C) a crown bud cluster on the base of the primary shoot with visible vascular connection to vascular ring, (D) a bud cluster developing in between two shoots at the transition zone, (E) an intact plant with a new shoot emerging from the cluster located at the base of a shoot, (F) transverse section through the eventual transition zone of that plant indicating remaining buds within the cluster still enveloped within scale leaves (red circle), (G) bud clusters at the root end of eventual transition zone from two positions, one at a distance from the main shoot (red circle) and other with connection to primary shoot, indicating position magnified in (H), illustrating vascular connection with the shoot (white arrow). cb - crown bud, cbc - crown bud cluster, ps - primary shoot, vr - vascular ring, x - xylem strands.

5.4.3 Experiment 2; Crown buds within a cluster; origin and ontogeny

5.4.3.1 Macroscopic features of the arrangement of buds within a cluster

On plants of 'Diva', crown buds were typically co-located as a cluster, i.e. a crown bud cluster (Figure 5.14A). Sequential removal of scale leaves of the bud cluster revealed more buds covered with scale leaves, becoming progressively smaller in size (Figure 5.14A-D). The most apical meristem of the cluster (Figure 5.14C & D) therefore, was presumed to be the apical bud of the first bud initial, which developed adventitiously (refer Section 5.4.2.3). Within the cluster, as each new crown bud became large enough to become macroscopically visible, the position of the apical bud was altered, and it was, therefore, not in the centre of the cluster. This displacement of the apical bud, due to the growth of the subsequent buds, resulted in a spiral arrangement as described below (refer Section 5.4.4.4; Figure 5.19 & Figure 5.22).

5.4.3.2 Histological studies of ontogeny of buds within the cluster

In the following paragraphs the development of the crown bud cluster is described according to the different maturity stages of the cluster, i.e. from the presence of two buds through to many buds.

Stage 1 – two buds in the cluster. At *Stage 1* the crown bud cluster was not yet macroscopically visible, but comprised two buds. At this stage an acropetal sequence of transverse sections revealed a mass of tissue present below the cluster (Figure 5.10F-I), which could not be clearly identified as stem. Above this position the primordial structures of the two buds were evident (Figure 5.10J). The smaller bud appeared to be axillary (Figure 5.10J), but a vascular connection between the two buds was not evident. Longitudinal sections of another bud cluster at *Stage 1* clearly showed both buds sharing the same scale leaf, confirming that the smaller and less differentiated bud was likely to be axillary to the apical bud (Figure 5.15A & B), albeit evidence of a vascular connection between these buds was not obtained.

Stage 2 - three buds in the cluster. At *Stage 2* the crown bud cluster was not yet macroscopically visible, but comprised three buds. The three buds were only visible when observed along different planes of axis (Figure 5.15C-E). Based on the presence of a scale leaf enveloping the other two crown buds present, the bud located at the proximal end was most likely to have been the first crown bud initiated from the apical bud of the cluster, i.e. which developed adventitiously (Figure 5.15D & F). The two

buds at the distal end were also enveloped by the same scale leaf (Figure 5.15D & E). While organised vascular connections were not visible between buds within a cluster (Figure 5.15E & F), as evident within *Stage 3*, it is possible that this was due to the buds not being arranged on a single plane of axis within the sections (Figure 5.15C-E), resulting from the distortion of the central axis as additional buds are initiated (refer Section 5.4.3.1).

Stage 3 - Four to five buds in the cluster. The crown bud cluster was macroscopically visible as a single bud at *Stage 3* (Figure 5.11A), but contained between four and five crown buds (Figure 5.11I-J). Based on the identification of a vascular ring, the cluster could be identified as having a stem axis of its own (Figure 5.11F & G). With sections progressing acropetally from the base of the cluster, the presence of three buds of varying sizes were revealed (Figure 5.11H-I). The bud in the middle, presumably the apical bud (Figure 5.11J), had visible vascular differentiation below it, connecting to the two buds at either side (Figure 5.11I). Not all buds were visible in a single longitudinal section of a crown bud cluster at *Stage 3*, as buds were located on different planes of axis (Figure 5.16). Buds were connected to the transition zone (Figure 5.16C) with visible vascular connection from the central bud to this tissue (Figure 5.16B). In contrast, vascular connections between the individual buds were not visible (Figure 5.16C & D).

Stage 4 - Six or more buds within the cluster. Although six or more buds were present within the cluster at *Stage 4* (Figure 5.17), the entire cluster was enveloped by a scale leaf (Figure 5.17A) and, macroscopically it was visible as a single, enlarged, bud. Based on the presence of the vascular ring at the distal end of the cluster, the base of the cluster could be identified as a stem (i.e. cluster stem) (Figure 5.17C & D). As noted previously, the axis of the initial bud was positioned at the side in this cluster, rather than at its centre (Figure 5.17A & B). Two buds were visible at the proximal end of the cluster stem and, with acropetal sectioning the presence of several more buds became evident (Figure 5.17B-E). Hence it was interpreted that the buds produced initially in the cluster diverged from the apex of the cluster's axis (Figure 5.17D & E). The earliest formed crown buds had well-developed primordia (Figure 5.17F & G), and more buds from the axillaries (Figure 5.17F). Vascular connections to the main axis of the first bud were not visible at the proximal end, but were visible at the distal end of the cluster's axis (Figure 5.17H).

5.4.3.3 Buds within the cluster - axillary or adventitious?

Although the first bud in the cluster initiated adventitiously, buds within the cluster had several buds enclosed within a single scale leaf, being typical therefore of axillary buds. While clear vascular connection to a main axis was not evident during early development of a cluster (*Stages 1-3*), they were evident at later stages (*Stages 3 & 4*; Figure 5.11 & Figure 5.17) and, together with developed scale leaves and vascular gaps in the main axis (Cutler et al., 2008), this observation confirmed the origins of these subsequent crown buds as axillary. Similar sized buds were apparent however, from the same mass of cells in these earliest formed buds in the cluster (Figure 5.10, Figure 5.15 & Figure 5.16). These earliest formed buds therefore appear to develop from the same meristematic tissue of the apical bud. Thus based on these sequential sections, it appears that these earliest formed buds were not connected to the apical bud via vascular connections, as found with typical axillary buds on a floral shoot (Figure 5.2). Whether this was due to compactness of the cluster stem, or whether they are different from axillary buds on a floral shoot, could not be clarified within this experiment, and was further explored in Experiment Three.

Floral shoots of gentians present an opposite leaf arrangement at a node, and an opposite decussate phyllotaxy (Bell and Bryan, 2008). At each node therefore, two axillary buds were located (Figure 5.2). Within the crown buds of the cluster it was not possible to identify a similar hierarchical arrangement however. Since the compactness of the cluster limited clear identification of hierarchical arrangement of crown buds, as presented within Experiment Three, the whole bud cluster was elongated.

Figure 5.14 Sequential dissections of a crown bud cluster on a plant of 'Diva' derived from tissue culture during its second growth cycle, i.e. 15 months from deflasking; (A - D) progressive removal of individual crown buds and scale leaves from oldest (1) to most recent (6). Numbering and age based on distance from apex and location of scale leaves.

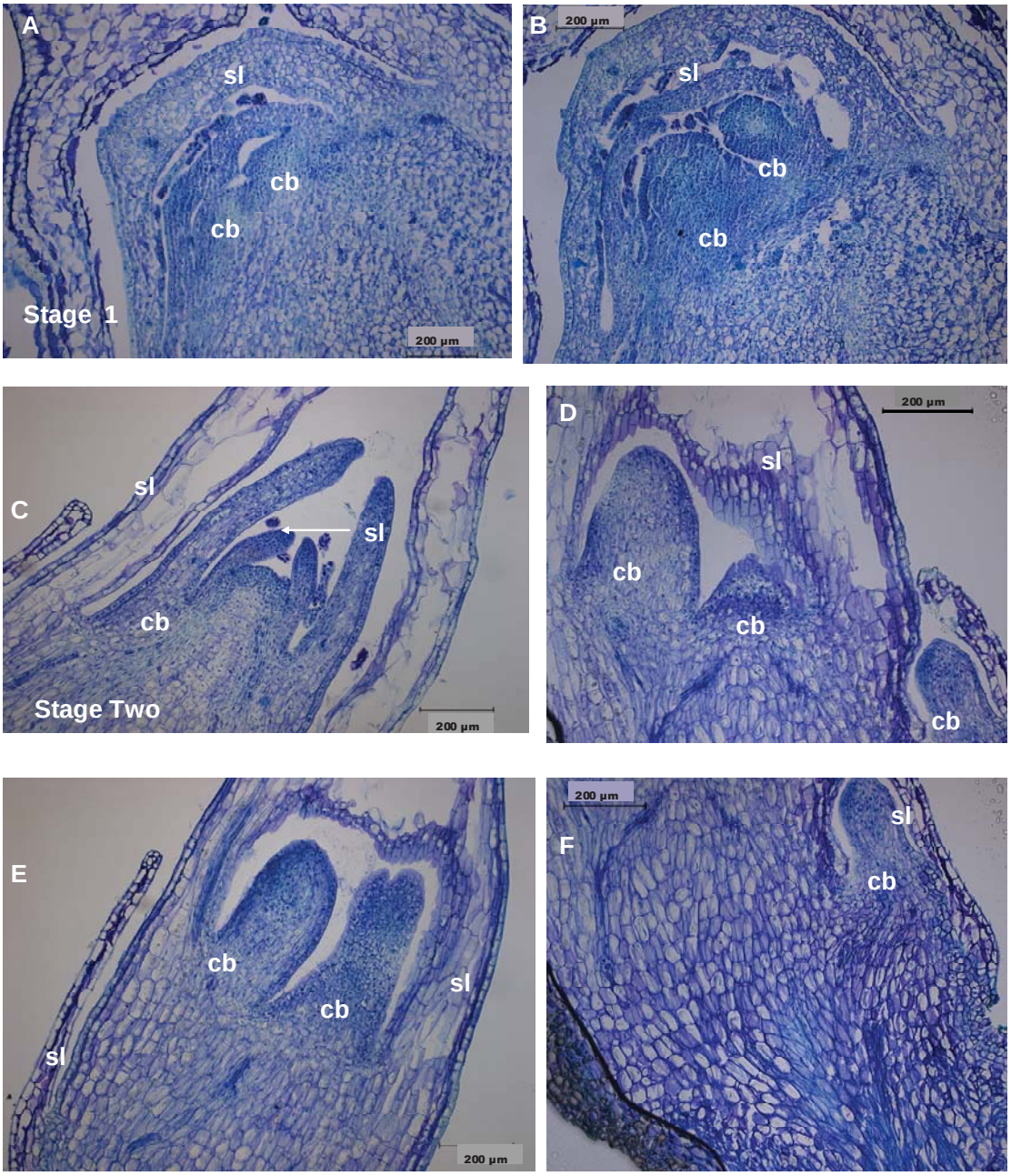

Figure 5.15 Longitudinal sections through crown bud clusters of 'Diva' during the first growth cycle following deflasking. The same bud cluster viewed along different planes of axis are presented in A-B, and another cluster in C-F, so as to show the association between buds and scale leaves, from plants with crown bud clusters at; (A-B) '*Stage 1*', i.e. two crown buds in cluster, (C-F) '*Stage 2*', i.e. three crown buds in cluster. cb - crown bud, sl - scale leaves.

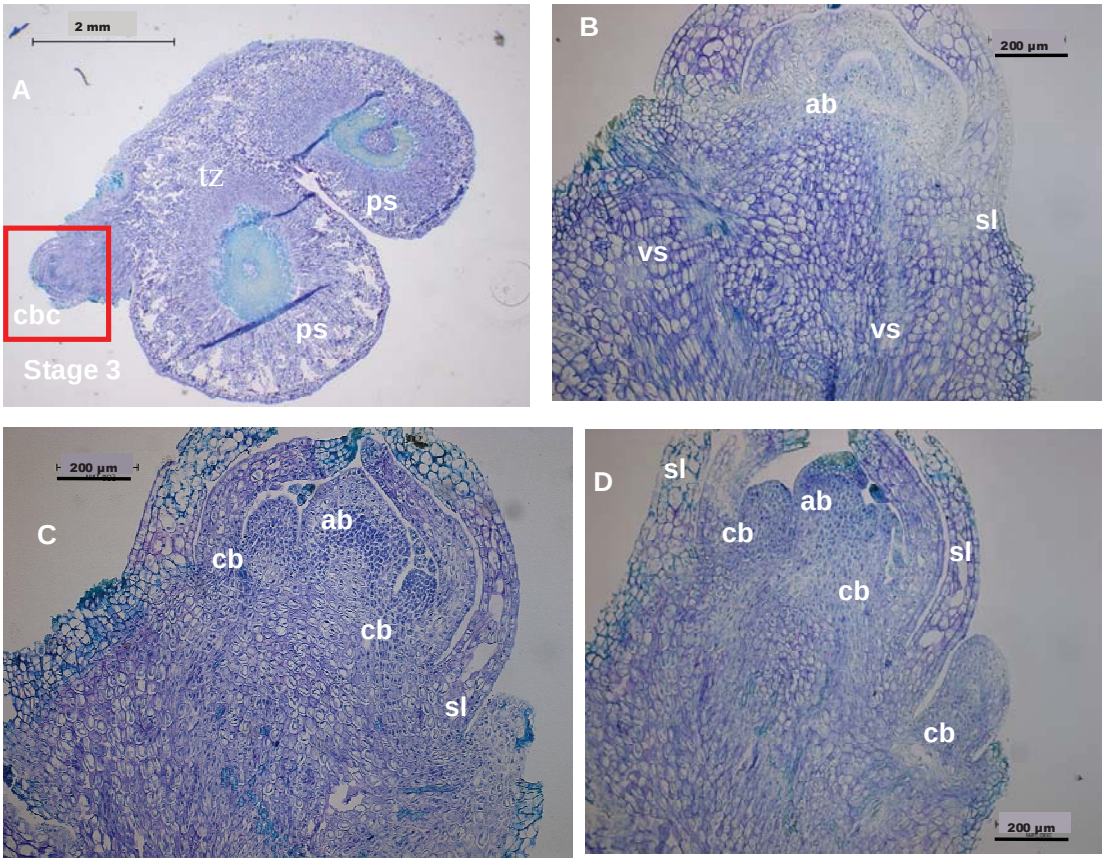

Figure 5.16 Transverse section through the transition zone of 'Diva' six months following deflasking; (A) indicating position of a crown bud cluster at *Stage 3* of development and magnified in, (B-D) a sequence of longitudinal sections indicating vascular connections between the buds and arrangement of scale leaves at different planes of axis. As indicated by staining with Toluidine Blue, tissue stained light blue are xylem vessels. ab - apical bud, cb - crown bud, ps - primary shoot, sl - scale leaves, tz - transition zone, vs - vascular strands.

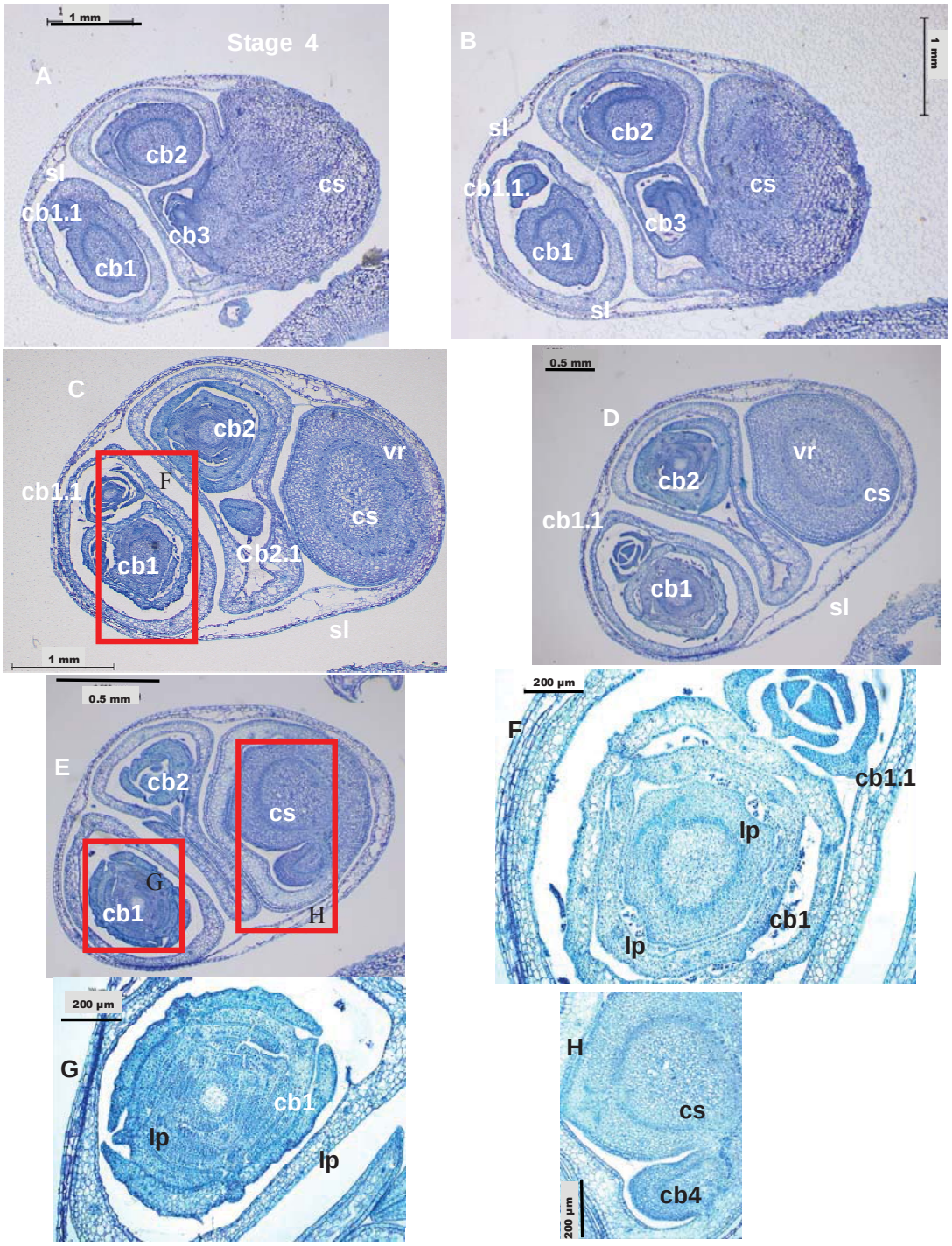

Figure 5.17 (A-E) Acropetal sequence of transverse sections of the crown bud cluster of the plant in Figure 5.12A, illustrating the arrangement of buds within a cluster at *Stage 4* of cluster development, with early initiated crown buds numbered as; cb1, cb2, cb3 and axillary buds of these buds as cb1.1 and cb2.1. Positions indicated in (C) and (E) are magnified in (F) & (G) to indicate axillary buds and illustrate the greater number of primordia within the earliest initiated bud (cb1) of the cluster and, (H) to indicate latest bud initiated from the cluster stem. cb - crown bud, cs - cluster stem, lp – leaf primordium, sl - scale leaf, vr - vascular ring

5.4.4 Experiment 3; Hierarchical arrangement of crown buds within the cluster

5.4.4.1 Macroscopic features of the crown bud cluster ± GA_3

After seven months of growth, plants of '03/04-114' developed bud clusters, with some instances of bud break and shoot emergence within the Control treatment (Figure 5.18A & D). In contrast, no shoot emergence occurred in plants treated with GA_3 but, individual buds within the cluster became spread apart on an elongated cluster stem (Figure 5.18B). In plants from the Control treatment, crown bud clusters were compact, being tightly enveloped with scale leaves (Figure 5.18D). In comparison, when treated with GA_3 plants developed an elongated, stem-like structure (i.e. cluster stem), with crown buds present along its length (Figure 5.18E). Most crown buds were present towards the distal end of the elongated cluster stem (Figure 5.18F - I), while in a few stems buds were also present in the middle. On these elongated cluster stems the scale leaves were elongated and loosely arranged along its length, and covered the crown buds at the distal end (Figure 5.18G, H & I).

By elongating the cluster stem, and separating the individual buds within a cluster, application of GA_3 facilitated determination that even the early initiated crown buds were axillary in origin within a cluster. Hence only the initial apical bud of a cluster was adventitious. As observed on elongated cluster stems (Figure 5.18), the rest of the buds within the cluster were always located in the axil of a scale leaf, being typical of axillary buds (McConnell and Barton, 1998). It is concluded therefore, that all buds within the cluster, except the initial bud, are axillary in origin.

5.4.4.2 Confocal microscopy of shoot, storage root and cluster stem ± GA_3

Within the Control treatment, transverse sections from the proximal end of the floral shoot showed secondary thickening, with xylem vessels arranged as a complete ring (Figure 5.20A and B). This vascular arrangement was considered typical of that seen in floral shoots in other species (Bowes, 1996; Chaffey et al., 2002; Evert, 2006), confirming observations made with light microscopy (Figure 5.1). Similarly, as indicated by fluorescence of the xylem vessels, storage roots in plants within the Control treatment had the expected arrangement of centrally positioned vascular tissues (Clegg and Cox, 1978; Evert, 2006) (Figure 5.20C and D), also confirming observations made with light microscopy (Figure 5.1). By contrast in the regions of the stem with bud clusters, the xylem ring had rays of xylem vessels radiating out of the ring (Figure

5.21A-C). At the proximal end of the elongated stem, lignified tissues were arranged as a circle with a larger cortex. At the distal end, stem lignification was limited to a few scattered cells only (Figure 5.21D & E), and the development of vascular connections to a bud was visible (Figure 5.21F). Compared to that evident in elongated stems, the base of the crown bud cluster in plants from the Control treatment was more disorganised. This comparative disorganisation resulted from several buds developing from the same apparent plane of axis, leading to distortion of the tissues within the stem. In contrast however, the arrangement of lignified cells was the same in both Control and GA_3-treated plants (Figure 5.21B & G). Thus it could be hypothesized that gibberellin would have activated the sub-apical meristem (Sachs, 1965; Sachs et al., 1959) to produce an elongated stem, whereas under natural conditions the sub-apical meristem would be compressed in the cluster stem.

5.4.4.3 Cluster stem

Macroscopically the tissue to which crown buds are attached, i.e. cluster stem, presented typical characteristics of a shoot when elongated with GA_3 (Figure 5.18);

- Firstly, scale leaves were present along the cluster stem, and individual crown buds were associated with scale leaves (Figure 5.18 & Figure 5.19). All scale leaves were not, however, associated with crown buds, as only one bud was present at each node (Figure 5.19).

- Secondly, transverse sections of the elongated stem presented typical secondary thickening as a ring, as observed previously on a floral shoot (Figure 5.20 & Figure 5.21). In contrast, stems were typically compressed at the base of the cluster, so a vascular ring was not clearly visible under natural conditions. It is hypothesized that several buds develop from the same position causing distortion to the cluster stem when compact. This explains the disorganised arrangement of buds visible on the microscopic sections of a bud cluster under natural conditions (Figure 5.11 & Figure 5.12).

Lignified tissue occurred as a circle at the proximal end of the cluster stem, but in samples taken further up the cluster stem, the amount of lignification was reduced (Figure 5.21) and, based on the presence of scattered xylem vessels, was most likely the latest formed (Chaffey et al., 2002). In comparison with the section at the base of the typical floral shoot (Figure 5.20), the base of the cluster stem was less organised.

Comparatively the area of non-lignified cells, especially the cortex which represents a greater amount of paranchyma cells, was larger in the stem of the cluster than a floral shoot. Given that the parenchyma cells typically serve a storage function (Bowes, 1996), it is hypothesized that the stem of the cluster is a modified form of stem, used to store carbohydrate reserves. Although not described histologically or in terms of presence of bud clusters, the structure to which crown bud clusters were attached in *Gentiana* spp has been referred to as a vertical rhizome (Ho and Liu, 2001; Ho and Pringle, 1995; Ohwi, 1965). As evident in the current study the transition zone and the cluster stem could be referred to as a vertical rhizome and, in a clonally propagated plant, this originally is derived from part of the shoot and root.

5.4.4.4 Cluster stem and the hierarchy of buds in the cluster

Acropetal dissection of bud clusters on the elongated cluster stem revealed a hierarchical arrangement (Figure 5.19) which resembled that observed in Experiment Two with non-GA_3 treated plants (Section 5.4.3.1). The elongated cluster stem however, made it visually easier to identify the relationship between buds in the cluster when treated with GA_3. While the earliest initiated buds were present individually on the cluster stem, smaller crown buds were present towards the apex as a single unit covered in scales (Figure 5.19A-C). As each bud scale was removed on this unit, two buds were visible (Figure 5.19C, D & E). One was a separate and well-developed crown bud, and the second comprised a bud containing more buds and scale leaves. It was concluded therefore that the apical meristem of the cluster was located within the latter complex.

The comparative maturity and order of arrangement of crown buds within a cluster was determined by the location of scale leaves and buds on the elongated stem, i.e. the buds at the proximal end were considered most mature. Buds were arranged in a spiral pattern along the stem axis of the cluster (first bud initial which developed adventitiously), with a single bud developing at each node at an approximate 90º angle of phyllotaxy (Figure 5.19F). Floral shoots of gentians present an opposite leaf arrangement at a node, and an opposite decussate phyllotaxy (Bell and Bryan, 2008), hence at each node, two axillary buds each of similar level of development were located (Figure 5.2). In contrast, only one axillary bud at a node was stimulated to differentiate during the development of crown buds in a cluster (Figure 5.19). Hence arrangement of two equally sized axillary buds at a node was not observed in sequential sections

(Figure 5.11, Figure 5.16 & Figure 5.17). These individual buds developed sequentially as a spiral from the proximal to distal end of the cluster stem (Figure 5.19). Each bud was positioned at an approximate 90º angle to the preceding bud. This progressive development of buds from the apex of the cluster stem, resulted in the presence of buds of different sizes and stages at any single point in time. Hence gentian plants possess two different types of phyllotaxy; one within the bud cluster and the other on floral shoots. Such variation in phyllotaxy was also noted in other plants (Bell and Bryan, 2008), e.g. *Wollemia nobilis* (Tomlinson and Murch, 2009) and *Populus deltoides* (Larson, 1975), and in some species such differences denotes a change from vegetative to reproductive phases (Poethig, 1990; Poethig, 2003). In contrast to changes in phyllotaxis within the same shoot observed in these species however, the phyllotactic arrangements within clusters and floral shoots appear to be predetermined in gentians. On the other hand, the abortion of the axillary meristem can occur even when bud primordia are at the very early stages of their formation (Bell and Bryan, 2008). If this occurred within the bud cluster, the phyllotaxy would be the same as that in the floral shoot. This latter scenario is however is considered unlikely, as the bud cluster appears consistent in developing one bud per node. While this angle of investigation was inconclusive, and was not an objective of the current study, the reason for differences in phyllotaxis within the crown bud cluster and floral shoot will be of interest for study further.

Figure 5.18 Plants of '03/04-114' following treatment application of either, (A) control or (B) foliar GA_3, after seven months. (C) control and treated plants after 10 months (7/12/09). (D) Compact bud clusters (red circle) in control treated plant. (E-I) Elongated stem with separated bud cluster, (E & F) at the distal end (red circle and white arrows), (F & G) with scale leaves (white arrows) and, (H & I) buds/shoots arising at the distal end (white arrows).

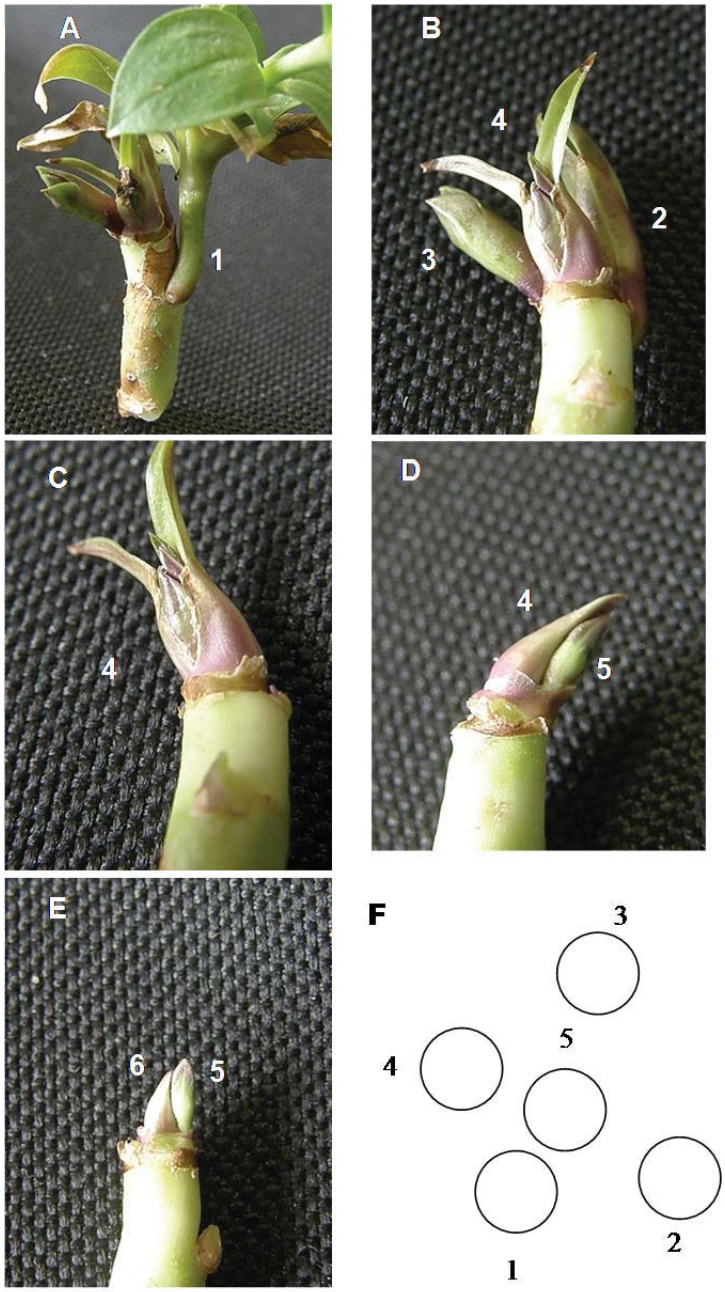

Figure 5.19 Progressive dissection of a bud cluster on an elongated stem of '03/04-114' following foliar application of GA_3; (A) non-dissected with most distal-positioned crown bud/shoot (1) emerging, (B) crown buds 2-4 revealed, (C-E) crown buds 4-6 revealed. (F) Diagrammatic illustration of arrangement of buds within the cluster (cross-sectional view); earliest formed axillary bud (1) to the last (5). More buds, including the apical bud of the stem cluster, were present within crown bud 5.

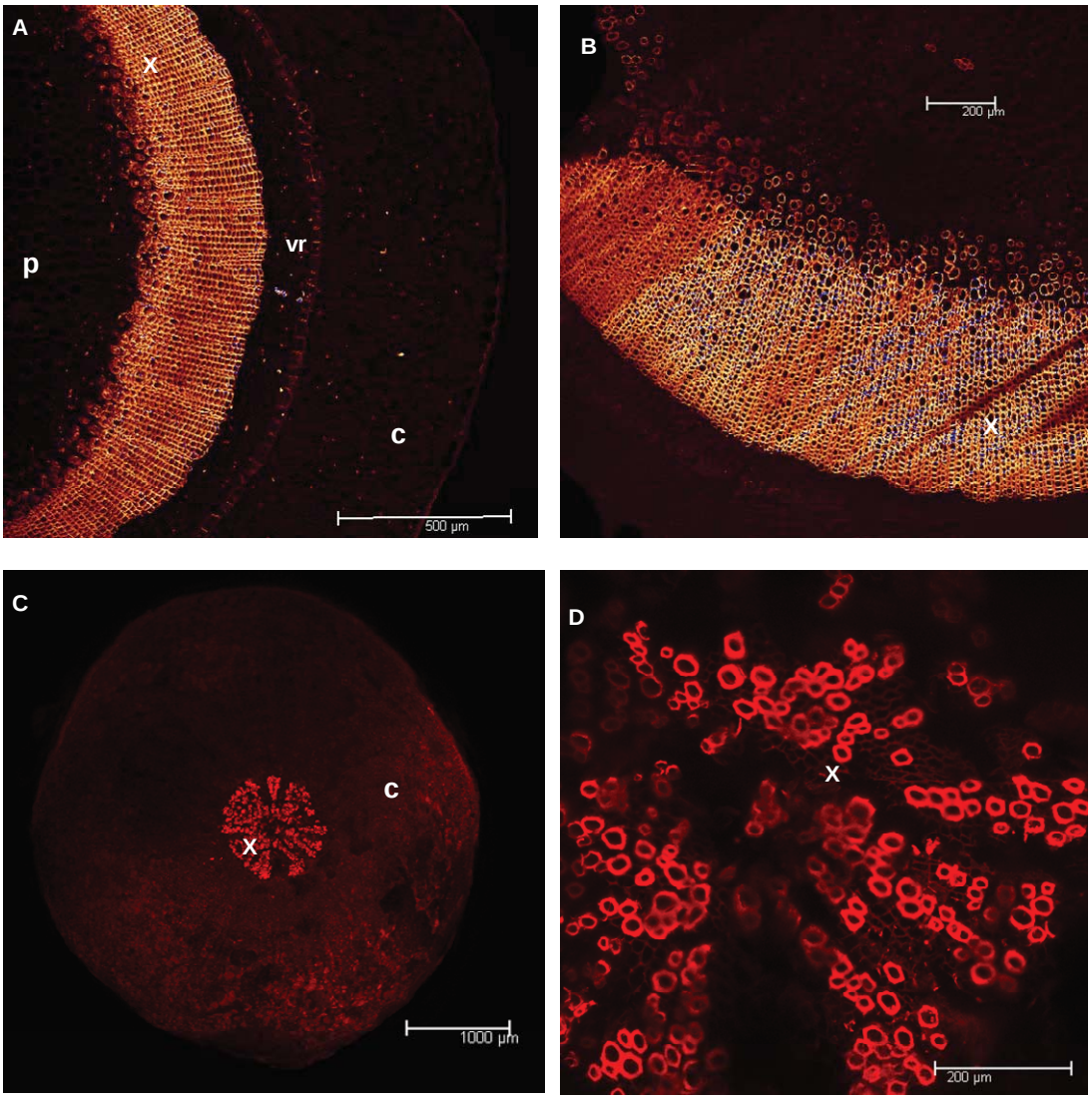

Figure 5.20 Transverse section of (A & B) floral shoot and (C & D) storage root of 'Diva' at increasing magnification; (A) section at the base of the shoot illustrating vascular ring, (B) magnified image illustrating lignified cells, (C) full section of the storage root indicating xylem vessels in the centre, and magnified in (D). Orange and red fluorescence indicates the xylem vessels. c - cortex, p -pith, vr - vascular ring, x – xylem.

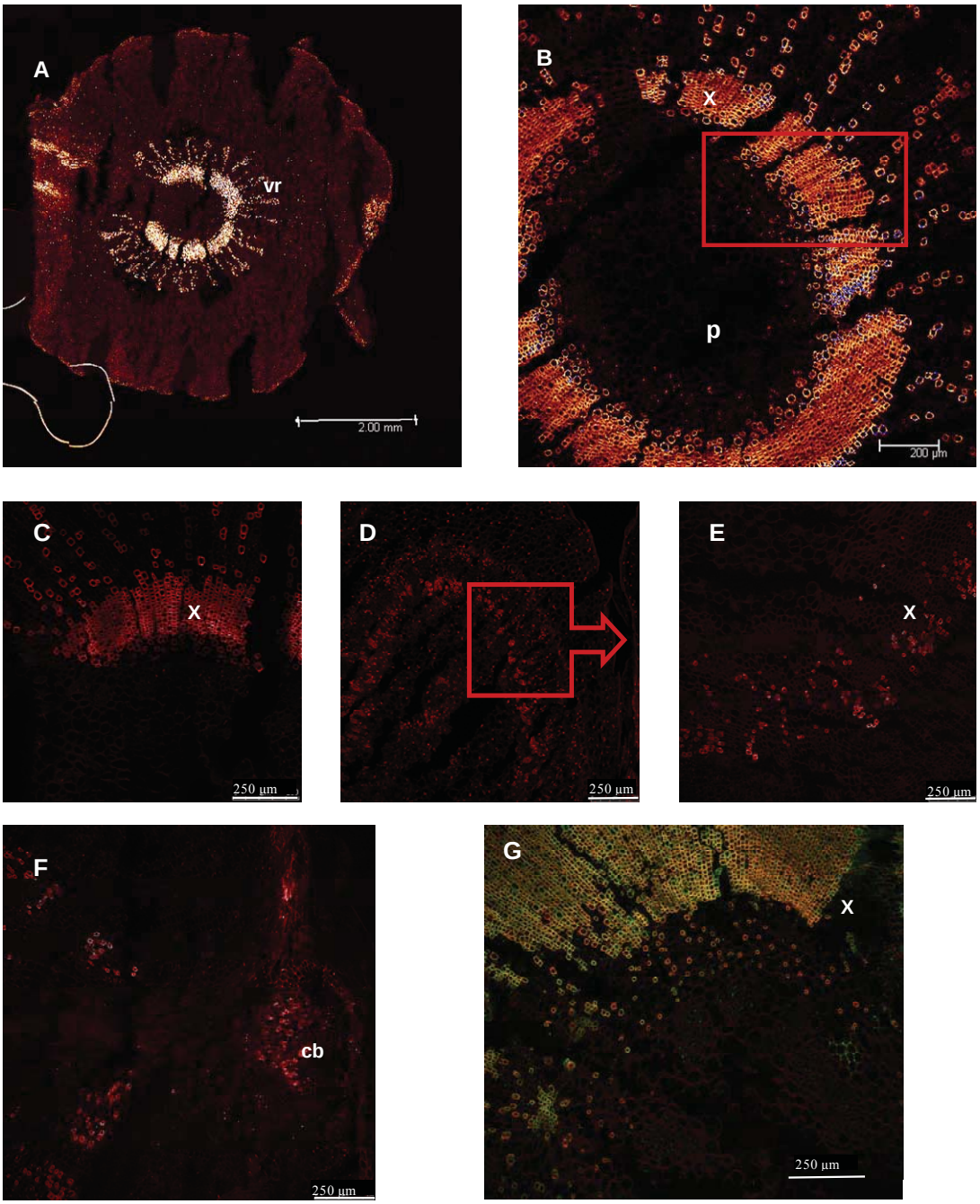

Figure 5.21 Transverse sections of the elongated cluster stem following treatment with GA_3 and control 'Diva'; (A) proximal end of the elongated stem with the centre magnified in (B) to illustrate the vascular ring similar to stem, indicating position of further magnification presented in (C), indicating xylem vessels. (D) Distal end of the elongated stem indicating position of magnified image presented within (E), indicating the arrangement of xylem vessels and, (F) possible initiation of a bud. (G) Arrangement of xylem vessels at base of the cluster in control treatment. Orange and red fluorescence indicates the xylem vessels. cb - crown bud, p - pith, vr - vascular ring, x – xylem.

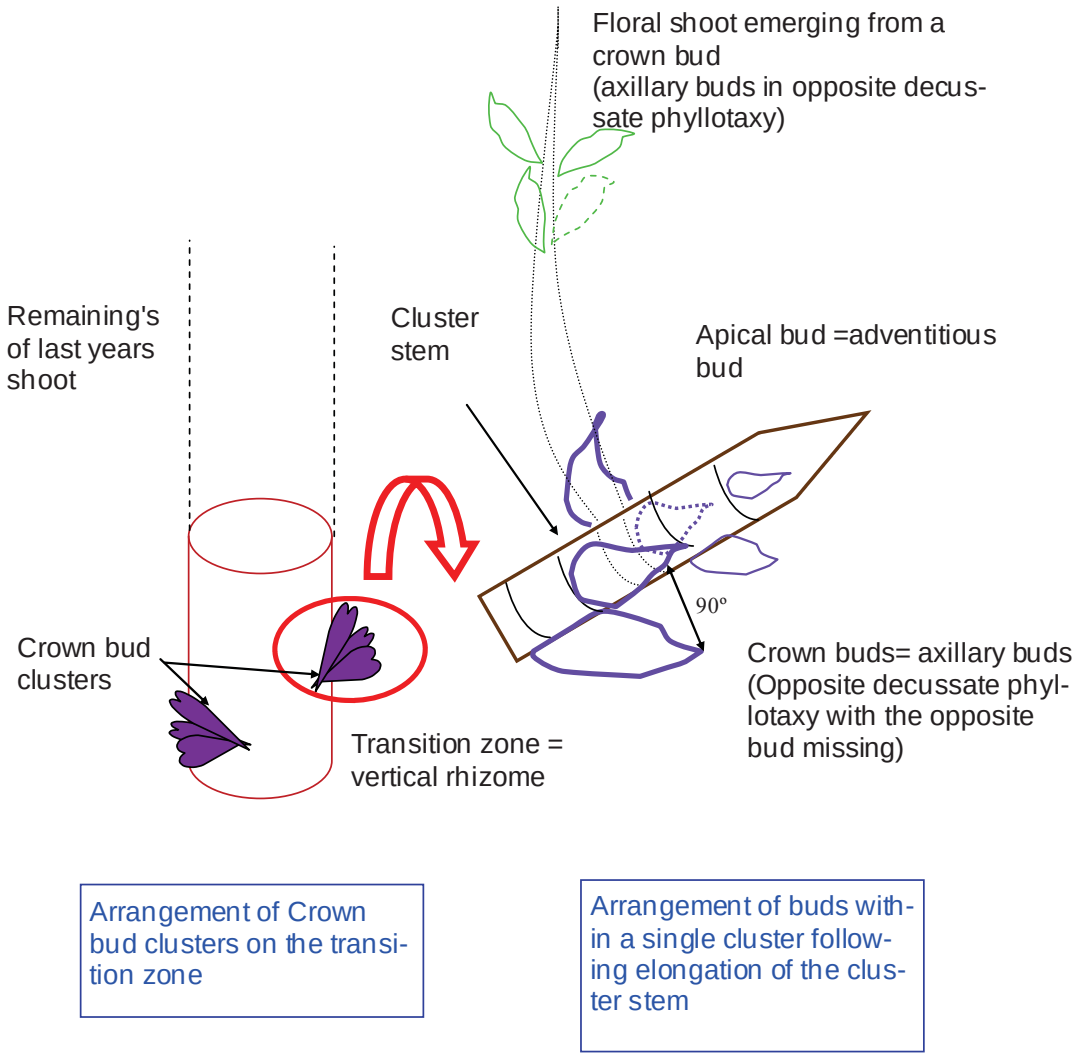

Figure 5.22 Schematic diagram illustrating the arrangement of crown bud clusters on the transition zone, and hierarchical arrangement of buds within the cluster, as apparent following elongation of the compressed cluster stem (i.e. when GA treated).

5.5 Overall Conclusion

Crown bud clusters primarily originate from the transition zone of both seedlings and clonally propagated plants (Figure 5.22). This region has been referred to as a rhizome or crown in previously published articles. Development of a crown bud cluster is a multistep process, which in subsequent chapters of this thesis will be referred to as the 'crown bud formation process'. The development of the first bud initial within the bud cluster was adventitious, but subsequent buds within the cluster developed as axillary buds from this first bud initial, resulting in the formation of a compact stem. Crown buds were arranged spirally at 90° on this compact stem, with a single bud developing at each node. Unlike in seedlings, in clonally propagated plants one extra step, i.e.

development of the transition zone from the intersection of shoot/stem and root tissues, was part of the crown bud formation process. This additional requirement during crop establishment, for plants produced via tissue culture or possibly cuttings, may also be important in other herbaceous perennials that propagate naturally with the aid of storage organs such as rhizomes in the following season. Because of the importance in establishment of an adequate number of crown buds during clonal propagation of gentians, potential factors influencing this process, and strategies to manipulate bud formation, require further study (Chapter 6 & 7).

Acknowledgement that a multistep process exists for crown bud formation, also leads to the suggestion that there could be multiple factors influencing initiation and development of buds at each step. If initiated at different times within a plant therefore, individual buds/clusters will be at different developmental stages, and will experience different environmental factors during the annual growth cycle. As a result, presumably there could be differences in time of harvest maturity for individual shoots arising within these buds differing in prehistory (Chapter 2) and, therefore, spread in time to flower. Further to that, in clonally propagated plants the time taken to develop the transition zone may differ between individual plants. If so, subsequent stages of the multistep process may also vary, leading to further differences in the timing of initiation and development of crown buds between plants. Any differences in time taken to develop the transition zone therefore, could be another factor that contributes to variation in time to flower observed between plants (Chapter 2). Hence it will be important to achieve an understanding of the factors influencing the crown bud formation process, in order to establish uniformity of planting materials, which could potentially led to narrower spread in time to flower.

At the beginning of the current study, it was not known if the origin of buds that develop into floral shoots were axillary or adventitious. From the current study it can be concluded that floral shoots develop from axillary buds, however the developmental fate of the apical buds in the cluster in subsequent growth cycles needs further investigation (Chapter 9). Although crown buds are axillary, their hierarchical arrangement within the cluster (Figure 5.22), suggests the possibility for the existence of paradormancy from the apical bud and/or from other axillary buds within the cluster and, therefore, individual buds may vary in their response. Thus the time to reach

developmental end points within the cluster and, therefore, the time to reach harvest maturity, may vary between buds, and warrants further investigation (Chapter 9).

The findings of the current research related to identification of anatomy and ontogeny of crown buds and the associated storage tissue may also be utilized to investigate morphological features of other herbaceous perennials with storage organs. It is interesting to note that the location of adventitious buds in the transition zone was associated with internal locations of vascular strands (distance to the periderm), but how this is determined within the plant will be of interest for further study (Section 5.4.1.4). Secondly, the existence of two types of phyllotaxis within the same plant was observed with differences between floral shoots and the crown bud cluster (Section 5.4.4.4); is it due to a change in phyllotaxy based on vegetative and reproductive phase change, or is it due to abortion of one bud in the axil? Hence further study is warranted on the physiological factors determining the location of adventitious bud formation, control of rhizome development and control of phyllotaxis/orthostichy. It would appear that gentians would be an interesting model plant for such studies.

Chapter 6 Crown bud formation process: preliminary screening for influence of photoperiod, temperature and growth regulators

6.1 Abstract

During the vegetative propagation of gentians there is a delay in formation of crown buds or even death of planting material, which leads to a delay in cut flower production. In order to achieve a shorter time from propagation to flower production, and to reduce plant losses, it was considered necessary to increase the certainty for formation of crown buds on the transition zone earlier in the season. Two experiments were carried out to identify the potential influence of environmental factors (photoperiodic regime and temperature) and application of growth regulators (GA_3, TDZ, ethephon, NAA and NPA) on the crown bud formation process using the gentian cultivar 'Diva'. The transfer of plant material previously devoid of any visible crown buds to naturally occurring short days, or application of either TDZ (100 ppm) or ethephon (864 ppm), increased the number of crown buds, whereas GA_3 (100 ppm) and long days inhibited bud formation. Exposure to the cooler temperatures experienced outside did not influence the crown bud formation process compared to that occurring within a heated greenhouse.

6.2 Introduction

Flowering shoots of hybrid gentians develop from overwintering crown buds produced in the previous growing season (Chapter 3). Anecdotal observations from commercial growers led to the conclusion that formation of an adequate number of crown buds in plants propagated from either tissue culture or cuttings can take longer than desired, causing a delay in the harvest of commercial cut flower production (Ed Morgan, Pers Com. Takashi Hikage, Pers Com.). Further to this, anecdotal observations have also associated the incidence of plant death, during establishment of vegetative propagules, with the failure to form these crown buds. As initiation of crown buds primarily occurs within the transition zone (Chapter 5), it was hypothesized this plant death and/or the delay in establishing an adequate number of crown buds could be attributed to either the failure or delay in initiation of crown buds in the transition zone. If, therefore, establishing plants with an adequate number of crown buds within a short timeline, and increased plant survival, was to be achieved, it was considered desirable to identify

factors influencing the crown bud formation process within young vegetative propagules.

In contrast to seedlings of gentian, for vegetative propagules the formation of the transition zone was an additional prerequisite for initiation and development of crown buds (Chapter 5). Crown buds were found to originate adventitiously as clusters on the transition zone, with buds within the cluster being axillary. Prior to the actual appearance of these crown buds therefore, a multistep process is involved, starting from development of the transition zone, initiation of an adventitious bud initial for each cluster, initiation of axillary buds within the cluster, and development of the individual crown buds (Chapter 5). As a result of this being a multistep process, multiple factors related to either the plant and/or environment could influence initiation and development of crown buds. During the current study appearance of individual crown buds (end product of the crown bud formation process) was used in evaluation of potential factors influencing this multistep process.

While environmental conditions could potentially be one factor influencing initiation and development of crown buds, statistically validated data pertaining to gentians has not previously been published. In contrast, the environmental influences on the induction of dormancy (presumably endodormancy) in woody perennials has been reported widely (Anderson et al., 2010; Arora et al., 2003; Faust et al., 1997; Rohde and Bhalerao, 2007), and has generally been associated with short photoperiods and declining temperature in autumn (Ruttink et al., 2007). Most of these studies have however focussed on apical and axillary buds which were pre-existing prior to dormancy induction and, therefore, do not directly pertain to the multistep process encompassing induction of the bud initial through to development of individual crown buds, which is of relevance in the current thesis. Despite this limitation, the above studies present evidence of the potential requirement for environmental stimuli in the final stages of the multistep process of development of a crown bud. Since with hybrid gentians crown buds were also found to be dormant prior to emergence (Chapter 3 & 4), and the buds being formed in the previous growth cycle, it was considered worthy of investigation whether any such environmental factors regulating development of dormant buds in other species could have an impact on initiation and development of crown buds in hybrid gentians. Within the series of experiments presented in this

chapter, the potential involvement of photoperiod and temperature on the crown bud formation process was, therefore, investigated.

As evident in other plants, the response to photoperiod is typically dependent on the latitude that the plant has adapted to grow at, i.e. ecotype (Reeves and Coupland, 2000). *In vitro*, genotypes of *Gentiana scabra* Bunge, initiated more crown buds compared to varieties of *Gentiana triflora* Pall (Anonymous, 1988), indicating a potential genetic source of variation in the number of crown buds in gentians. The hybrid gentian cultivars 'Diva', 'Starlet' and 'Spotlight', used for experiments presented in this thesis, have *G. scabra* or *G. triflora* as dominant parental lines. *G. scabra* is endemic to latitudes of 30°N to 54°N (Ohwi, 1965), with corresponding maximum photoperiods of 14:00 h:min to 17:00 h:min in June, and a minimum of 7:00 h:min to 10:00 h:min in December (Lammi, 2005). In contrast *G. triflora* is endemic to latitudes of 34°N to 54°N with, maximum photoperiods of between 14:25 h:min and 17:00 h:min in June and, a minimum between 7:22 h:min and 10:00 h:min in December (Lammi, 2005). At the commencement of the current research the critical photoperiod for initiation and development of crown buds in parental lines, or presently cultivated varieties, were not known and, therefore, the potential influence of photoperiod was considered logical for investigation. Based on the preceding information however, the minimum photoperiod for both parental lines encountered during short days, i.e. 7 h to 10 h, translates to a critical duration of dark ranging from 14 h to 17 h. Assuming the hybrids used in the current experiments have inherited any photoperiodic responses characteristic of their parental lines, a period of 2 h of night-break lighting (between 23:00 HR and 01:00 HR) was considered sufficient to achieve a long day environment in Palmerston North (i.e. shortest natural day length of 9 h translates to 15 h dark, which splits into two periods of 6 h 30 min with night-break lighting). While it was not possible to identify the critical photoperiod for all hybrid gentians during the current study, the potential influence of the naturally occurring shorter days and longer days, if any, on the crown bud formation process, was considered worthy of investigation in at least one cultivar of interest to growers in New Zealand (NZ).

Photoperiod was found to influence assimilate partitioning, with increased partitioning below-ground during short days compared to long days in both *Asparagus officinalis* L. (Woolley et al., 2001) and *Gladiolus grandiflorus* Andrews (Shillo and Halevy, 1981). Although a correlation between photoperiod and partitioning of photo-assimilates has

not been reported in gentians, increased root growth has been associated with an increased number of buds (presumably crown buds) (Kawakami and Shimonaka, 1996). Since, formation of the transition zone was the first stage of the multi-step process of initiation of crown buds (Chapter 5), it was considered possible that assimilate partitioning to below-ground organs could promote development of the transition zone and, therefore, crown buds. Thus as evaluated within the experiments presented within this chapter, it could be hypothesized that the conditions that promote assimilate partitioning to below-ground growth, may also promote the crown bud formation process either directly or indirectly.

There is no existing consensus regarding the influence of temperature on the crown bud formation process in gentians. Based on technical reports from Japan, conditions that promoted formation of crown buds in gentian plants while *in vitro* was 20 °C compared to 25 °C (Sato, 1988). While these reports did not report the influence of these conditions on subsequent growth, i.e. after deflasking, this temperature response is in contrast to the greater number of crown buds at relatively higher greenhouse temperatures for plants in their second growth cycle *in vivo* (Chapter 3). Hence, so as to identify any influence of growing environment temperature on the crown bud formation process, within the current study utilisation of two different temperature regimes was considered worthy of further examination.

Gibberellic acid (GA_3) has repeatedly been reported to promote growth of gentians *in vitro* (Fiuk and Rybczyski, 2008) and to reduce the period following propagation *in vitro* till establishment (at 100 ppm) in the open field (Abe and Nishimura, 2001; Odaira, 1999; Okayama-Ken and Nogyo, 2003). Despite these previous reports, the role of GA_3 in influencing initiation and development of crown buds on vegetative propagules either *in vitro* or *in vivo*, has not been specifically addressed nor quantified. In contrast however, as reported with true seedlings of gentians *in vivo*, the application of GA_3 (100 ppm) resulted in increased growth of plants and, increased number of *"over-wintering buds"* (presumably crown buds) (Okayama-Ken and Nogyo, 2003). Given the non-existence of peer-reviewed publications related to application of GA_3 to vegetative propagules, during the current study the potential role of exogenous application of GA_3 on the crown bud formation process was investigated. A rate of 100 ppm was chosen as gentians have previously been shown to respond to this concentration (Okayama-Ken and Nogyo, 2003).

Cytokinin is the most frequently reported growth regulator with regard to initiation and development of adventitious buds, both *in vivo* and *in vitro* (Murashige, 1974; Tezuka et al., 2011; Villalobos et al., 1985). During the current study therefore, the potential influence of exogenous application of cytokinins was investigated. Compared to other synthetic cytokinins, TDZ has been found to promote initiation of adventitious roots/buds, both *in vivo* and *in vitro*, in a range of plants including gentians. In cultivars of *G. triflora,* production of adventitious buds was increased from leaf, shoot or root explants *in vitro* when thidiazuron (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-yl urea; TDZ) was applied, compared to other common cytokinins like benzyladenine (BA), or zeatin (Hosokawa et al., 1996). Peer-reviewed publications pertaining to the response of gentians *in vivo* were not present, but with *A. officinalis*, N_1-(2-chloro-4-pyridyl)-N_3-phenylurea (CPPU,a synthetic, cytokinin-like compound) applied as a foliar spray (5 ppm) promoted initiation of buds forming on rhizomes (Ku et al., 2005). Additionally, TDZ applied to 3-yr-old plants of *Panax quinquefolium* L., as a foliar spray (125 ppm) *in vivo*, promoted root growth and initiation of adventitious buds from roots (Proctor et al., 1996). With the absence of reports about the use of TDZ on gentians *in vivo*, TDZ at 100 ppm was used initially, as reported in *P. quinquefolium* (Proctor et al., 1996).

The direct influence of auxin on initiation and development of crown buds has not been reported, but in the presence of cytokinins the auxin, 1–naphthaleneacetic acid (NAA), promoted initiation of adventitious buds on gentians *in vitro* compared to the other common auxins, indole-3-acetic acid (IAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) (Hosokawa et al., 1996). Although *in vivo* application of auxin transport inhibitors has not been reported in gentians, in *Euphorbia esula* L. 1–N–naphthalphthalamic acid (NPA 10000 ppm) applied to leafless plants resulted in increased initiation of adventitious buds compared to plants with leaves (Horvath 1999). It was suggested that there was a potential inhibition of buds in *E. esula* due to auxin derived from leaves. Given that the bud initials for crown buds in gentians are adventitious (Chapter 5), in order to identify whether auxins inhibit initiation and development of crown buds, the application of both NAA and NPA were investigated during the experiments reported here. NPA was used as a foliar spray (Horvath, 1999), as application of NPA (1000 ppm) as a ring with lanolin did not influence initiation of adventitious buds in *Populus tremuloides* Michx. (Wan et al., 2006). In the absence of any previous study relating to

gentians, a concentration of 100 ppm was used in application of both NAA and NPA during these preliminary investigations.

Compared to other growth regulators, exogenous application of ethylene has not commonly been reported as influencing initiation of buds in other plant species, but has been noted in some preliminary technical reports regarding gentians (Morgan and Debenham, 2008; Sato et al., 1988). Application of the ethylene-releasing compound ethephon (2-chloroethylphosphonic acid; 100 ppm applied as a foliar spray) increased the number of crown buds and advanced the date of appearance of crown buds (Morgan and Debenham, 2008). Similarly, concentrations of 1000 ppm and multiple applications (seven times fortnightly) has also been reported to increase the rate of appearance of buds (presumably crown buds) in gentians (Sato et al., 1988). During the current experiments exogenous application of ethephon at a concentration of 100 ppm (as also used previously), and at the recommended dosage for commercial application (864 ppm), were applied as a single application, in order to identify any potential impact on the crown bud formation process.

Within the series of experiments presented in this chapter, a range of growth regulators and key environmental factors (photoperiod and temperature) were screened to determine their potential involvement in the crown bud formation process in vegetative propagules of at least one cultivar of interest to growers in NZ. Using the cultivar 'Diva', the specific objectives were to identify the influence of;

- photoperiod, temperature and GA_3 on the crown bud formation process.
- application of TDZ, NAA, ethephon or NPA on the crown bud formation process, under long days.

6.3 Materials and methods

6.3.1 General management of plants

Plants of the gentian cultivar 'Diva' were propagated by tissue culture at The New Zealand Institute for Plant and Food Research (Palmerston North, NZ; 40°20'S175°60'E) using nodal cuttings. Plants were kept in a culture room at 20 ºC with 16 h of lighting from cool white fluorescent bulbs (41 μmol s^{-1} m^{-2} PAR at foliage height). Plants were deflasked between 19th and 26th March, 2008, into 60-cell trays (45 ml cell volume) containing a bark:pumice media and placed in a fog tent with base

heating (20 ºC) for 13 days before transferring to an open mist bed. Cell trays were transferred to one of two greenhouses after four weeks growth (natural progression of day length during this period was from 12:11 h:min to 11 h) between 9th and 14th April (heated at 15 ºC, ventilated at 20 ºC) so as to avoid accumulation of chill units. The two greenhouses supplied one of two photoperiodic regimes (refer Section 6.3.2).

Throughout the two experiments plants were irrigated using a drained capillary system, which supplied between 50 and 60 ml of water per plant per day, supplemented with one overhead watering per week. A liquid feed of half strength Peters General Purpose® foliar fertilizer (20N–8.7P–16.6K; Scott's Australia, NSW) was supplied once a week, while in cell trays. After a root plug was established (21st May 2008), plants were potted into black polythene bags (1.7 L) using a growing medium of (CAN fines A grade bark 50%; bark fibre 30%; pumice 7 mm 20%) with 1 kg m^{-3} serpentine super, 150 g L^{-1} dolomite, 200 g L^{-1} 8-9 month Osmocote® (16N–3.5P–10K, Grace-Sierra International, The Netherlands), and 100 g L^{-1} 3-4 month Osmocote® (15N–4.8P–10.8K), prior to treatments with growth regulators on 28th May 2008.

For both experiments plants were selected for evenness of size and no visible crown buds on the region destined to become the transition zone. At the time of commencement of treatment application, plants typically comprised of a single primary shoot with adventitious roots and no visible transition zone or flower buds.

6.3.2 Experiment One: Effect of photoperiod, temperature and GA_3

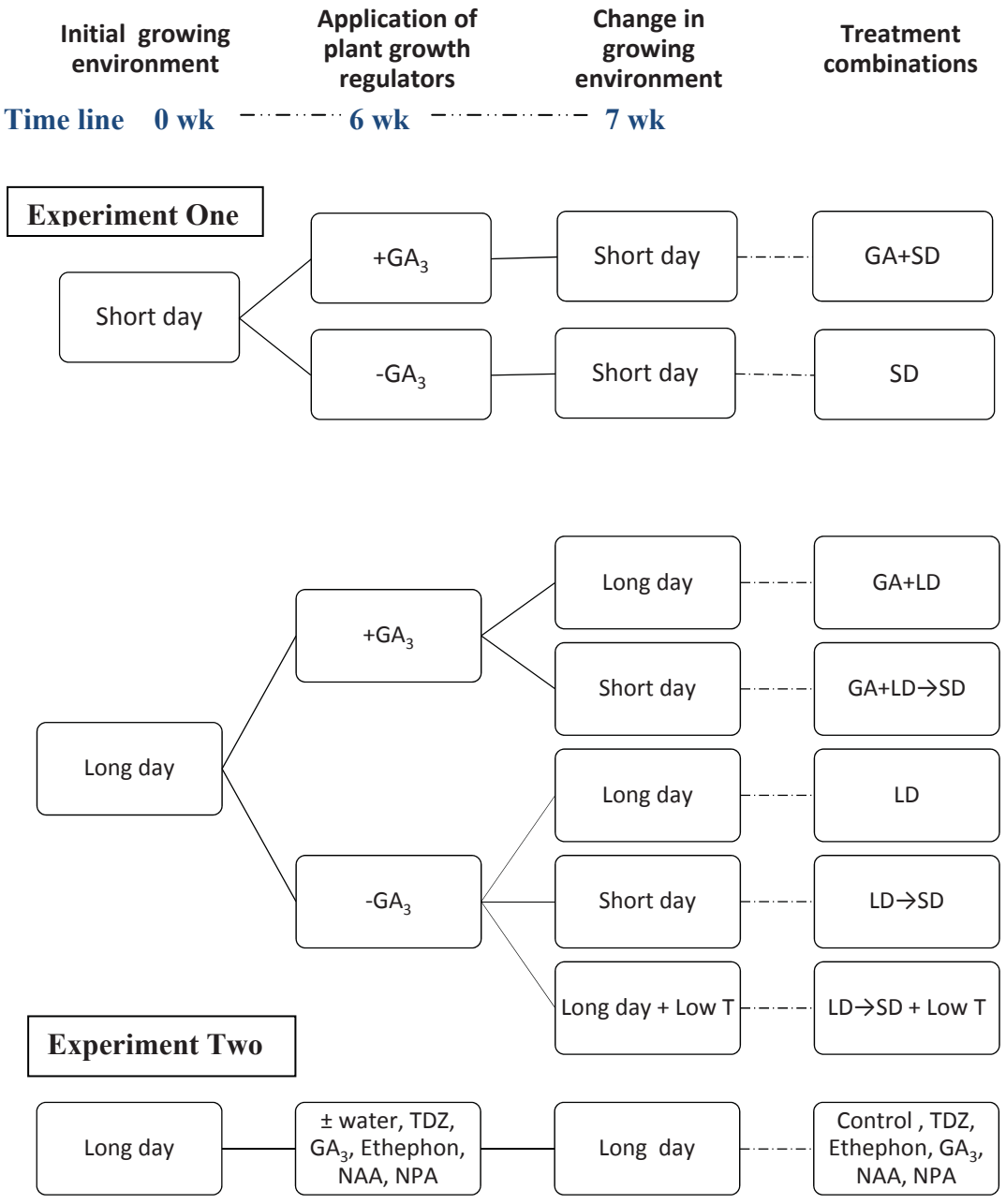

Figure 6.1 Schematic representation illustrating the composition of treatments applied within Experiments One and Two, commencing from the transfer to greenhouses at 0 wk following deflasking and growth in fog tent.

Between 9th and 14th April 2008 plants were grown under one of two photoperiodic regimes (Figure 6.1), either;

- Short photoperiodic regime (SD; natural progression of day length experienced in autumn through to spring ranging from 11 h to a minimum 9:18 h:min in June, and subsequently increasing to 13 h in October) (Lammi, 2005), or

- Long photoperiodic regime (LD; 2 h night break lighting at 4.585 µmol s^{-1} m^{-2} from 23:00 to 01:00 HR during the same natural progression of day length experienced in autumn through to spring).

After six weeks of growth under one of the two photoperiods (28th May 2008), one of two concentrations of GA_3 (OlChemIm Ltd., Czech Republic) were applied (Figure 6.1). GA_3 was applied onto the foliage, exposed crown and the surface of the growing medium, as a spray to run-off (≈ 50 ml per plant) at either 100 ppm (+GA) or 0 ppm (-GA; Control). GA_3 was prepared by first being dissolved in 10 ml 95% ethanol, and made up to the required volume with distilled water together with 0.05% Tween 20 (Sigma CAS No. 9005-64-5) as a surfactant. The Control treatment (-GA) comprised water, ethanol and Tween 20.

One week following the foliar application of GA_3 (4th June 2008; 7 wk), plants under LD were transferred to either the SD (LD→SD) or LD regime, while those under the SD regime remained within the SD regime (Figure 6.1). The natural photoperiod at the commencement of this change was 9:24 h:min. At the same time, additional to LD→SD treatment which was in the greenhouse, a set of plants from the LD environment, treated with 0 ppm GA_3, was transferred outside to experience the comparatively low temperatures and natural photoperiodic regime (LD→SD + Low T). During the course of the experiment mean monthly temperature in the outside environment varied between 6.8 °C in June to 17 °C in November, while the temperature in the greenhouses remained above 16 °C throughout the experiment.

6.3.3 Experiment Two: Effect of GA_3, TDZ, NAA, ethephon and NPA under long days

All plants were grown in a greenhouse under long days for six weeks prior to application of one of five plant growth regulators (refer Section 6.3.2; Figure 6.1). The five growth regulator treatments comprised either; GA_3, TDZ (Sigma-Aldrich), NPA (synthesized in the lab using 1-naphthylamine) or NAA (Sigma- Aldrich), each at rates

of either at 0 ppm or 100 ppm, or ethephon (commercial preparation of Ethrel 48™; 480 g L^{-1} ethephon; May & Baker Agrochemicals, NZ) at either 0 ppm, 100 ppm or 864 ppm. GA_3 was prepared or applied as described in Section 6.3.2. The treatment solutions of NPA and NAA were prepared by first being dissolved using 10 ml ethanol, before adding distilled water to the required volume. All growth regulators were applied as a spray to the foliage, exposed crown and the surface of the growing medium, to run-off (≈ 50 ml per plant) with 0.05% Tween 20 as a surfactant.

6.3.4 Experimental design, data collection and analysis

Plants were destructively harvested 5 months (3rd November 2008) after treatment with growth regulators, i.e. 6½ months after commencement of photoperiodic treatments. The original shoot of the plant at the time of deflasking was identified as the primary shoot, axillary shoots arising from the primary shoot were identified as secondary shoots, and any axillary shoots arising from these secondary shoots as tertiary shoots. During the course of the two experiments crown buds which formed on the transition zone sometimes developed into shoots, and were identified as crown shoots (Figure 3.2).

When destructively harvested, for each plant the number of crown buds, crown shoots, secondary and tertiary shoots were counted. Due to the fact that in some treatments crown buds developed into crown shoots, the combined number of crown buds and crown shoots per plant was also analysed as a variable. Primary storage roots, which developed at the proximal end of the transition zone, were also counted. For the primary shoot, at the time of destructive harvest the length, number of nodes, and nodes with secondary (i.e. axillary) shoots were recorded. Dry weight measurements were recorded of; primary storage roots, primary shoots, secondary shoots, tertiary shoots, crown shoots and crown buds. The quality of crown buds was also assessed based on their diameter.

All experiments comprised a Completely Randomised Design, utilising six single-plant replicates. Data were analysed using the general linear models procedure of the Statistical Analysis System (SAS) software version 9.2 (SAS Institute, Cary, NC, USA). Data were either normally distributed or approached normality to the extent that transformations did not alter the statistical outcome. Mean comparisons were conducted using the LSD test (5%).

6.4 Results

6.4.1 Overview

While there were no visible crown buds evident at the beginning of the study, as detailed in the following sections, in some treatments a variable number of crown buds developed and emerged as crown shoots. The treatment influence on the crown bud formation process was evaluated based on the final step, i.e. appearance of crown buds and/or crown shoots on the transition zone.

6.4.2 Experiment One: Effect of photoperiod, temperature and GA_3

Within non-GA treated plants, those remaining continuously under LD had the lowest combined number of crown buds and crown shoots (Figure 6.2; $P < 0.05$). In contrast, numbers were greatest within plants under the LD→ SD regime in both the heated greenhouse (LD→SD) and those moved outside (LD→SD + low T). While the combined number of crown buds and crown shoots was more than twice as great in plants continuously grown within naturally occurring short day regime (SD), compared with the LD regime it was significant only at $P < 0.1$. Crown buds produced in the LD→SD treatment, at either temperature (i.e. both in greenhouse and outside), were of similar in size and colouration of bud scales (Figure 6.7) to primary-crown buds observed previously (Chapter 3).

For plants treated with GA_3, a lower number of crown buds or crown shoots were evident under LD, LD→SD or SD environments, compared to treatments comprising LD→SD without GA_3 ($P < 0.05$) which had at least 2 more. In all treatments without GA_3 a proportion of crown buds emerged as crown shoots, however none of the treatments with GA_3 resulted in crown buds emerging as shoots (Figure 6.2). In contrast, as evident by the combined dry weight of primary, secondary and tertiary shoots, application of GA_3 to plants continuously under either the SD or LD regime resulted in more above-ground growth compared to all other treatments ($P < 0.001$; Figure 6.3). This increase was mainly due to the development of secondary and tertiary shoots, as evident by the number (Figure 6.4 and Table 6.1) and dry weight (Figure 6.3; $P < 0.001$), which was 7 to 8 times greater than treatments without GA_3 under the same environmental conditions. In contrast to the weight of secondary and tertiary shoots, the weight of primary shoots did not differ between treatments, except in plants grown

outside (LD→SD + low T), which also had a lower dry weight of total above ground shoots ($P > 0.05$).

Plants grown continuously within the naturally occurring SD photoperiodic regime, and those treated with GA_3 under either the SD or LD photoperiodic regime, resulted in the development of greater dry weight of storage roots (Table 6.1; $P < 0.05$). The number of storage roots was also greatest in plants grown under the SD photoperiodic regime. Within those plants not treated with GA_3, those which remained within the LD photoperiodic regime had half the number of storage roots compared to treatments under SD and outside (LD→SD + low T) growing environments (Table 6.1). Among plants treated with GA_3, those within the LD→SD photoperiodic regime had less dry weight above ground, fewer storage roots, and a lower number of secondary shoots ($P < 0.05$).

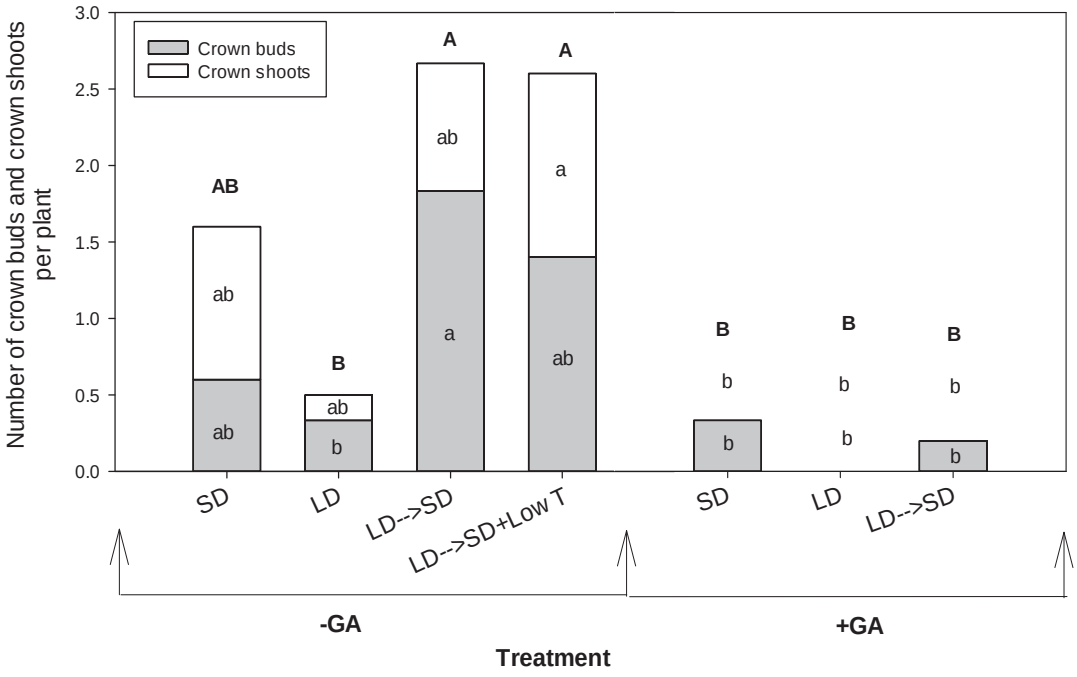

Figure 6.2 Number of crown buds, crown shoots, and their combined total per plant for 'Diva' 6½ months from the start of treatment application (Experiment One). Treatment abbreviations as detailed in Figure 6.1. Means, with the same letter are not significant at $P \leq 0.05$ (LSD) for each variable located in the order from the base of the stack bar; i.e. lower case letters for number of crown buds or crown shoots, and upper case letters for the combined number.

Table 6.1 Number of; storage roots, secondary and tertiary shoots, and the dry weight of storage roots, per plant for 'Diva' 6½ months from the start of treatment application (Experiment One).

Treatment[x]	Number of storage roots [z]	Dry weight of storage roots (g) [z]	Number of secondary shoots [z]	Number of tertiary shoots [z]
SD	13 ± 2 [a]	0.59 ± 0.18 [a]	1 ± 1 [b]	0.2 ± 0 [b]
LD	5 ± 1 [c]	0.17 ± 0.0 [c]	1 ± 1 [b]	1 ± 1 [b]
LD→SD	8 ± 1 [bc]	0.28 ± 0.08 [bc]	2 ± 0 [b]	0.5 ± 0.5 [b]
LD→SD + Low T	11 ± 2 [ab]	0.30 ± 0.12 [abc]	2 ± 1 [b]	0 [b]
GA+SD	10 ± 2 [abc]	0.59 ± 0.14 [a]	7 ± 1 [a]	2 ± 1 [ab]
GA+LD	10 ± 2 [abc]	0.52 ± .04 [ab]	8 ± 1 [a]	3 ± 1 [a]
GA+LD→SD	6 ± 1 [c]	0.17 ± 0.03 [c]	3.6 ± 1 [b]	1 ± 1 [ab]

[z]within the same column, treatment values followed by the same letter are not significantly different at $P \leq 0.05$ (LSD)

[x] Treatment abbreviations as detailed in Figure 6.1

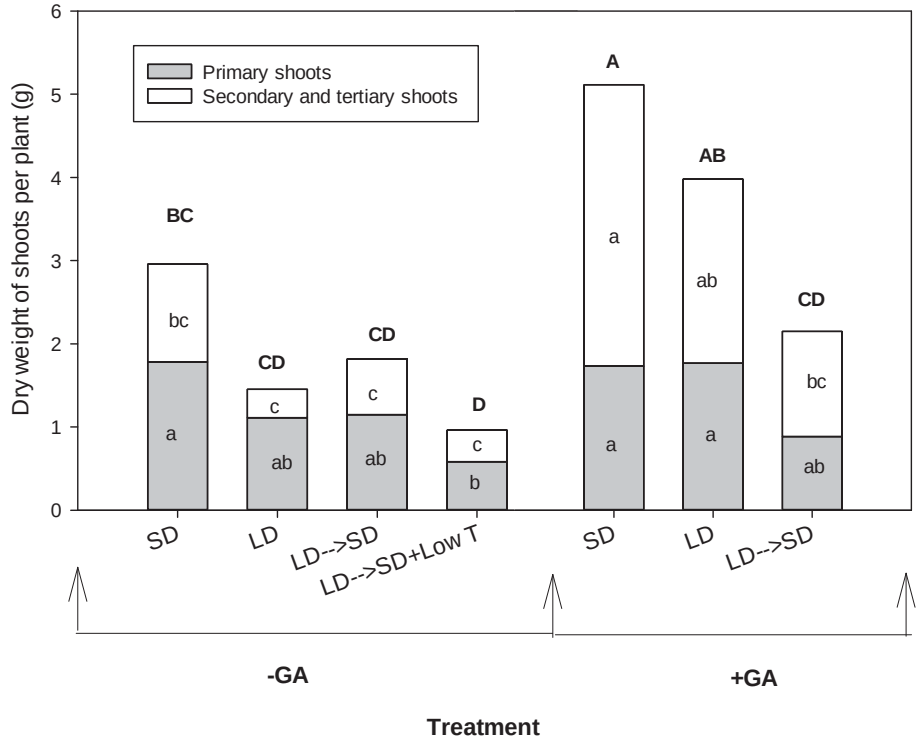

Figure 6.3 Dry weight of primary, secondary and tertiary shoots (combined) per plant for 'Diva' 6½ months from the start of treatment application (Experiment One). Treatment abbreviations as detailed in Figure 6.1. Means with the same letter are not significant at $P \leq 0.05$ (LSD) for each variable located in the order from the base of the stack bar; i.e. lower case letters for dry weight of primary or secondary and tertiary shoots, and upper case letters for the combined weight of all shoots.

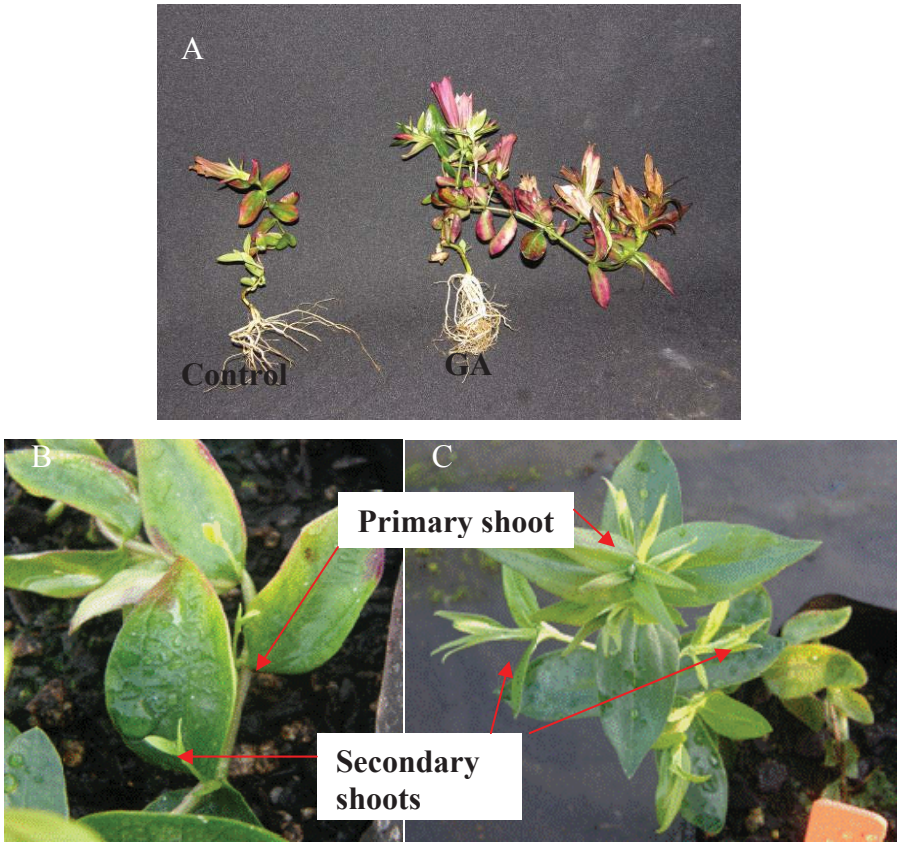

Figure 6.4 (A) Typical levels of growth for plants of 'Diva' 6½ months from treatment with (GA) or without (Control) GA_3, under long days. (B & C) Level of development of secondary shoots from the axillary buds on the primary shoot, as apparent from two positions along the primary shoot, 4 weeks following application of GA_3 (Experiment One).

6.4.3 Experiment Two: Effect of GA_3, TDZ, NAA, ethephon and NPA under long days

Within the LD photoperiodic regime, 5 months following treatment application, plants treated with plant growth regulators, as well as those within the Control treatment, developed crown buds at the transition zone. None of the plants treated with NAA or ethephon at 100 ppm, however, survived. As a result, therefore, only data from one of the ethephon treatments (i.e. 864 ppm) were subsequently used for data analysis.

Compared to all other growth regulators, application of TDZ produced the greatest combined number of crown buds and crown shoots, resulting in 10 times more buds than those within the Control treatment (Figure 6.5; $P < 0.001$). Although not significantly different, the combined number of crown buds and crown shoots produced by plants treated with ethephon was also high, being six times greater than that achieved by those within the Control treatment. Development of crown shoots was also greater in

plants treated with TDZ or ethephon than in Control and GA_3 treatments (Figure 6.5). Although the number of crown buds was greater in plants treated with TDZ, the buds were typically all less than 1 mm in diameter (Figure 6.7).

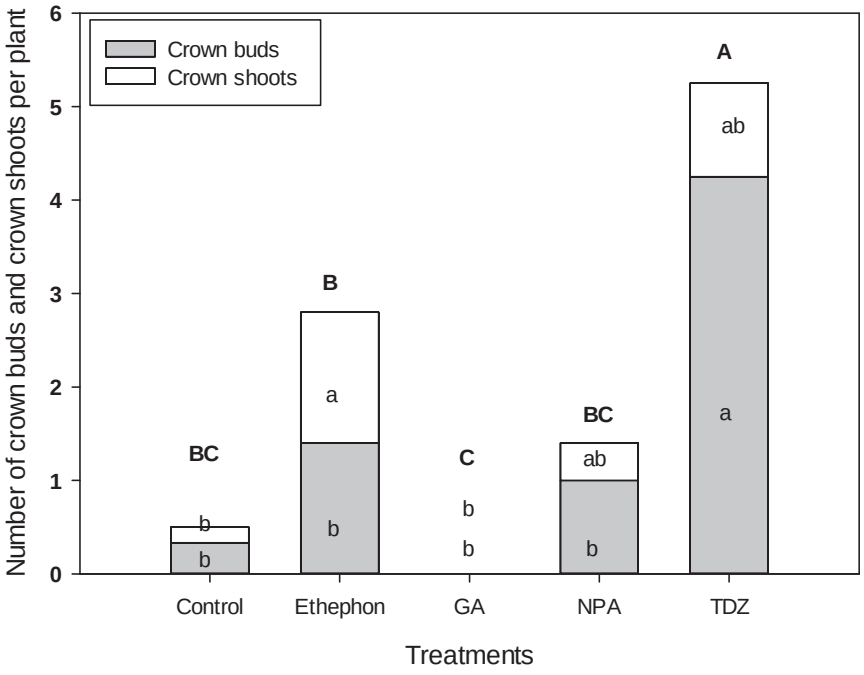

Figure 6.5 Number of crown buds, crown shoots, and their combined total per plant for 'Diva' 5 months following application of plant growth regulators under long days (Experiment Two). Treatment abbreviations as detailed in Figure 6.1. Means, with the same letter are not significant at $P \leq 0.05$ (LSD), for each variable located in the order from the base of the stack bar; i.e. lower case letters for number of crown buds or crown shoots, and upper case letters for the combined number.

Plants treated with GA_3 produced the lowest number of crown buds and/or crown shoots, i.e. none (Figure 6.5), but also the greatest number of secondary and tertiary shoots, than other treatments (Table 6.2; $P < 0.05$). The combined dry weight of primary, secondary and tertiary shoots was significantly different between treatments ($P < 0.05$), as within plants treated with GA_3 the dry weight of secondary and tertiary shoots was 6.5 times greater than in the Control treatment ($P < 0.05$; Figure 6.6).

The greatest number of storage roots was observed in the TDZ treatment, but none of the other treatments with growth regulators were different from that achieved in the Control treatment ($P < 0.05$; Table 6.2).

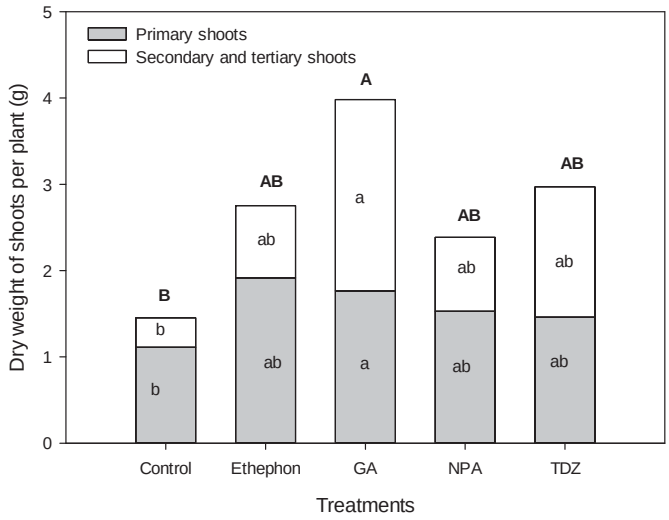

Figure 6.6 Dry weight of primary and secondary shoots (combined) per plant for 'Diva' 5 months following application of plant growth regulators under long days (Experiment Two). Treatment abbreviations as detailed in Figure 6.1. Means with the same letter are not significant at $P \leq 0.05$ (LSD) for each variable located in the order from the base of the stack bar; i.e. lower case letters for dry weight of primary or secondary and tertiary shoots, and upper case letters for the combined weight of all shoots.

Table 6.2 Number of; storage roots, secondary and tertiary shoots, and the dry weight of storage roots per plant for 'Diva' 5 months following application of plant growth regulators under long days (Experiment Two).

Treatment	Number of storage roots # [z]	Dry weight of storage roots (g) [n.s.]	Number of secondary shoots [z]	Number of tertiary shoots [z]
Control	5 ± 1^{b}	0.17 ± 0.52	1 ± 1^{b}	1 ± 1^{b}
ethephon	11 ± 3^{ab}	0.68 ± 0.5	2 ± 1^{b}	0^{b}
GA_3	10 ± 2^{ab}	0.52 ± 0.65	8 ± 1^{a}	3 ± 1^{a}
NPA	8 ± 1^{ab}	0.48 ± 0.65	2 ± 1^{b}	0^{b}
TDZ	12 ± 3^{a}	0.56 ± 0.75	2 ± 1^{b}	1^{b}

[z] within the same column, treatment values followed by same letter are not significantly different at $P \leq 0.05$ (LSD)

[n.s.] not significantly different at $P \leq 0.05$ (LSD)

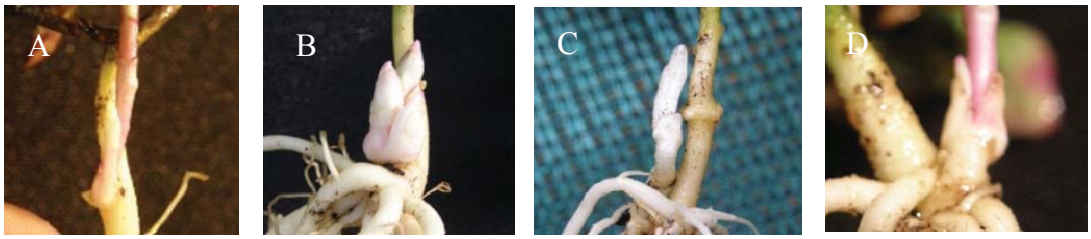

Figure 6.7 Representative samples of crown buds in the transition zone of 'Diva' 6½ months from the start of treatment application in Experiment One and Two; (A) Outside, (B) LD→SD, (C) TDZ and, (D) ethephon.

6.5 Discussion

6.5.1 Influence of environmental factors; photoperiod and temperature

The crown bud formation process in the cultivar 'Diva' was promoted by the short photoperiodic regime and inhibited under the long photoperiodic regime (Figure 6.2). As similar responses were found in plants under the LD→SD photoperiodic regime, both within the heated greenhouse and in the cooler temperatures experienced outside (LD→SD + Low T), the crown bud formation process in the transition zone can be regarded as having a greater response to photoperiod than to temperature (Figure 6.2). In the current study however, the critical photoperiod for development of crown buds was not determined, but will be of interest to determine in the future using specific durations of short and long photoperiods instead of the natural progression utilized in the current study. Additionally it will provide greater understanding of whether it is a specific photoperiod or perhaps the direction of change of photoperiod that contributes to the crown bud formation process.

While the naturally occurring short photoperiodic regime promoted crown bud formation, a few buds were also present on plants under the long photoperiodic regime (Figure 6.2). It was also noted within the natural growth cycle of established plants of the cultivar 'Spotlight', the first evidence of bud initiation (i.e. appearance of crown buds) was seen in summer, when comparatively long days and higher temperatures existed (Chapter 3). Based on these observations, it could be hypothesized that, initiation and development of crown buds could possibly be a facultative response to photoperiod. Alternatively it is also possible that under a long photoperiodic regime as compared with short photoperiodic regime, greater photo-assimilates are prioritized for use in development of above-ground biomass (Shillo and Halevy, 1981) and, in doing so formation of crown buds is inhibited. This would be contrasted by the situation under shorter photoperiods resulting in allocation of photo-assimilates to stimulate both

below-ground biomass (Shillo and Halevy, 1981) and initiation of crown buds within the transition zone. In the current study this hypothesis was further supported by a greater number and dry weight of storage roots under the short photoperiodic compared under the long photoperiodic regime (Table 6.1). Because of the significance of the photoperiodic regime on the crown bud formation process, this hypothesis of the indirect influence of photoperiod via assimilate partitioning was also further explored in Chapter 7. Accumulation of photo-assimilates in the roots under short photoperiods could be an important component in the initiation and development crown buds. The greatest number of crown buds (Figure 6.2) was however formed when the plants were shifted from the long to short photoperiodic regime (LD→SD), compared with continuous exposure to naturally occurring short photoperiodic regime (SD). As the number of storage shoots or their dry weights was not greater within the LD→SD treatment compared with SD, no correlation was observed between the number of storage roots (Table 6.1) and number of crown buds. This was in contrast to previous technical reports *in vitro* that report increased root growth was associated with an increased number of buds (Kawakami and Shimonaka, 1996). In the current study, this lack of any correlation between greater below-ground growth and more crown buds, resulted in consideration of an alternative hypothesis, i.e. that allocation of photo-assimilates to the development of the transition zone, instead of storage roots, may be more of an important determinant for initiation of crown buds. In the following chapter (Chapter 7) this hypothesis was explored further.

Based on preliminary technical reports, an 18 h photoperiod promoted initiation of crown buds in gentians *in vitro* more than 12 h, and 20 ºC more than 25 ºC (Sato, 1988). In contrast, as evident in the current study, transfer to the short photoperiodic regime (SD; 11 h to a minimum 9:18 h:min in June, and subsequently increasing to 13 h in October) resulted in a comparatively greater response than the long photoperiodic regime (LD; 2 h night break lighting during the same natural progression of day length experienced in SD), with temperature having little or no effect. These technical reports however, consist of data that were not statistically validated and are based on seedlings, which have an established transition zone (Chapter 5), and were not of the same parental line as ‘Diva’. The results from these technical reports therefore, may not be applicable to the vegetative propagules used in the current study.

A shorter photoperiod promoted development of buds and endodormancy in woody perennials like *Populus* spp (Ruttink et al., 2007) and herbaceous perennials like *Nelumbo nucifera* Gaertn. (Masuda et al., 2006). During the current study the dormancy status of crown buds was not analysed, but the presence of crown shoots indicates that not all newly produced crown buds remained dormant. Given paradormancy over crown buds has been previously noted in 'Spotlight' (Chapter 4), within the current experiment it is possible that the crown buds that did not emerge could have been under correlative inhibition, endodormancy or both. If exposing plants to a period of shorter/shortening photoperiods promotes the crown bud formation process, identifying what causes the crown buds to develop and sustain endodormancy remains to be investigated.

6.5.2 Influence of exogenous growth regulators

6.5.2.1 Application of GA_3 and its interaction with photoperiod

Compared to other growth regulators, application of GA_3 stimulated development of secondary and tertiary shoots, leading to an increase in above-ground growth (Figure 6.6; Table 6.1), but inhibited the number of crown buds (Figure 6.5). This was in contrast to preliminary technical reports suggesting a greater number of crown buds following application of GA_3 (Okayama-Ken and Nogyo, 2003). However, this technical report utilised different cultivars and seedlings. Seedlings are faster to form the transition zone than vegetative propagules, and typically would have already initiated bud clusters (Chapter 5) prior to treatment application. If so, the response may differ from vegetative propagules which still require completion of the initial stages in the multistep process of crown bud formation, i.e. formation of the transition zone. In contrast to this preliminary technical report, and similar to what was found within the current study, suppression of formation of storage organs and associated buds were reported in response to GA_3 in other herbaceous perennials (Araki et al., 1993; Biran et al., 1974). Hence the role of GA_3 was further investigated in relation to the crown bud formation process (Chapter 7).

In the current experiment the failure of GA_3 to increase the number of crown buds in 'Diva' could potentially be due to temporal changes in assimilate partitioning. As the weight of the primary shoot was not affected by any treatment, the increased above-ground growth of plants treated with GA_3 was clearly attributed to greater development of secondary and tertiary shoots (Figure 6.2 and Table 6.1), i.e. assimilate partitioning

to these organs. Promotion of shoot growth by exogenous GA_3 was also reported with *Gentiana triflora in vitro* (Zhang and Leung, 2002), as well as with *Solanum tuberosum* L. (potato) as whole plants and cuttings *in vivo* (Menzel, 1980). It is also possible however, that GA_3 indirectly promotes initiation and development of crown buds later in the growth season via directing these above-ground assimilates to stimulate growth in below-ground structures. In the current experiment the plants were destructively harvested 5 months after application of GA_3, and the possibility remains therefore, that the consequent redirection of photo-assimilates to greater below-ground growth, and initiation and development of crown buds, would occur later in the season. The existence of a potential delayed effect of exogenous application of GA_3 was, therefore, assessed in subsequent experiments (refer Chapters 8 and 9).

Application of exogenous GA_3 inhibited development of crown buds under the three photoperiodic regimes examined. Stimulation of greater above-ground growth by application of GA_3 occurring under both the naturally occurring short photoperiodic (GA+SD) and long photoperiodic (GA+LD) regimes however, was not evident when plants were transferred to the naturally occurring short photoperiodic regime (GA+LD→SD). It could be hypothesized that the transfer from the long photoperiodic regime to short photoperiodic regime stimulates conditions that are inhibitory to gibberellin activity, as also reported in *Begonia* × *cheimantha* Everett (Oden and Heide, 1989), potato (Jackson, 1999) and *Fragaria* × *ananassa* Duch. (strawberry) (Hytönen et al., 2009). This inhibition may have stimulated assimilate partitioning to the transition zone (as the number of storage roots were also not high), reducing the emergence and growth of axillary buds from shoots (Table 6.1). If so, it is possible that the physiological changes, triggered by changing the photoperiodic regime, would be capable of inhibiting GA activity and, therefore, making the plant less responsive to exogenously applied GA_3. While this interaction between application of exogenous GA_3 and photoperiodic regime was evident during the current study, involvement of endogenous GAs was not analysed, and highlights this as worthy of investigation in the future. In strawberry anti-gibberellins were capable of promoting typical short photoperiod responses under a long photoperiod, with exogenous GA_3 being capable of reversing such effects (Hytönen et al., 2009). Based on the current study therefore, and as evident in other plants, GA activity could vary in response to photoperiodic regime, and could be inhibitory to formation of crown buds within the normally promoting SD photoperiodic regime. It is hypothesized therefore, that inhibition of GA could

potentially promote the crown bud formation process, even under inhibitory photoperiodic regimes. This hypothesis was investigated further in subsequent experiments (refer Chapter 7).

6.5.2.2 Influence of exogenous growth regulators (TDZ, ethephon and NPA) under inhibitory long days

Under the inhibitory long photoperiodic regime, the crown bud formation process was promoted by exogenous application of TDZ (Figure 6.5; Table 6.2). Although the photoperiod was not specified, promotion of adventitious buds or shoots in response to TDZ has previously been noted with gentians *in vitro* (Hosokawa et al., 1996), as well as in other species (Proctor et al., 1996). Application of TDZ therefore, may have promoted formation of adventitious bud initials for formation of clusters on the transition zone. Although the number of crown buds produced was higher with TDZ, buds were visually assessed to be of poor quality (Figure 6.7), and unlikely to contribute to floral shoot production (Chapter 3). As reported in Chapter 3, only the larger diameter, primary-crown buds contribute to formation of floral shoots. It is, therefore, considered important to identify the effect of such differences in quality. To this end, the emergence and subsequent development of crown buds as shoots were investigated in the next cycle of growth following application of growth regulators (refer Chapter 7).

Although the number of crown buds was not significantly different, ethephon at 864 ppm produced better quality buds than TDZ (Figure 6.7). The inability to determine the increased number of crown buds as being statistically significant between ethephon and the Control treatment, as previously reported (Morgan and Debenham, 2008), may have been due to the greater variability of plants and the limited number of replicates used for the current experiment. This wide variability among vegetative propagules could also be a reason for wide variability in reaching duration to harvest maturity between mature plants of same cultivar (Chapter 2), however this hypothesis requires further investigation. Further, relative to application of ethephon, the preliminary technical report (Sato et al., 1988) mentions the use of a dosage of 1000 ppm, and frequency of application greater than that used in the current experiment. Hence in future experiments it may be important to identify the optimum dosage and frequency of ethephon to be used, and increase the replication used.

Involvement of ethephon and/or ethylene in the initiation and development of buds has not been widely reported in other herbaceous perennials, but the synthesis of ethylene during endodormancy was noted in *Euphorbia esula* L. (Horvath et al., 2006), *Betula pendula* Roth (Ruonala et al., 2006) and in *Chrysanthemum morifolium* Ramat (chrysanthemum) (Sumitomo et al., 2008). In chrysanthemums, dormancy induction by application of exogenous ethylene was also noted. Since in 'Spotlight' crown buds also present a period of endodormancy prior to emergence (Chapter 3 & 4), it is possible that exogenous ethylene promotes the crown bud formation process in 'Diva' via induction of endodormancy. As evident however by the emergence of crown shoots, not all buds remained dormant in the current experiment. The role of ethephon and/or ethylene in endodormancy of crown buds was, therefore, investigated further in subsequent experiments (refer Chapter 7).

Due to reduced survival of plants treated with NAA, it was not possible to evaluate the effect of the application of auxins on the crown bud formation process. At the dosage used however, a greater number of crown buds was not evident when plants of 'Diva' were treated with the auxin transport inhibitor (NPA). The synergistic effect of both auxin and cytokinin on formation of adventitious shoots *in vitro* in gentians (Hosokawa et al., 1996), illustrates the need to explore the interactive effects of these growth regulators upon initiation and development of crown buds, rather than individually. Further to that it is well known that a greater cytokinin to auxin ratio was required for initiation of adventitious buds, and has been used in regeneration of plants *in vitro* (Murashige, 1974). Due to the limitations of time, this angle of investigation was not carried out during the current thesis and, therefore, remains to be investigated in the future.

As hypothesised previously, the short photoperiodic regime potentially fulfilled the requirement of early development of the transition zone by diverting assimilates to both the transition zone and below-ground growth. Although treatment with TDZ and ethephon promoted initiation and development of crown buds, how this was achieved was not clear, since similar storage root weight and numbers were observed irrespective of treatment effect (Table 6.2). Based on the multistep process however, these plant growth regulator treatments could potentially be influential in development of the transition zone, bud initials for clusters and subsequent buds within the clusters. It was

therefore considered worth investigating the influence of TDZ and ethephon further for possible effects under different photoperiodic regimes (Chapter 7).

As noted previously, formation of crown buds was visible during long photoperiods within established plants of 'Spotlight' in their second growth cycle (Chapter 3). However the plants used in the current experiment were in their first growth cycle from tissue culture. In addition to potential cultivar differences, it is expected that plants in their second growth cycle would have already developed a transition zone and contained initials of buds and bud clusters (Chapter 5). In contrast, those in the first growth cycle did not have a fully developed transition zone. Based on the crown bud formation process, it could therefore also be hypothesized that formation of the transition zone with adventitious bud initials, may require specific environmental conditions such as a shortening photoperiod. Once the transition zone has developed, the requirement of individual crown buds within the cluster (i.e. axillary) may occur even during long days and high temperatures. During the current study however, the number of bud clusters and buds within the cluster were not separately evaluated. In the following chapter, therefore, the potential significance of these separate steps, or how the specific environment and plant growth regulators may act on these steps, were taken into consideration in evaluation of treatment effects (Chapter 7). Factors influencing the crown bud formation process in plants in their second growth cycle, was further investigated in subsequent chapters (refer Chapter 8 & 9).

6.5.3 Conclusions

From the current experiments it was evident that both environmental factors and exogenous growth regulators can influence the crown bud formation process on vegetative propagules of 'Diva' previously devoid of any visible crown buds. An increase in the number of crown buds was observed both under naturally occurring short photoperiodic regimes and by application of the growth regulators TDZ or ethephon. In contrast, exposure to either a long photoperiodic regime or application of GA_3 was inhibitory.

It was hypothesized that the naturally occurring short photoperiodic regime could potentially fulfill the requirement of early development of the transition zone by partitioning assimilates to both the transition zone and storage roots. However how the growth regulators were effective in this phenomenon was not clear during the current

study. In part this was due to the lack of information related to the individual steps within the crown bud formation process. As formation of adventitious bud initials and subsequent formation of individual axillary buds may have separate requirements, in future experiments it will be crucial to address these separately, by evaluating both bud initials and buds within the clusters (Chapters 7 & 8).

Chapter 7 Crown bud formation process: Influence of photoperiod, exogenous growth regulators & their interactions

7.1 Abstract

A promotive effect on the crown bud formation process, of changing from a long photoperiodic regime to a naturally occurring short photoperiodic regime, was confirmed. When applied alone ethephon tripled the number of crown buds and crown shoots compared to the normally inhibiting long photoperiodic regime, with the promotional effects doubled when paclobutrazol (PBZ) or thidiazuron (TDZ) was also applied. Exogenous growth regulators that promoted the crown bud formation process under the long photoperiodic regime, when applied under the naturally occurring short photoperiodic regime, resulted in 3-5 times greater buds on roots instead of the normal position on the transition zone. The responses to ethephon and TDZ were observed as early as 4 weeks from the last treatment application, but did not persist through to the 20th week in treatments applied with TDZ alone. Gibberellic acid and 1-methylcyclopropene alone or in combination reduced the appearance of crown buds by three times, compared to PBZ and/or ethephon. Based on these results potential factors influencing the crown bud formation process relative to the growing environment and plant growth regulators, and potential manipulative strategies to achieve a greater number of high quality buds earlier in the growing season, were identified.

7.2 Introduction

Inadequate initiation of crown buds has been perceived as a limitation in the vegetative propagation of gentians (Chapter 1). Additionally, as encountered during cultivating plants in preparation for experiments within this thesis, plants exhibited approximately 10-50% survival rates beyond their first growth cycle (personal observations) based on whether plants were propagated using cuttings or tissue culure. It was hypothesized that this failure to survive to the next growth cycle could have been due to inadequate initiation of crown buds. As presented in Chapter 6, transfer of plants from a long photoperiodic regime to a naturally occurring short photoperiodic regime, application of either thidiazuron (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-yl urea; TDZ) or ethephon (2-chloroethylphosphonic acid), increased the number of crown buds in plants of the gentian cultivar ‘Diva’ (Chapter 6). In contrast, the application of gibberellic acid (GA_3)

and/or continuous exposure to a long photoperiodic regime was found to inhibit the number of crown buds.

During the first growth cycle, the multistep process of formation of crown buds starts with development of the transition zone (Chapter 5). This is followed by initiation of adventitious buds, that later develop into individual bud clusters with preformed axillary buds. It was proposed the treatments that were effective in the production of a greater number of visible crown buds (Chapter 6) may have acted by influencing the development of the transition zone and/or bud initials. While the preliminary investigations provided an indication of potential factors influencing the crown bud formation process (Chapter 6), the qualitative aspects of crown buds or bud clusters that were produced, or their timing of initiation and emergence, remained to be quantitatively described. It was therefore envisaged that such treatments needed to be evaluated for the quantity, quality and timing of formation of crown buds, as presented within this chapter. Hence the crown bud formation process, the potential control of this process by photoperiod, exogenous growth regulators, and their interaction, was further investigated, with a view to developing strategies to achieve an adequate number of crown buds.

As evident previously with 'Diva', a greater number of crown buds were observed following exposure to a naturally occurring short, rather than long, photoperiodic regime (Chapter 6). Due to the changes in partitioning of photo-assimilates and storage carbohydrates reported in response to photoperiodic changes (Shillo and Halevy, 1981), it was proposed that the crown bud formation process in 'Diva' might similarly be an indirect result of such changes in partitioning. Since the transition zone was the primary site for initiation of crown bud clusters (Chapter 5), it was hypothesized that the partitioning of photo-assimilates towards the transition zone, in response to changes in photoperiod, could have an impact on the crown bud formation process and, therefore, their appearance. This hypothesis was further investigated in the current study as a confirmation of these earlier findings. Additionally, due to this influence of photoperiod on the crown bud formation process, the response to exogenous growth regulators may also vary based on photoperiod. In the previous experiment however, the influence of exogenous growth regulators was investigated only under a long photoperiodic regime which, by itself, was inhibitory (Chapter 6). In order to determine whether these growth regulators were similarly effective under both long and naturally occurring shorter

photoperiods, within the current experiment the interaction between photoperiod and exogenous growth regulators was investigated.

Promotive effects of a naturally shortening photoperiod on the crown bud formation process were counteracted by exogenous application of GA_3 in 'Diva' (Chapter 6). Concentrations of endogenous gibberellins (GAs) reduced with exposure to shorter photoperiods in plants such as *Populus* spp (Rohde and Bhalerao, 2007), *Begonia* × *cheimantha* Everett (Oden and Heide, 1989) and *Euphorbia esula* L. (Horvath et al., 2006). As also observed in *E. esula*, GA synthesis inhibitors, such as paclobutrazol (PBZ; 16 mg L^{-1}) led to adventitious buds forming on roots (Chao et al., 2007). Based on these results from other species, and the response of 'Diva' to exogenous GA_3 (Chapter 6), it was hypothesized that inhibition of GAs could promote the crown bud formation process in 'Diva'. Since, there were no previous reports on the application of PBZ to gentians, in order to evaluate this hypothesis, 20 mg L^{-1} PBZ was chosen for use during the current study, based on the concentrations successfully used in *E. esula* (Chao et al., 2007).

Plants of 'Diva' treated with TDZ developed a greater number of crown buds as compared to those not treated (Chapter 6), and TDZ has also been used effectively for initiation of adventitious buds *in vitro* with *Gentiana* spp (Fiuk and Rybczyski, 2008; Hosokawa et al., 1996) and other species (Proctor et al., 1996). In the current experiments it was hypothesized that during the multistep process of initiation and development of crown buds, TDZ could initiate adventitious bud initials for the subsequent development of crown bud clusters. Application of TDZ however, produced buds which were of poor quality (Chapter 6), being similar in size to buds which failed to develop into floral shoots (Chapter 3). Buds developed on plants treated with ethephon were of a better quality, being similar in size to crown buds which have the potential to develop into floral shoots. Additionally, as buds with a greater diameter developed longer, i.e. higher quality, shoots (Chapter 3), in the current study it was considered important to identify any treatment effect on bud size and resulting shoot quality.

Ethephon may have promoted the crown bud formation process when applied to 'Diva' under conditions which were inhibitory, i.e. long photoperiodic regime, however the number of crown buds was not different statistically compared to the Control treatment (Chapter 6). While ethephon has previously been reported to promote the number of

crown buds in gentians, this has only been within preliminary technical reports (Morgan and Debenham, 2008; Sato et al., 1988), not peer-reviewed literature and, therefore, these findings are considered to be at best indicative only. Although a direct influence of ethylene initiating buds has only been noted with *Lilium speciosum* Thunb (Van Aartrijk et al., 1985), it has been reported to be involved in the induction of endodormancy by environmental triggers (Ruonala et al., 2006; Ruttink et al., 2007). Supporting the hypothesis for a regulatory role of ethylene in bud dormancy, exogenous application of ethephon (1000 mg L^{-1}) or 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor (50 µg L^{-1}), caused dormancy in *Chrysanthemum morifolium* Ramat, but not in ethylene insensitive mutants (Sumitomo et al., 2008). Further to that, ethylene has been used commercially to prevent shoot emergence in potato (Prange et al., 1998). Within the current study, it was considered plausible that the application of ethephon could initiate formation of endodormant crown buds and, therefore, its application was reinvestigated. Additionally, by application of ethylene inhibitors, it was considered possible to explore whether a more direct role existed for ethylene in the crown bud formation process. With this in mind, 1-methylcyclopropene (1-MCP) was used in the current study as an inhibitor of ethylene action, applied as recommended at low concentrations (1 ppm) and a temperature range of 20-25 ºC (Blankenship and Dole, 2003).

With the potential involvement of TDZ, ethephon and PBZ in initiation and development of crown buds, and/or ethephon in development of dormancy, these growth regulators may act in different steps in the multi-step process of initiation and development of crown buds. With this in mind, it was hypothesized that with sequential application of TDZ or PBZ followed by application of ethephon, it would be possible to identify which if any sequential combination induces a greater number of high quality crown buds earlier in the growth cycle. In the current study therefore, the influence of these growth regulators was investigated individually as well as in sequential combination. Given the apparent photoperiodic response on the crown bud formation process (Chapter 6), determining any interaction of these growth regulators under environmental conditions that promote or inhibit the process, was also considered worthy of investigation. While previous experiments (Chapter 6) only evaluated the overall number of crown buds, during the current study treatments were evaluated in relation to the number of clusters as well as buds/shoots within the clusters. This change

in evaluation methodology reflected the progressive evolution in understanding during this thesis of the multistep process.

As plants were destructively harvested at one point in time during the previous experiments, progressive changes in the crown bud formation process with time were not investigated (Chapter 6). Since initiation and development of crown buds earlier in the season was considered potentially important for the successful propagation and survival of this crop, any influence of photoperiod or exogenous growth regulators on the duration to appearance of crown buds was investigated during the current study. Additionally, during previous experiments the status of endodormancy was not analysed, but the presence of shoots derived from newly formed crown buds indicated that not all the crown buds that were produced in 'Diva' were dormant (Chapter 6). In contrast, in the cultivar 'Spotlight' none of the crown buds emerged as shoots in the subsequent growth cycle without exposure to cold, suggesting the existence of endodormancy and an obligate requirement for chilling (Chapter 3). As reported in other species, the physiological processes of induction, maintenance and release of dormancy could be two independent processes or occurring concurrently with induction, initiation and development of a bud (Ruttink et al., 2007). In *E. esula* the transition to endodormancy was induced by a reduction in both temperature and photoperiod (Dogramac et al., 2010), but what the inductive trigger is in gentians like 'Diva' is not known. In the current study therefore, in addition to determining the factors that promote the crown bud formation process, it was also considered relevant to identify what causes the crown buds to develop endodormancy, if any. Since clipping of shoots removed correlative inhibition in 'Spotlight' (Chapter 4), in the current experiment the ability of the treatments to induce endodormancy during exposure to a naturally occurring short photoperiodic regime was analysed after clipping of any existing shoots.

Within the series of experiments presented in this chapter, the overall aim was to identify the influence of growth regulators and photoperiodic regimes on the crown bud formation process, leading to the timely production of high quality crown buds. For this purpose, vegetative propagules of the gentian cultivar 'Diva' were utilized with the specific objectives to quantify the influence of;

- photoperiod on; duration to appearance, quality and quantity of crown buds, and implications in the subsequent growth cycle,

- a range of exogenous growth regulators and their antagonists as a single application or additively on; duration to appearance, quality and quantity of crown buds, and implications in the subsequent growth cycle,

- any interaction between photoperiod and a range of exogenous growth regulators and their antagonists as a single application or additively on; duration to appearance, quality and quantity of crown buds and implications in the subsequent growth cycle.

7.3 Materials and methods

7.3.1 General management of plants

Plants of the gentian cultivar 'Diva' were propagated by tissue culture at The New Zealand Institute for Plant and Food Research (Palmerston North, New Zealand; 40°37'S 175°60'E) using nodal cuttings. Plants were kept in a culture room at 20 ºC with 16 h of lighting from cool white fluorescent bulbs (34.46 µmol s^{-1} m^{-2} PAR at foliage height), and subsequently transferred to 15 ºC with 16 h lighting during August 2008. Plants were deflasked (29th October 2008) and managed as described previously (refer Section 6.3.1). Cell trays were transferred to a greenhouse (heated at 15 ºC, ventilated at 20 ºC) so as to avoid accumulation of chill units. Prior to commencement of all experiments, plants experienced a long photoperiod, provided either by the natural day length or 2 h night-break lighting (4.585 µmol s^{-1} m^{-2} PAR at foliage height) from 23:00 HR to 01:00 HR each day.

After plants had developed an established root system (i.e. root-ball held the volume of the plug-cell), they were re-potted and managed as described previously (refer Section 6.3.1). In each of the following experiments, at the commencement of treatment application, plants were at a stage of development where the primary shoot displayed opened/opening flower buds, and had an average dry weight of above ground organs of 6.2 ± 1.5 g, and of primary storage roots (secondary roots and fine feeding roots not included) of 0.44 ± 0.12 g (Figure 7.1). The plants were, therefore, in their first growth cycle, i.e. 5 months following deflasking, with few plants showing the presence of crown buds.

7.3.2 Experiment One: Photoperiod, TDZ, PBZ, and ethephon

7.3.2.1 Treatment application

The timing and identity of treatment applications was as illustrated in Figure 7.2 and Table 7.1. Plants within the heated greenhouse were transferred to a heated greenhouse with one of the two photoperiodic regimes on 31st March 2009, either;

- Short photoperiodic regime (LD→SD; natural progression of day length experienced in autumn through to spring, ranging from 12 h to 9:18 h:min, and subsequently increasing to 12:18 h:min by September) (Lammi, 2005) or,
- Long photoperiodic regime (LD; 2 h night break lighting at 4.585 μmol $s^{-1}m^{-2}$ from 23:00 to 01:00 HR during the same natural progression of day length experienced in autumn through to spring described above).

Immediately following transfer to the photoperiodic treatment environment, the first treatment with growth regulators were applied, i.e. TDZ (Sigma-Aldrich) at either 0 ppm or 100 ppm, or PBZ (commercial preparation of Pay back™; 250 g L^{-1} paclobutrazol; Nufarm Limited, Auckland, NZ) at either 0 ppm or 20 ppm (Figure 7.2). Subsequently after 3 weeks, a last set of treatments were applied, i.e. ethephon (commercial preparation of Ethrel 48™; 480 g L^{-1} ethephon; May & Baker Agrochemicals, NZ) at either 0 ppm or 864 ppm (20th April 2009), to plants previously treated with each growth regulator under each of the two photoperiodic regimes. Application of all growth regulators was as a spray to the foliage, exposed crown and the surface of the growing medium, to run-off (≈ 50 ml per plant) including 0.05% Tween 20 (Sigma CAS No. 9005-64-5) as a surfactant. The Control treatment within each photoperiodic regime comprised water and Tween 20.

At the beginning of the second growth cycle, 22 weeks following the last treatment application (i.e. application of ethephon; 23rd September 2009), any shoots remaining from the first growth cycle (i.e. primary shoots) were clipped (leaving crown buds and any newly emerging crown shoots). Plants were then placed into a cool-store (5 ºC) for one week and, upon removal, subsequently repotted into polythene bags (1.7 L; refer Section 7.3.1). Following potting up, all plants were grown in a greenhouse (heated at 15 ºC, ventilated at 20 ºC) under a long photoperiod, provided by night-break lighting (refer above), continuously through to the end of the second growth cycle. Hence plants

from all treatments were in the same growing environment during the second growth cycle.

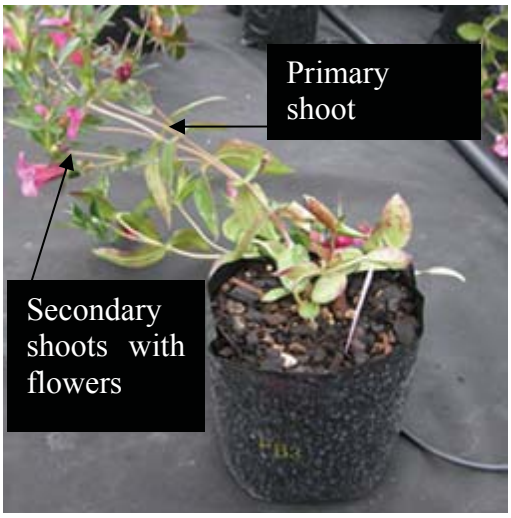

Figure 7.1 A representative plant of 'Diva' at the start of treatment application within each of three experiments (First growth cycle; 31st March 2009). The primary shoot and secondary shoots carried opened/opening flowers.

7.3.2.2 Experimental design

Experiment One was conducted as a Completely Randomised Design (CRD) comprising a factorial arrangement of treatments (2×6×4). The treatments (Figure 7.2) comprised two photoperiodic regimes (LD, LD→SD), six growth regulator treatments, and four sampling periods. The growth regulator treatments comprised an initial application of TDZ, PBZ or Control, followed by application of either of two concentrations of ethephon three weeks later (i.e. ethephon at 0 ppm or 100 ppm). Data were collected at four sampling periods (4th, 8th, 12th, and 20th week following the last application of plant growth regulators).

In the first growth cycle there were 7 individual plant replicates for each treatment combination. In the second growth cycle the number of replicates for each treatment combination varied, as determined by the number of plants remaining alive from the first growth cycle (3-13 individual plants).

Table 7.1 Abbreviations used for presentation of environmental and plant growth regulator treatment combinations within figures and tables detailing results from Experiment One, Two and Three.

Description of treatment combinations	Abbreviation
Long photoperiodic regime	LD
Short photoperiodic regime	SD
Naturally occurring temperature in outside environment	Low T
all plant growth regulators at 0 ppm	Control
20 ppm PBZ	PBZ
864 ppm Ethephon	ETH
100 ppm TDZ	TDZ
20 ppm PBZ followed sequentially by 864 ppm Ethephon	PBZETH
100 ppm TDZ followed sequentially by 864 ppm Ethephon	TDZETH
100 ppm GA_3	GA
1 ppm 1-MCP	MCP
100 ppm GA_3 followed sequentially by 1 ppm 1-MCP	GAMCP

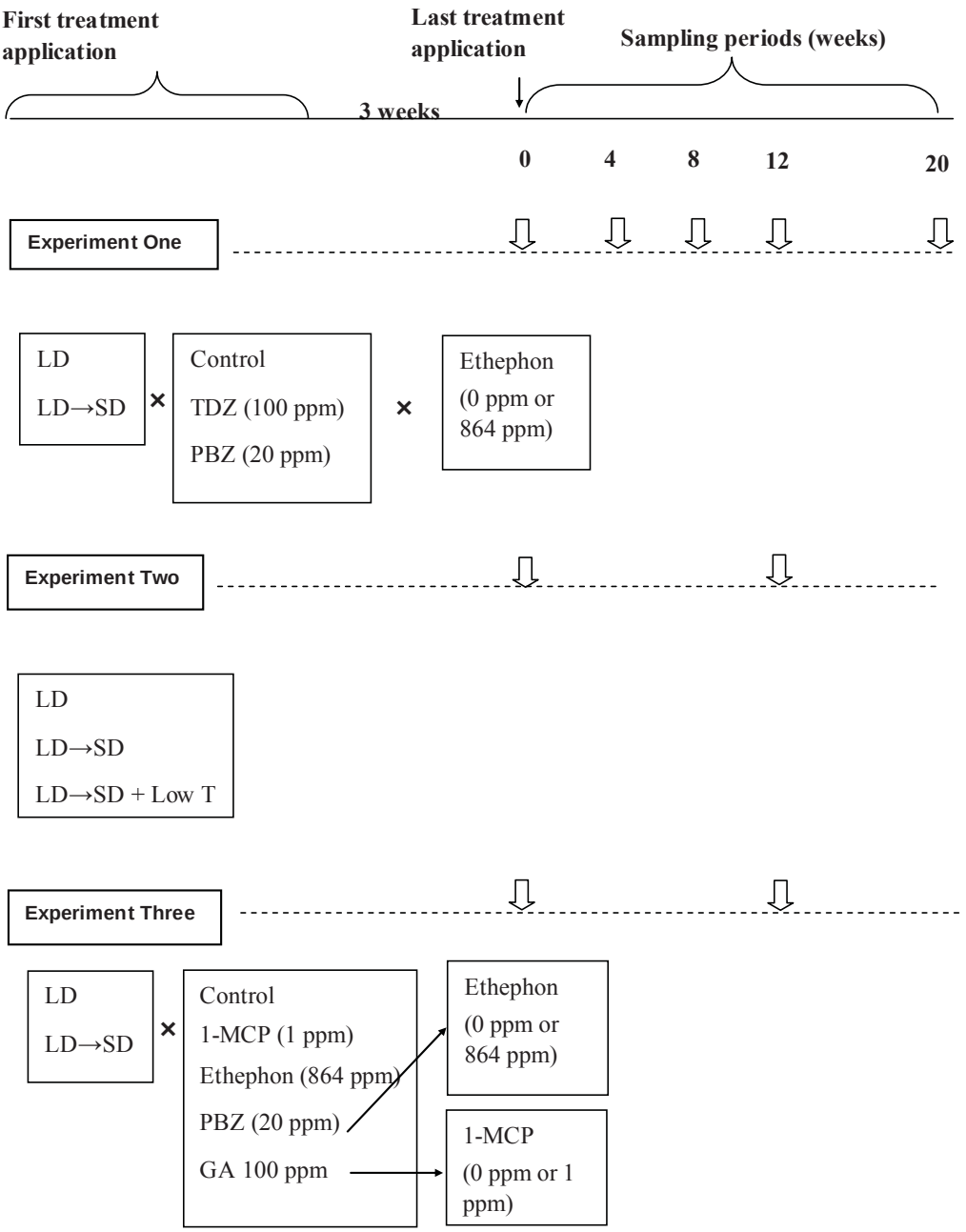

Figure 7.2 Schematic representation illustrating the composition of treatments applied within Experiments One, Two and Three during the first growth cycle. Left to right presents the time line for both treatment application and sampling dates commencing 31st March through to 9th September 2009. As indicated by the open arrows, the timing of sampling refers to the duration from the last treatment application (0 to 20 weeks).

7.3.2.3 Variables recorded

Seven replicate plants were selected randomly from each treatment combination, and were destructively harvested at the 4th, 8th, 12th and 20th week following the last application of growth regulators (Figure 7.2). The original shoot evident at the time of deflasking was identified as the primary shoot, and any axillary shoots arising from this were identified as secondary shoots. Buds and shoots other than the original primary shoots and secondary shoots were categorised based on their point of origin (Section 6.3.4), i.e. transition zone (crown buds and crown shoots) or root (root buds and root shoots). At each sampling period the number and fresh weight of buds or shoots, based on their location of origin, were recorded. As an additional measure of assimilate accumulation, the diameter of the transition zone was measured at the lowest internode at three positions of each plant at the 4th, 8th and 20th weeks. In addition, at the 12th week data were recorded of; shoot length, number of nodes and dry weight of; crown buds and crown shoots, as well as dry weight of; primary and secondary shoots, and primary storage roots. At the 20th week, data were also collected on the diameter of crown buds, the number of crown bud clusters, and the combined number of visible buds and/or shoots within the cluster.

Data collected in the second growth cycle were the number of shoots emerged from either the transition zone or roots 8 weeks after removal from cold storage. At flower harvest maturity, the number of nodes, number of nodes with floral axillaries, shoot length, and date of harvest maturity, were recorded for each shoot.

7.3.3 Experiment Two: Photoperiod and temperature

The timing of treatment applications was as illustrated in Figure 7.2 and Table 7.1. Plants within the heated greenhouse under the long photoperiodic regime were transferred (31st March 2009) to a greenhouse with one of either of the two photoperiodic regimes (LD or LD→SD), as in Experiment One (Section 7.3.2), or outside to experience the comparative cool temperatures and LD→SD (Figure 7.2; LD→SD + Low T). During the course of the experiment the monthly daily average air temperatures in the outside environment varied between 7 and 17 °C and, within the heated greenhouses, between 15 and 29 °C.

Experiment Two was conducted as a CRD comprising three treatments with 6 individual plant replicates. The treatments (Figure 7.2) comprised the two photoperiodic

regimes (LD or LD→SD) and, within the LD→SD regime, there were the two treatments differing in temperature of the growing environment (i.e. LD→SD or LD→SD + Low T). Following 15 weeks of growth in each treatment environment, plants were destructively harvested and the following data were collected; the dry weight of; primary and secondary shoots together, primary storage roots, and the number of crown buds and crown shoots.

7.3.4 Experiment Three: Photoperiod, GA_3, PBZ, ethephon, 1-MCP alone or in combination

7.3.4.1 Treatment application

The composition, timing and identity of treatment application were as illustrated in Figure 7.2 and Table 7.1. Plants within the heated greenhouse, under the long photoperiodic regime, were transferred to greenhouses with one of the two photoperiodic regimes as in Experiment One (Section 7.3.2) on 30th March 2009. Immediately upon transfer either PBZ (20 ppm), GA_3 (100 ppm), 1-MCP (1 ppm), ethephon (864 ppm) or water (Control) was applied. Three weeks later, half of the plants previously treated with GA_3 were also treated with 1-MCP (1 ppm) and, half of the plants previously treated with PBZ were also treated with ethephon (864 ppm).

Application of ethephon and PBZ were as described in Experiment One (refer Section 7.3.2). GA_3 was applied onto the foliage, exposed crown and the surface of the growing medium (≈ 50 ml per plant) at 100 ppm. GA_3 was prepared by first being dissolved in 10 ml 95% ethanol, and made up to the required volume with distilled water together with 0.05% Tween 20 (Sigma CAS No. 9005-64-5) as a surfactant. 1-MCP was prepared using the commercial preparation SmartFresh™ as a powder (a.i. 0.14% AgroFresh, Philadelphia, USA) by mixing the required weight with nano-water to obtain 1 ppm in a 0.129 m^3 airtight chamber. Plants were first placed in the air tight chamber and 1-MCP was placed in a 100 ml screw-top bottle with water. The bottle was opened inside the chamber, with plants exposed for six hours at an air temperature of 20 ºC (as per manufacturer's protocol) and lighting of 5.8 μmol s^{-1} m^{-2} PAR at foliage height, provided by fluorescent bulbs. The Control treatment comprised foliar application of water, ethanol and Tween 20, followed by placing the plants in an airtight container for the same duration as for those treated with 1-MCP.

7.3.4.2 Experimental design

The experiment was conducted as a CRD comprising a two factor factorial arrangement of treatments (2×7). The treatments (Figure 7.2) comprised the two photoperiodic regimes LD or LD→SD (refer Experiment One) and seven growth regulator treatments, i.e. application of PBZ, 1-MCP, ethephon, GA_3, Control, treated with PBZ followed by an application of ethephon, or treated with GA_3 followed by an application of 1-MCP. There were 6 individual plant replicates for each treatment.

7.3.4.3 Variables recorded

Plants were destructively harvested 12 weeks following application of the last treatment (Figure 7.2). At this time data were recorded of the number of buds or shoots categorised based on their point of origin, i.e. transition zone or root.

7.3.5 Data Analysis

For all three experiments data were analysed using the General Linear Model's procedure in the Statistical Analysis System software version 9.13 (SAS Institute, Cary, N.C.). If data were not normally distributed, then either a square root or log transformation was carried out to improve the normality.

Within Experiment One and Three, data were analysed as a factorial arrangement of treatments. When one or more of the main effects were significant and an interaction was not, only significant main effects were presented. If there was a significantly greater main effect than that of a significant interaction involving the main effect, both the main effect and interaction effects were presented (Mead, 1990). If the significance of the main effect was less than that for the interaction effect, then only the interaction effect was presented. For such significant interactions, LSMEANS or preplanned contrasts were used to compare means where appropriate. Some variables with significant interactions between the application of growth regulator and photoperiodic regime or each sampling period (i.e. duration from the last treatment application), were also analysed separately under each photoperiodic regime, as well as at each sampling period. Means were compared using Duncan's Multiple Range Test to evaluate treatment effects within each sampling period or photoperiodic regime.

Prior to treatment application crown buds were observed on a few plants, albeit infrequently. Data for the number of crown buds and/or crown shoots observed were

therefore, adjusted for any noted prior to the treatment application. As some of the crown buds emerged into shoots during the first growth cycle, the number of crown buds and crown shoots were also analysed as a combination in order to interpret treatment effects on the crown bud formation process. For some variables, results were only presented for the sampling conducted at the 20th week, as the treatment effects were most evident at this time.

7.4 Results

7.4.1 Experiment One: Photoperiod, TDZ, PBZ, and ethephon

7.4.1.1 Overview

Over the course of the 20 week period of observation following the last treatment application, both the photoperiodic regime and application of growth regulators influenced the number of crown buds, crown shoots, and their combined total (Table 7.2). Crown buds and/or crown shoots were evident at the date of first sampling, i.e. 4th week following the last treatment application, and had increased further by the 20th week (Figure 7.3A).

On their own, the number of crown buds followed a similar pattern of change over time, but since some of the crown buds started to emerge as shoots there were no significant differences between plant growth regulator treatments and/or photoperiodic regime later in the growth cycle (Table 7.2; Figure 7.3A). In contrast, the combined number of crown buds and crown shoots increased significantly overtime and, therefore, forms the basis of the presentation of results in most of the subsequent sections.

Due to the range of responses to the photoperiodic regime, plant growth regulators, and their interaction, at the end of the first growth cycle (at the 20th week from the last treatment application), plants within different treatments were at different stages of growth and development, ranging from no visible buds, bud initials on the transition zone or roots, through to bud clusters with crown buds emerged as shoots with floral axillaries (Figure 7.9 & Figure 7.10). As a consequence therefore, by the last sampling date plants within some treatments had already started their second growth cycle.

Table 7.2 Probability values indicating significance of main factors and interactions determined for key variables in Experiment One.

Factor(s)	Combined number of crown buds and shoots	Number of crown shoots	Combined number of root buds and shoots
Photoperiod	n.s.	$P < 0.05$	$P < 0.05$
Application of plant growth regulators	$P = 0.05$	$P < 0.0001$	$P < 0.001$
Duration	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Photoperiod × Application of plant growth regulators	$P < 0.0001$	$P < 0.0001$	$P < 0.01$
Photoperiod × Duration	n.s	$P < 0.0001$	$P < 0.001$
Application of plant growth regulators × Duration	n.s	$P < 0.0001$	$P < 0.0001$
Photoperiod × Application of plant growth regulators × Duration	n.s	$P < 0.0001$	$P < 0.02$

7.4.1.2 Quantitative changes in crown buds and crown shoots

Across the entire experiment an interaction was evident for the combined number of crown buds and crown shoots, between the photoperiodic regime and the application of plant growth regulators ($P < 0.0001$). The same general treatment response was also evident for fresh weight of these organs (Appendix V). The combined total of crown buds and crown shoots across all treatments reduced at the 8th week, but subsequently increased through to the 20th week (Figure 7.3A). The reduction in 8th week was due to death of some of the buds. At four weeks after the last application of treatments, differences were primarily due to the application of growth regulator treatments ($P < 0.01$; Figure 7.3B), as the effect of the photoperiodic regime was not prominent at this time (Table 7.2). In contrast, by the 20th week the interaction between the photoperiodic regime and the plant growth regulators became more evident ($P < 0.05$;Table 7.3).

Table 7.3 Probability values indicating significance of main factors and interactions within each week for the variable "combined number of crown buds and shoots" in Experiment One.

	Duration from last treatment application (weeks)			
Factor(s)	**4**	**8**	**12**	**20**
Photoperiod	n.s.	n.s.	n.s.	n.s.
Application of plant growth regulators	$P < 0.01$	n.s.	n.s.	n.s.
Photoperiod × Application of plant growth regulators	n.s.	n.s.	n.s.	$P < 0.05$

When sampled at the 4th week, plants treated with TDZ alone ($P < 0.001$), ethephon alone ($P < 0.01$), or TDZ followed by ethephon ($P < 0.05$), had up to two more crown buds and/or crown shoots than those in the Control treatment. By the 20th week, within the long photoperiodic regime, plants receiving ethephon either alone or in combination with any other treatment, resulted in the greatest increase. Plants treated with PBZ followed by ethephon ($P < 0.01$) or TDZ followed by ethephon ($P < 0.05$), presented up to six more crown buds and/or crown shoots than those in the Control treatment (Figure 7.3C & Figure 7.9). The number of crown buds within plants treated with PBZ followed by ethephon, was three times greater than PBZ alone ($P < 0.05$) and, TDZ followed by ethephon was five times greater than TDZ alone ($P < 0.01$). Treatment with TDZ however resulted in the development of clusters with crown buds which were thin, pale in colour, and less than 1 mm in diameter (Figure 7.9E). An increase in the number of crown buds by three more than in the Control treatment was apparent within plants treated with ethephon alone under the long photoperiodic regime, however this was not significantly different ($P = 0.1$). In contrast to what was observed under the long photoperiodic regime, the effects observed from the application of growth regulators were not apparent within the short photoperiodic regime, with plants in all treatments averaging 4 ± 1 crown buds and/or crown shoots (Figure 7.3D & Figure 7.10). By the 20th week, within the short photoperiodic regime, plants within the Control treatment had five times greater crown buds and/or crown shoots than those within the long photoperiodic regime ($P < 0.05$; Figure 7.3C & Figure 7.3D).

Over the course of the experiment, an interaction was evident between the photoperiodic regime and the application of growth regulators for the number of crown buds alone ($P < 0.01$), i.e. excluding crown shoots. The effect was evident only at the

4th week however, with no significant effect of the photoperiodic regime, and only the application of growth regulators being significant ($P < 0.01$). The order of magnitude of treatment effects was the same as the combined total of the crown buds and crown shoots (Figure 7.3B), therefore this data were not presented.

Across the entire experiment, the number of crown shoots varied in response to the duration from the last treatment application, photoperiodic regime, application of growth regulator and their interaction (Table 7.2). The effect of the photoperiodic regime or the application of growth regulators was not evident at the 4th and 8th week ($P > 0.05$), however it was evident from the 12th week for application of the growth regulators, and their interaction with the photoperiodic regime ($P < 0.05$; Figure 7.6A & B). At the 20th week a significant influence was evident on the number of crown shoots in response to the photoperiodic regime ($P < 0.01$), application of growth regulators ($P < 0.0001$; Figure 7.6C) and their interaction ($P < 0.01$). Under the long photoperiodic regime, plants in all treatments using ethephon produced between two and four more crown shoots than those in the Control treatment (Figure 7.6A) i.e., ethephon alone ($P < 0.01$), PBZ followed by ethephon ($P < 0.001$) and, TDZ followed by ethephon ($P < 0.0001$). In contrast however, under the short photoperiodic regime growth regulators did not affect the number of crown shoots ($P > 0.05$; Figure 7.6B). Treatments that resulted in a greater number of crown shoots under the long photoperiodic regime resulted in plants achieving between two and four shoots less than in the Control treatment when applied under the short photoperiodic regime, i.e. application of PBZ followed by ethephon ($P < 0.0001$) and, TDZ followed by ethephon ($P < 0.001$).

An interaction was evident between the photoperiodic regime and the application of growth regulators for the number of crown bud clusters arising from the transition zone, 20 weeks following the last treatment application ($P < 0.05$; Figure 7.4A & B). Under the long photoperiodic regime the number of clusters was three times greater than in the Control treatment, when plants were treated with either PBZ followed by ethephon ($P < 0.01$) or TDZ followed by ethephon ($P < 0.05$). Application of TDZ followed by ethephon also resulted in three times greater clusters than from TDZ alone ($P < 0.01$). Differences in the number of crown bud clusters following single applications of growth regulators however, were not evident compared to the Control treatment within the long photoperiodic regime. In contrast however, under the short photoperiodic regime, the influence of either the application of a single growth regulator, or interaction between

growth regulators, was not evident (Figure 7.4B). Plants within the Control treatment under the short photoperiodic regime had two more bud clusters than under the long photoperiodic regime by the 20th week ($P < 0.05$). Application of PBZ followed by ethephon resulted in the development of three times as many crown bud clusters under the long photoperiodic regime than evident under the short photoperiodic regime ($P < 0.01$).

Plants within all the treatments comprising ethephon tended to present a greater combined total of crown buds and crown shoots per cluster than the rest of the treatments by the 20th week. Application of ethephon alone, or subsequent to the application of TDZ, resulted in twice the combined total of crown buds and crown shoots per cluster than plants within the Control or TDZ only treatments (Figure 7.4C). The effect of application of PBZ alone or followed by ethephon however, were similar, achieving 1 ± 0.45 buds and/or shoots per cluster.

The proportion of the crown buds emerging as shoots, calculated relative to the combined total of crown buds and crown shoots, was influenced by the photoperiodic regime ($P < 0.01$), the application of growth regulator ($P < 0.0001$; Figure 7.6D) and their interaction ($P < 0.001$), when measured at the 20th week following the last treatment application. The proportion of shoot emergence was greater in all plants treated with ethephon (41 ± 19%) than the rest of the treatments (5 ± 5%), irrespective of whether applied subsequent to PBZ or TDZ. This effect of plant growth regulators however, was only evident under the long photoperiodic regime, and not under the short photoperiodic regime. At the 20th week the proportion of flowering shoots, out of the total number of crown shoots emerged, was primarily influenced by the application of growth regulators ($P < 0.001$), with all treatments comprising ethephon achieving a greater proportion of floral shoots (i.e. 42 ± 16%) than in all other treatments (4 ± 4%).

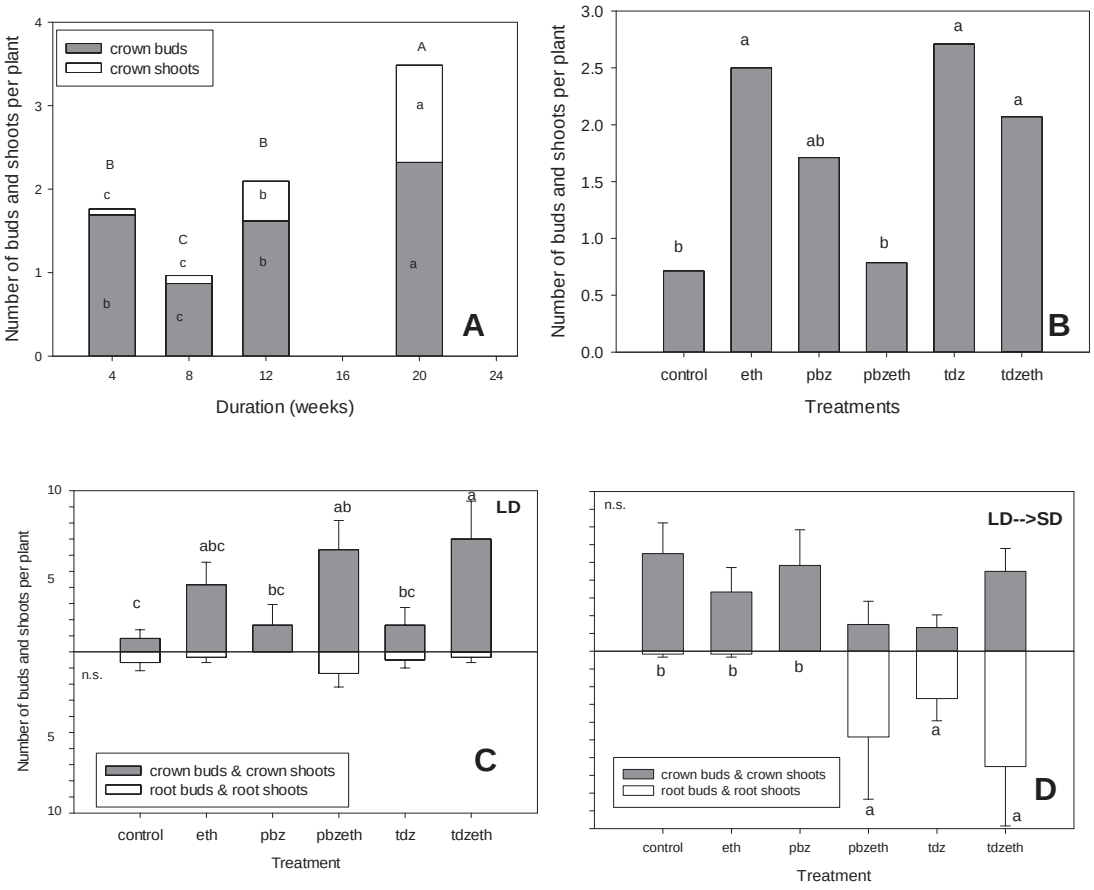

Figure 7.3 Combined number of visible crown buds and crown shoots per plant of 'Diva' (Experiment One) for the; (A) main effect of duration from last treatment application[x], (B) main effect of treatment with growth regulators 4 weeks following the last treatment application, (C & D) combined number of buds and shoots developed from the transition zone or storage roots 20 weeks following the last treatment application within two photoperiodic regimes (C, LD; D, LD→ SD). Abbreviations for treatments as described in Table 7.1. Vertical lines represent standard error values. Means followed by different letters were significantly different at $P \leq 0.05$; upper case letters apply to variables representing combined value within stack bar in Figure 7.3A. n.s. = not significantly different (Number of root bud and shoots at LD and number of crown buds and shoots at LD→ SD).

[x] as indicated at 8th week, some buds died during the 20 weeks of development

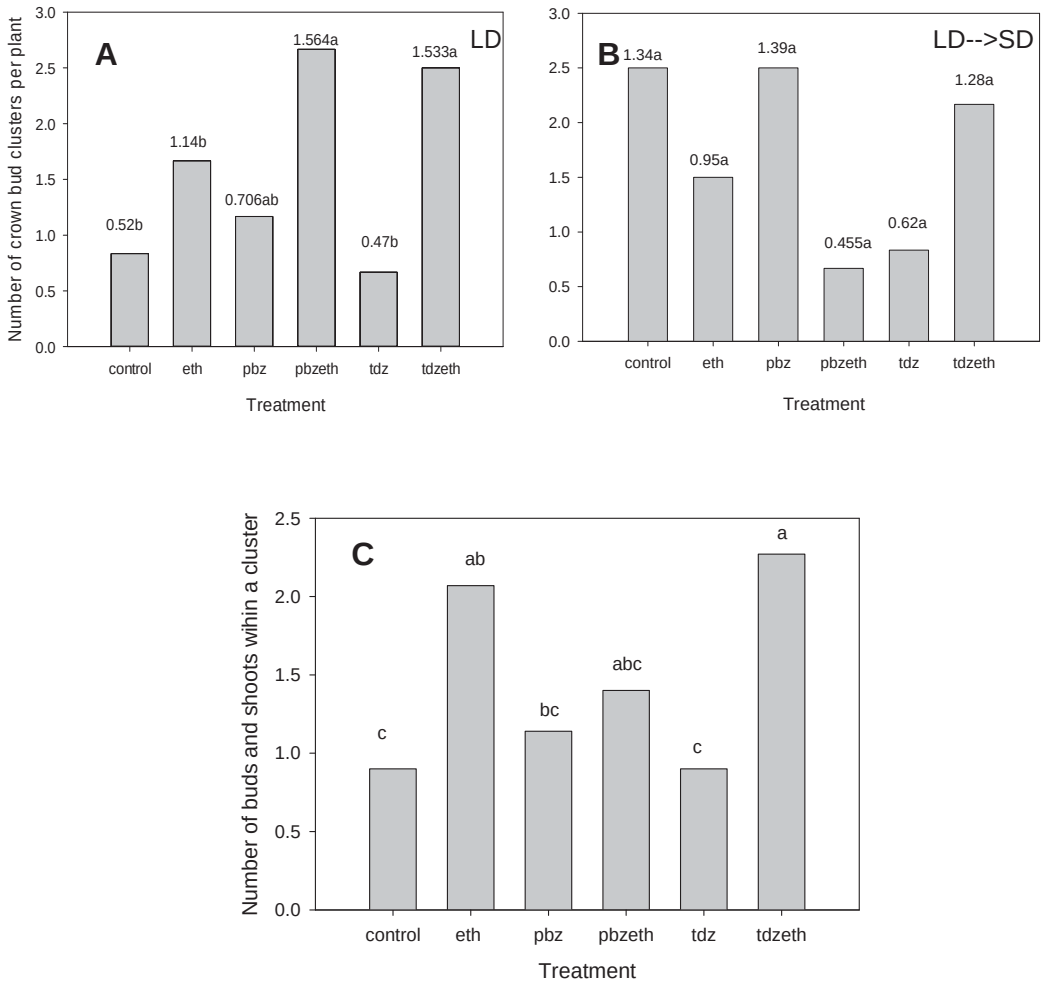

Figure 7.4 Number of crown bud clusters per plant of 'Diva' at 20 weeks following the last treatment application (Experiment One) under; (A) Long day or, (B) short day photoperiodic regimes and, (C) combined number of visible crown buds and crown shoots within a cluster, for the main effect of application of growth regulators. Abbreviations for treatments as described in Table 7.1. Means followed by different letters were significantly different at $P \leq 0.05$; for data that were sqroot transformed, transformed means and mean separation letter are presented at the top of each column.

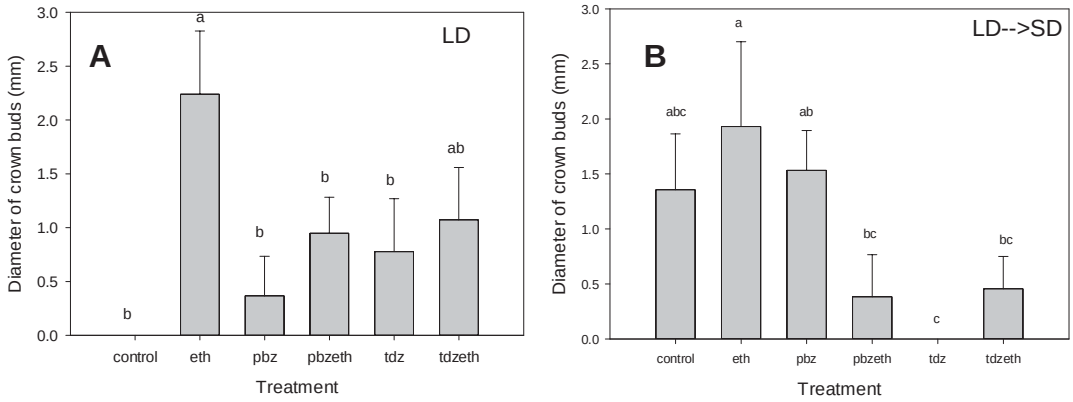

Figure 7.5 Diameter of crown buds on plants of gentian cultivar `'Diva' at 20 weeks following the last treatment application within two photoperiodic regimes (Experiment One); (A) LD, (B) LD→SD. Abbreviations for treatments as described Table 7.1. Vertical lines represent standard error values. Means followed by different letters were significantly different at $P \leq 0.05$.

7.4.1.3 Qualitative characteristics of crown buds and crown shoots

An interaction was evident between the photoperiodic regime and application of growth regulators for the average diameter of crown buds at the 20th week from the last treatment application ($P < 0.05$). Crown buds in plants within the Control treatment had greater diameter under the short photoperiodic regime than within the long photoperiodic regime ($P < 0.05$; Figure 7.5A & B). The effect of the application of growth regulators was evident under the long photoperiodic regime ($P < 0.05$), with application of ethephon alone resulting in crown buds being between two and four times greater in diameter than those from the Control, TDZ alone, PBZ alone, or PBZ followed by ethephon treatments. Under the short photoperiodic, bud diameter was greatest within the ethephon alone, PBZ alone or Control treatments ($P < 0.05$), all of which produced similar sized buds, which were between four and six times larger than in the rest of the treatments.

When measured at the 20th week the qualitative characteristics of; the length of crown shoots ($P < 0.0001$; Figure 7.7A), number of nodes ($P < 0.0001$; Figure 7.7B) and diameter of these shoots ($P < 0.0001$; Figure 7.7C), was increased by the application of ethephon irrespective of whether applied subsequent to TDZ or PBZ. Those plants treated with ethephon presented shoots which were; up to 6 to 8 cm longer in length, between 3 to 6 more nodes, and 1.4 to 2 cm greater in diameter, than those arising from all other treatments. Dry weight of crown shoots at the 12th week following the last treatment application, also presented a similar pattern of treatment differences ($P <$

0.01). Hence all these qualitative characteristics of shoots were primarily influenced by the application of ethephon, irrespective of the photoperiodic regime of the growing environment.

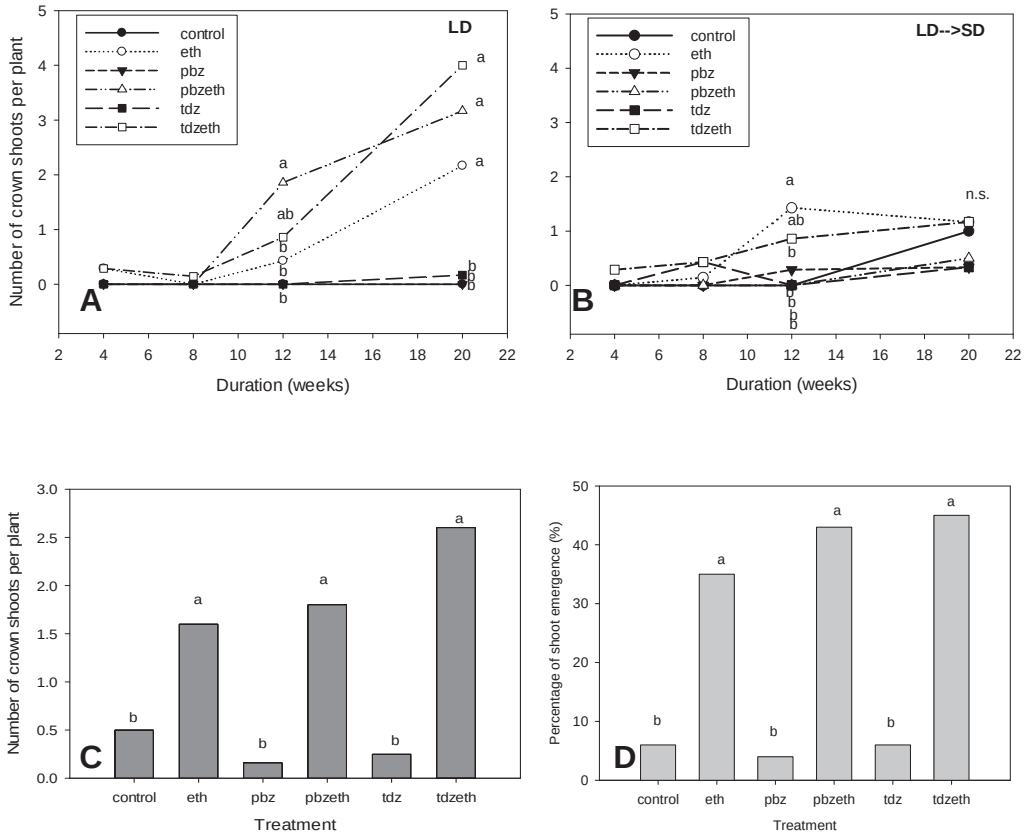

Figure 7.6 Number of crown shoots per plant of 'Diva' (Experiment One); (A & B) during the 20 weeks following the last treatment application within two photoperiodic regimes (A, LD; B, LD→SD) and, (C) for the main effect of treatment with application of growth regulators at 20 weeks following the last treatment application. (D) Percentage of crown shoots emerged as a proportion to the combined number of crown buds and crown shoots for the main effect of treatment with application of growth regulators 20 weeks following the last treatment application. Abbreviations for treatments as described in Table 7.1. Means followed by different letters were significantly different at $P \leq 0.05$. n.s. = not significantly different.

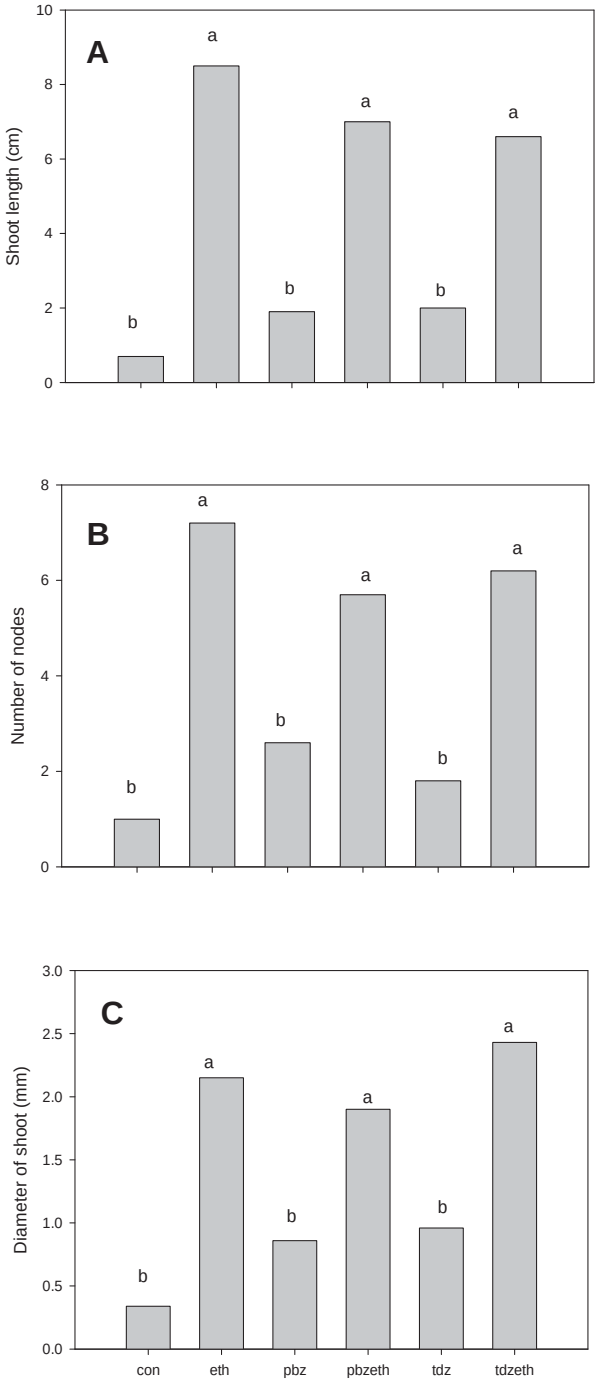

Figure 7.7 (A) Shoot length (B), number of nodes and (C), diameter of new shoots developed from crown, at 12 weeks following last treatment application for the main effect of treatment with application of growth regulators to plants of 'Diva' Experiment One). Abbreviations for treatments as described in Table 7.1. For each variable, means followed by different letters were significantly different at $P \leq 0.05$.

7.4.1.4 Buds and shoots from roots

The number of root buds and root shoots increased with the duration from the last treatment application, and varied in response to the photoperiodic regime and the type of growth regulators applied (Table 7.2; Figure 7.8). While not evident at earlier sampling dates, by the 20th week the influence of the photoperiodic regime, application of growth regulators, and their interaction was evident (Table 7.2; Figure 7.8A & B). Growth regulator effects were not evident under the long photoperiodic regime for the number of root buds and root shoots ($P > 0.05$; Figure 7.8A & B, Figure 7.9). In contrast, within the short photoperiodic regime, application of TDZ followed by ethephon had the greatest number of root buds and root shoots, being greater than those evident within the Control ($P < 0.001$), ethephon alone ($P < 0.001$) or PBZ alone ($P < 0.001$) treatments (Figure 7.10). Treatment with PBZ followed by ethephon also produced three more root buds than plants within the Control ($P < 0.05$), ethephon alone ($P < 0.05$) or PBZ alone ($P < 0.05$) treatments. Fresh weight of the combined total of the root buds and root shoots followed a similar pattern of change to their combined number, through to the 20th week ($P < 0.0001$), with the short photoperiodic regime (2.4 ± 1.4 g) having a greater influence than the long (0.5 ± 0.4 g), at the 20th week ($P < 0.05$; data not shown).

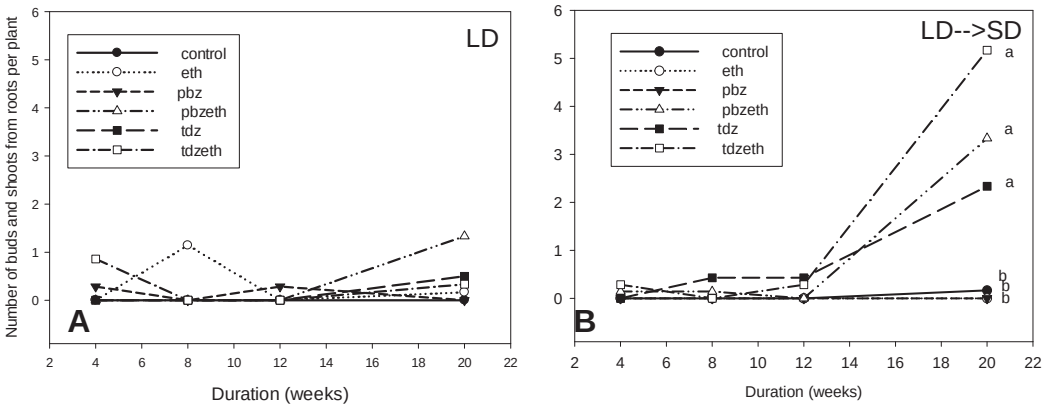

Figure 7.8 (A-B) Combined number of visible root buds and shoots per plant during the 20 week period following the last treatment application within two photoperiodic regimes (A, LD; B, LD→ SD) for plants of 'Diva' (Experiment One). Abbreviations for treatments as detailed in Table 7.1. Means followed by different letters were significantly different at P ≤ 0.05, n.s. = not significantly different.

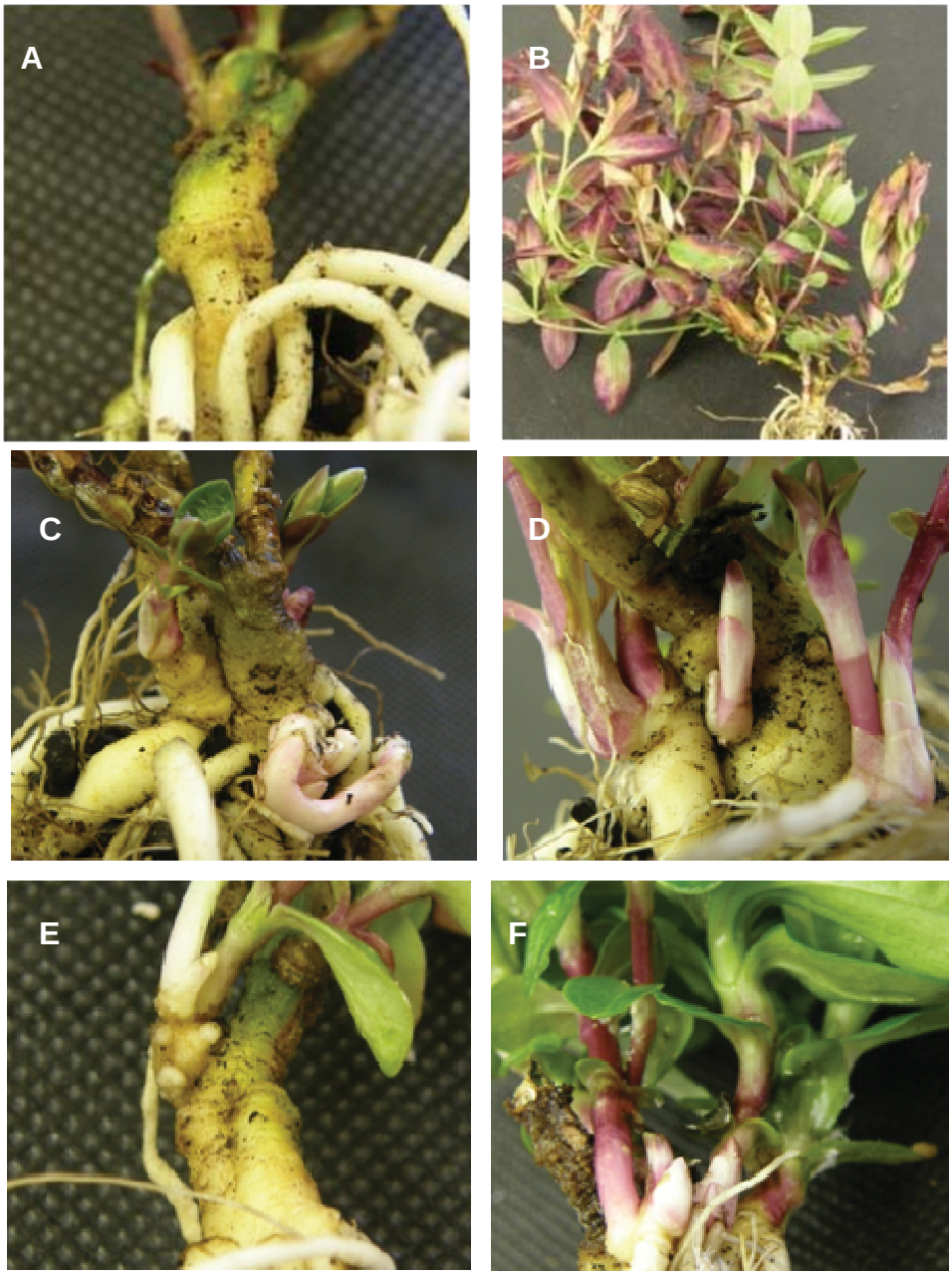

Figure 7.9 Macroscopic features near the transition zone of representative samples of plants of 'Diva' from treatments under the long photoperiodic regime 20 weeks following the last treatment application (Experiment One); (A) Control with no crown buds, (B) ethephon with clusters comprising both crown buds and crown shoots, (C) PBZ with clusters comprising crown buds, (D) PBZETH with clusters comprising both buds and shoots, (E) TDZ with small and thin crown buds and crown shoots, (F) TDZETH with clusters comprising both crown buds and crown shoots. Abbreviations for treatments as detailed in Table 7.1.

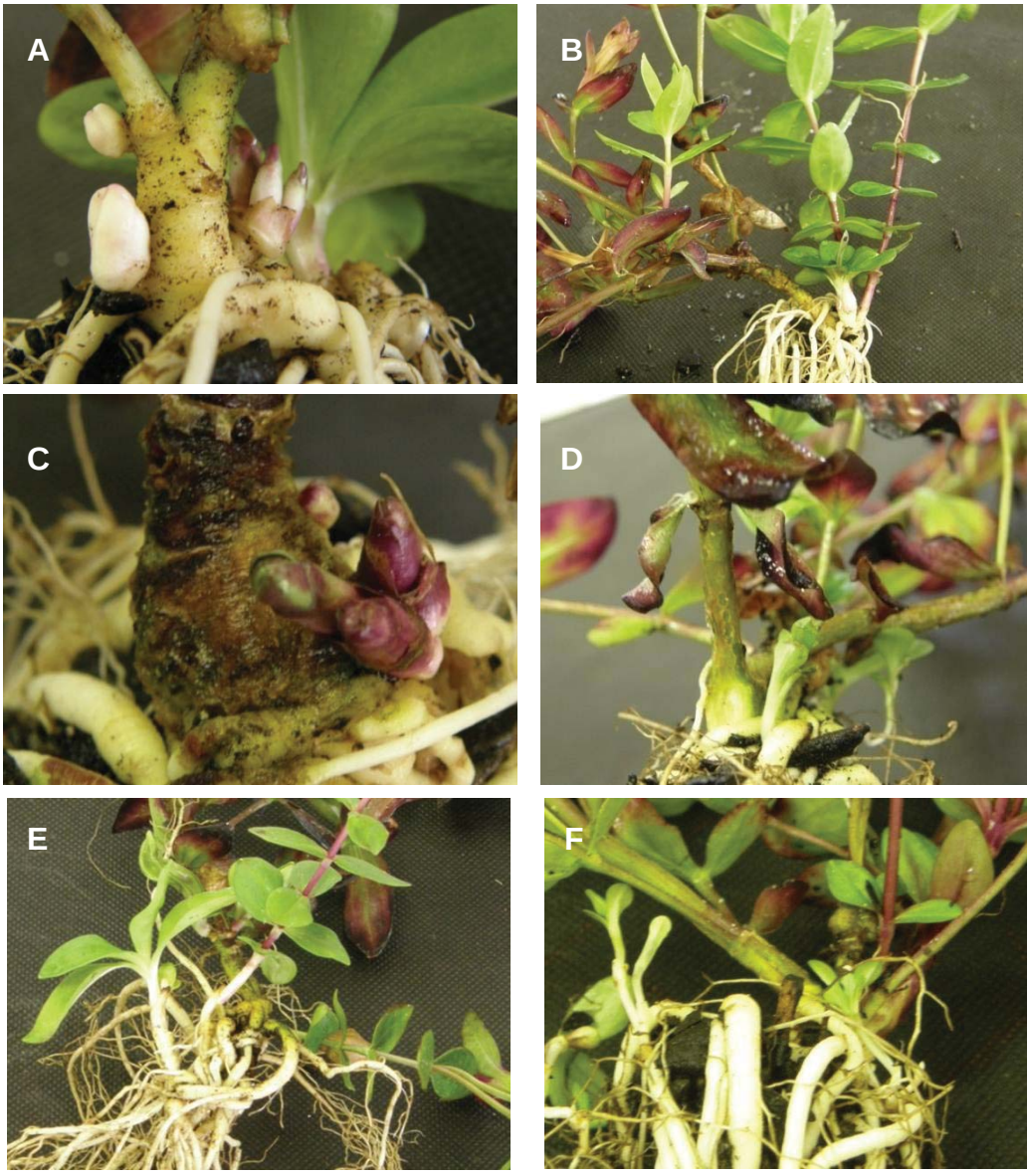

Figure 7.10 Macroscopic features near the transition zone of representative samples of plants of 'Diva' from treatments under the naturally occurring short photoperiodic regime 20 weeks following the last treatment application (Experiment One); (A) Control with clusters of crown buds, (B) ethephon with clusters comprising both crown buds and crown shoots, (C) PBZ with clusters of crown buds, (D) PBZETH, (E) TDZ, (F) TDZETH with clusters comprising buds and shoots from roots, which were small and thin. Abbreviations for treatments as detailed in Table 7.1.

7.4.1.5 Diameter of the transition zone

The application of growth regulators influenced the diameter of the transition zone at both the 4th and 8th week ($P < 0.05$), but not at the 20th week ($P > 0.05$) of sampling. An effect of the photoperiodic regime was not evident at any sampling date. At the 4th week, application of PBZ alone, ethephon alone or TDZ followed by ethephon, resulted in plants with between 50% and 75% greater diameter compared to within the Control

treatment (3.2 ± 0.26 mm). As evident at the 8th week, plants treated with PBZ developed the widest transition zone (7.5 ± 0.66 mm) followed by ethephon (6.1 ± 0.72 mm). Comparatively, at this time of sampling, plants within the Control treatment consisted of a 4.2 ± 0.61 mm diameter transition zone. Although not significant at the 20th week, plants treated with PBZ alone had a greater diameter at the transition zone (7.2 ± 0.76 mm) than evident within the rest of the treatments (5.6 ± 0.55 mm).

7.4.1.6 Growth of existing primary and secondary shoots and storage roots

As a measure of assimilate partitioning to above-ground foliage, the combined dry weight of primary and secondary shoots measured at the 12th week following the last treatment application was evaluated, and an interaction between the photoperiodic regime and the application of growth regulators was evident ($P < 0.05$). Within the Control treatment, combined shoot dry weights were 53% greater under the long photoperiodic regime than the short photoperiodic regime ($P < 0.05$). Under the short photoperiodic regime, treatment with ethephon alone ($P < 0.05$), PBZ alone ($P < 0.01$), PBZ followed by ethephon ($P < 0.05$) or TDZ ($P < 0.05$), resulted in the combined shoot dry weights being nearly 50% greater than achieved in the Control treatment (Figure 7.11A & B). In contrast however, there were no significant differences in the combined primary & secondary shoot dry weight between plant growth regulator treatments under the long photoperiodic regime ($P > 0.05$).

At the 12th week the dry weight of the storage roots was not significantly influenced by the main effects or their interactions ($P > 0.05$), with an average of 1.7 ± 0.11 g being attained.

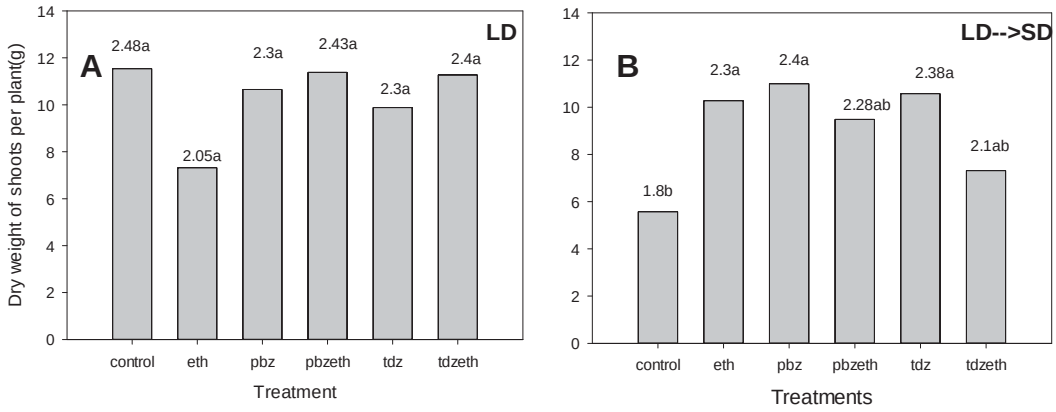

Figure 7.11 (A-B) Dry weight of primary and secondary shoots per plant as at 12th week following the last treatment application within two photoperiodic regimes (A, LD; B, LD→ SD) of 'Diva' (Experiment One). Abbreviations for treatments as described in Table 7.1. Means followed by different letters were significantly different at P ≤ 0.05; data were log transformed for analysis, hence both transformed means and mean separation letter are presented above each column.

7.4.1.7 Second growth cycle - survival and development

Due to poor plant survival through to the end of the first growth cycle, the number of plants varied greatly between treatments, leading to a variable number of replicates (3-13) for assessment of growth and development in the second growth cycle. Some caution therefore needs to be taken in interpreting the results from the second growth cycle, as a greater chance of falsely claiming non-significance is presumably likely.

During the second growth cycle, following 8 weeks of growth out of cold storage, the number of crown shoots which had emerged (including the shoots emerged prior to cold storage) did not differ between the treatments ($P > 0.05$), and averaged 2.1 ± 0.5 per plant. In contrast, an influence of the photoperiodic regime ($P < 0.01$) and the application of growth regulators ($P < 0.01$) was evident for the number of shoots arising from roots, with a greater number of root-shoots under the short photoperiodic regime (1.5 ± 0.62) than the long photoperiodic regime (0.4 ± 0.14). Compared to all other treatments, TDZ alone resulted in the greatest number of the root-shoots (5 ± 2), with effects more evident under the short photoperiodic regime.

The number of crown shoots that reached harvest maturity, irrespective of whether they were emerged prior to or following cold storage, were not influenced by the application of growth regulators, photoperiodic regime or, their interaction ($P > 0.05$). In contrast, the length of harvested shoots was influenced by the application of growth regulators ($P < 0.01$; Table 7.4) and its interaction with photoperiodic regime ($P < 0.001$). Of the

plants that were in the long photoperiodic regime in the first growth cycle, treatment application of PBZ followed by ethephon produced the longest shoots, being between 20 cm and 40 cm longer than from PBZ alone, TDZ alone or TDZ followed by ethephon. Under this photoperiodic regime the length of shoots arising from the PBZ followed by ethephon treatment were however, not different from those within the Control treatment or ethephon alone. Of the plants that were in the short photoperiodic regime, plants within the Control treatment had ≈ 40 cm longer shoots than the treatments with single application of ethephon or TDZ, but were not different from the rest of the treatments.

The number of nodes per shoot at harvest was influenced by the treatments, with the application of growth regulators ($P < 0.001$) and their interaction with the photoperiodic regime ($P < 0.01$) following a similar pattern to that evident for shoot length. Influence of the application of growth regulators ($P < 0.05$) and their interaction with the photoperiodic regime ($P < 0.01$) was also evident for the number of nodes with floral axillaries. Of the plants that were under the long photoperiodic regime ($P < 0.01$), application of PBZ followed by ethephon produced the greatest number of floral nodes, achieving 3-4 more floral nodes than on shoots arising from plants treated with PBZ alone or, when TDZ was applied followed by ethephon or not.

The effects of the application of growth regulators on shoot quality, i.e. length and node numbers was not prominent for treatments applied under the short photoperiodic regime. Shoots harvested from the Control treatment under the short photoperiodic regime however, produced 1 more floral node than under the long photoperiodic regime ($P < 0.05$).

Table 7.4 Quantitative and qualitative characteristics of crown shoots at harvest maturity (second growth cycle) of plants of 'Diva' (Experiment One), resulting from treatment within the first growth cycle. (All plants were grown in the long day environment during the second growth cycle).

Growth regulator	Number of crown shoots harvested [z]		Shoot length (cm)		Number of nodes		Number of floral nodes	
	LD	LD>SD	LD	LD>SD	LD	LD>SD	LD	LD>SD
Control	2^{a}	3.2^{ab}	42.8^{ab}	59.2^{a}	14.3^{b}	19.4^{a}	5.8^{ab}	7.2^{ab}
PBZ	3.6^{a}	1.4^{b}	39.8^{b}	41.4^{ab}	14.9^{b}	13.1^{ab}	4.9^{b}	7.4^{a}
TDZ	2^{a}	1^{b}	21^{b}	17^{b}	12^{b}	7.2^{b}	3.4^{b}	3^{b}
ETH	3.5^{a}	6.0^{a}	47.6^{ab}	23^{b}	14.3^{b}	14^{a}	5.7^{ab}	7^{ab}
PBZETH	3.3^{a}	1.7^{b}	71^{a}	34.9^{ab}	23.3^{a}	14.4^{a}	8.1^{a}	4.6^{ab}
TDZETH	1^{a}	0^{b}	19^{b}	34.3^{ab}	10^{b}	13.1^{ab}	5^{b}	5.3^{ab}

[z] Within the each column, means followed by different letters were significantly different at $P \leq 0.05$ for each variable using DNMRT groupings. Abbreviations for treatments as described in Table 7.1..

7.4.2 Experiment Two: Photoperiod and temperature

At 15 weeks following the change of growing environment, the combined number of crown buds and crown shoots was four times greater ($P < 0.05$) under the outside environment (LD→SD; Low T) than within the long photoperiodic regime in the greenhouse environment (Figure 7.12). Although not significantly different, within the greenhouse environment the combined number of crown buds and crown shoots was two times greater under the naturally occurring short photoperiodic regime (LD→SD) than in the continuous long photoperiodic regime.

The dry weight of shoots ($P < 0.01$) was 2-3 times greater in the long photoperiodic regime than either the short photoperiodic regime or outside (Figure 7.12). Dry weight of storage roots however, was not different ($P > 0.05$) between treatments, averaging 1.385 ± 0.24 g.

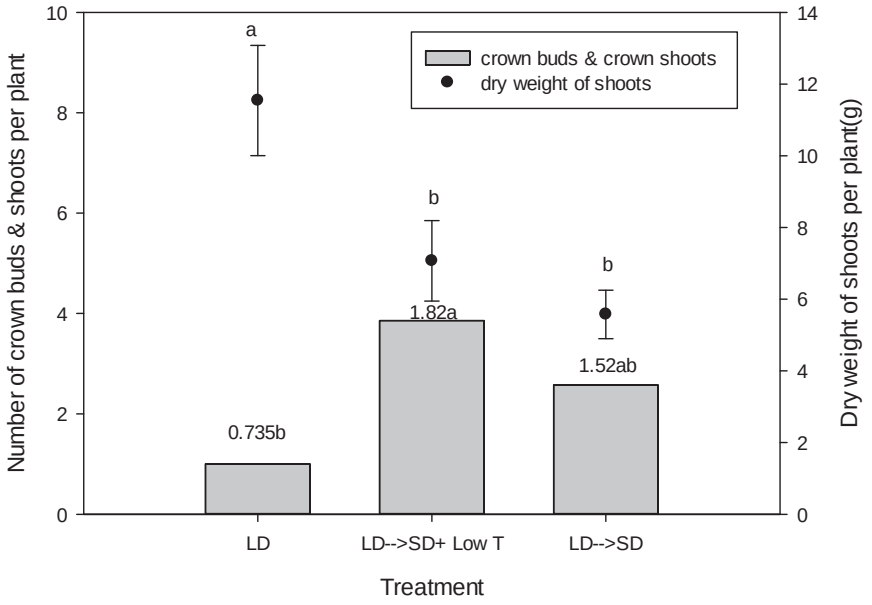

Figure 7.12 Combined number of visible crown buds and crown shoots and total above-ground dry weight per plant of 'Diva'15 weeks following the last treatment application (Experiment Two). Abbreviations for treatments as described in Table 7.1. Vertical lines represent ± standard error values. Means followed by different letters were significantly different at $P \leq 0.05$; number of buds and shoots data were sqroot transformed for analysis, hence both transformed mean and mean separation letter are presented above each column.

7.4.3 Experiment Three: Photoperiod, GA_3, PBZ, ethephon, 1-MCP alone or in combination

The application of growth regulators affected the combined number of crown buds and crown shoots recorded at the 12th week following the last treatment application ($P < 0.05$; Figure 7.13). Plants treated with PBZ followed by ethephon presented the greatest combined number, followed by ethephon alone, and each was three times greater than 1-MCP followed by GA_3 or 1-MCP alone. Application of GA_3 alone, 1-MCP alone or 1-MCP followed by GA_3 resulted in a similar combined number of crown buds and crown shoots, averaging 1 ± 0. Although not significant, treatment with PBZ alone resulted in twice the combined total of crown buds and crown shoots than evident when GA_3 was applied alone.

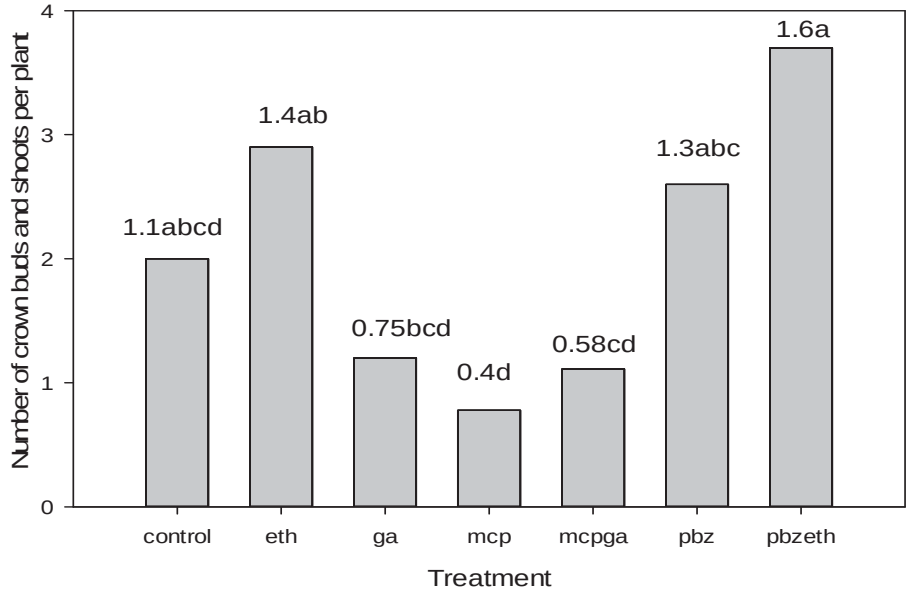

Figure 7.13 Combined number of visible crown buds and crown shoots per plant for the main effect of application with growth regulators at the 12th week following the last treatment application of 'Diva' (Experiment Three). Abbreviations for treatments as described in Table 7.1. Means followed by different letters were significantly different at $P \leq 0.05$; for data that were sqroot transformed, transformed means were presented along with mean separation letter.

7.5 Discussion

7.5.1 Influence of photoperiod

7.5.1.1 Quantity and quality of crown buds and resulting shoots

Similar to the findings reported in previous experiments (Chapter 6), during the current study the naturally occurring short photoperiodic regime promoted the crown bud formation process. Within experiments One and Two this was evident by the combined total of crown buds and crown shoots being five times greater and the number of bud clusters being three times greater, as compared with under the long photoperiodic regime (Figure 7.3C-D, Figure 7.4A-B, & Figure 7.12). A similar influence of short photoperiod on promotion of formation of vegetative propagules has been noted in other species (Heide, 2001; Hytönen et al., 2004), similarly confirming for these species that a short photoperiod led to increased production of buds for regrowth in the next growth cycle. Along with this quantitative influence, within plants in the Control treatment, the diameter of crown buds was also greater under the naturally occurring short than long photoperiodic regime, indicating the promotion of a qualitative influence of the photoperiodic regime on buds (Figure 7.5, Figure 7.9A & Figure 7.10A).

During growth in the subsequent second growth cycle, the non-significance of any treatment effect for the number of shoots emerged or harvested (Table 7.4) could be due to the variable number of plants remaining alive, particularly following exposure of plants with recently emerged shoots to chilling (Chapter 8). Although not statistically significant, the measures of floral shoot quality (i.e. shoot length, number of nodes, and number of floral nodes) were, however, greater at harvest maturity in plants that were grown in the naturally occurring short photoperiodic regime during the first growth cycle (Table 7.4). As also previously noted (Chapter 3), greater quality of shoots may be attributed to the greater quality of buds (i.e. diameter of buds) within this treatment. The results of the current study therefore, confirm the beneficial effect of the naturally changing photoperiodic regime on the crown bud formation process, both qualitatively and quantitatively.

7.5.1.2 Photoperiod induced photo-assimilate distribution

Based on the greater combined primary and secondary-shoot dry weight under the long photoperiodic regime (Figure 7.11C-D & Figure 7.12), it was concluded that the long photoperiodic regime promoted partitioning of photo-assimilates to above-ground growth. Since significant differences between photoperiods were not evident for the dry weight of storage roots (as also reported in Chapter 6), based on the greater number of crown buds and crown shoots arising from the transition zone under the naturally occurring short photoperiodic regime (Figure 7.3C-D), it was hypothesized that the short photoperiodic regime promoted partitioning of photo-assimilates to the transition zone. This inverse relationship between above-ground growth and the transition zone was also observed in Chapter 6. This relationship was further confirmed within Experiment Two, with the plants in the short photoperiodic regime having 2-3 more crown buds, but less dry weight of shoots, compared to the plants in the long photoperiodic regime (Figure 7.12). As found in *Gladiolus grandiflorus* Andrews, due to changes in the partitioning of assimilates, growth of corms continued throughout the growing period when under a short photoperiod, but not under a long photoperiod (Halevy, 1985; Shillo and Halevy, 1981). Hence in interpreting the results of the current study, it could be hypothesized that the transition zone, including crown buds, becomes a stronger sink under the short photoperiodic regime, whereas primary and secondary shoots become a stronger sink under the long photoperiodic regime.

The transition zone is used for translocation and/or storage of assimilates (Baird et al., 1992; Bellingham and Sparrow, 2000; Bowes, 1996; Del Tredici, 2001; Suzuki and Stuefer, 1999), as well as the site of attachment for crown bud clusters in gentians (Chapter 5). While the counteracting influence of long and short photoperiodic regimes was most eloquently shown using gladiolus (Halevy, 1985; Shillo and Halevy, 1981), in other plant species the promotion of partitioning of photo-assimilates to various underground storage organs was also observed with exposure to short photoperiod (Masuda et al., 2007; Rodríguez-Falcón et al., 2006; Snyder and Ewing, 1989; Stewart and Kane, 2006; Van Dam et al., 1996), as well as under low irradiance (Funnell et al., 2002a). Further support for the existence of a photoperiodic response similar to what was reported here with the gentian cultivar 'Diva', was evident by the inhibition of the number of buds and the promotion of shoot growth observed in *Dioscorea alata* L. (Vaillant et al., 2005) and *Sedum telephium* L. (Heide, 2001) under a long photoperiodic regime. Although distinct differences in variables indicative of size or weight of the transition zone and roots was not evident during the current study, a greater number of crown buds and shoots, as well as bud clusters, is indicative of assimilates having been utilized for their initiation and development (Marcelis, 1996). It is therefore hypothesized that gentians follow the common phenomenon observed in other herbaceous perennials in response to shortening photoperiod, i.e. accumulation of photo-assimilates within the transition zone/rhizome, which contributes either directly or indirectly to development of crown buds. This phenomenon will be discussed further in association with growth regulators in Section 7.5.6. As evident in the current study, however, some crown buds were initiated in the first growth cycle under the long photoperiodic regime (Figure 7.3, Figure 7.4, & Figure 7.12), leading therefore to the conclusion that a shortening photoperiod was a facultative requirement for the crown bud formation process in 'Diva'. The optimum photoperiod or the photoperiodic transition required therefore, remains to be determined in the future.

7.5.1.3 Cold vs photoperiod

When plants of 'Diva' were exposed to the naturally occurring short photoperiodic regime, the crown bud formation process was promoted with or without exposure to cold growing conditions (Figure 7.12; Chapter 6), which was similar to that reported for other herbaceous perennials like *S. telephium* (Heide, 2001). In contrast, some species require cold temperatures coupled with shorter photoperiod, or only cold temperatures

alone, to promote development of buds with dormancy, e.g. *Prunus* spp (Heide, 2008). Although in the current study the crown bud formation process was promoted by the naturally occurring short photoperiodic regime, even when crown buds were present, without cold exposure under the greenhouse environment, poor plant survival was noted during the current study with 'Diva', and in previous experiments with 'Spotlight' (Chapter 3). As found in *Populus deltoides* Bartr., short-day conditions activated some cold-resistant genes without exposure to low temperature, and enhanced endodormancy, but cold increased the degree of cold hardiness (Druart et al., 2007; Park et al., 2008). Hence in order to achieve greater survival along with a greater number of crown buds, as observed under the short photoperiod, lower temperature may also be required in 'Diva'. The presence of proteins associated with cold hardiness has been reported in gentians (Takahashi et al., 2006), although what triggers production of these proteins is not yet understood. Considering the promotive effects of cold on subsequent shoot emergence (Chapters 3, 8 & 9), future experiments might benefit from extending the focus to include the interaction of photoperiod and chilling on the crown bud formation process, endodormancy, plant survival, and their relation to these proteins responsible for cold resistance in gentians.

During the current study, the treatment differences in the degree of endodormancy of crown buds formed was difficult to determine, because plants carried crown buds and crown shoots at different stages of development at any given point in time. As a result of that, and also due to the variable number of plants surviving through to the second growth cycle, data related to shoot emergence in the second growth cycle was not statistically different among treatments. Based on the pattern of shoot emergence in the first growth cycle, although a greater number of crown buds was present, emergence of these as shoots was less in the short photoperiodic regime as compared with the long photoperiodic regime within the Control treatments (Figure 7.6 A-B). This presumably indicates the possible inhibitory effect of a short photoperiodic regime on shoot emergence, or imposition of dormancy in buds as found in poplar (Ruttink et al., 2007). It is possible that under natural conditions the crown bud formation process as well as induction of endodormancy is a facultative response to short photoperiod (Fennell and Hoover, 1991; Masuda et al., 2006). It is also possible however, that the crown bud formation process and endodormancy are two separate processes that occur concurrently under natural conditions.

7.5.2 Influence of gibberellins

As found in previous experiments (Chapter 6), during the current experiments GA_3 inhibited the crown bud formation process, but application of the GA synthesis inhibitor PBZ did not result in the development of a significantly greater number of crown buds/shoots as the Control treatment (Figure 7.13). This therefore was contrary to what was expected if GA-mediated photoperiodic control over the crown bud formation process was believed to be operating in 'Diva', as evident with potato where initiation of tubers, which was promoted by a short photoperiodic regime (Martinez-Garcia et al., 2001), was delayed by the application of GA_3 (Vreugdenhil and Sergeeva, 1999; Xu et al., 1998) and was enhanced by PBZ (Bandara and Tanino, 1995; Harvey et al., 1991). As the response to PBZ could vary with dosage (Nishizawa, 1993) and, lack of observation of an increased number of crown buds and/or shoots during the current study may have been attributed to insufficient dosage to counteract the endogenous GA activity. This was also noted in the failure to observe the commonly known response of growth retardation by PBZ (Vreugdenhil and Sergeeva, 1999), as also evident in the current study with the dry weight of primary and secondary shoots (Figure 7.11). Alternatively it could also be due to variation in sensitivity of tissues (Firn, 1986) or species-specificity (Rademacher, 2000) to applied PBZ and, therefore, a response may be apparent if applied at a different developmental stage. Although application of PBZ did not increase the number of crown buds, when applied under the naturally occurring short photoperiodic regime the crown bud formation process was not inhibited (Figure 7.3D), subsequently resulting in the crown buds (Figure 7.4B & Figure 7.5B) and shoots of similar quality to those within the Control treatment (Table 7.4). With potato under a non-inductive long photoperiodic regime, the concentration of endogenous GA was also high (Vreugdenhil and Sergeeva, 1999), and vice versa under a short photoperiodic regime (Jackson, 1999; Rodríguez-Falcón et al., 2006) and, therefore, assuming the same mechanism may apply with 'Diva', the dosage of PBZ used during the current study may have failed to counteract this increased GA level. Hence in any future experiments, utilising PBZ at a range of dosages at different developmental stages would enable clarification of what if any influence inhibition of GA synthesis has on the crown bud formation process. Further to that, the sequential application of both GA_3 and PBZ, as a full factorial of treatments, would also enable a better understanding of role of gibberellins in the crown bud formation process.

The promotive influence of PBZ on the quality of storage organs expressed as weight and period of dormancy (Tekalign and Hammes, 2004), and decreased quantity, was noted in other species (Ascough et al., 2008). In the current study a similar influence was noted with greater diameter of the transition zone (Section 7.4.1.5; Figure 7.9C & 10C). Based on this qualitative effect on the transition zone, and the increased quality and number of crown buds formed under the short photoperiodic regime, in future experiments it will be important to investigate these variables under the influence of different dosages of PBZ. Even under the long photoperiodic regime however, PBZ was more effective in quantitative as well as qualitative aspects when plants were treated with ethephon (Figure 7.3C, Figure 7.4A & Table 7.4). Hence, although not statistically evident during the current study, PBZ may have an impact on initiation and development of crown bud clusters but, as discussed in the following sections (refer Section 7.5.5) may involve crosstalk with other growth regulators. Alternatively due to the differences in mode of action of different anti-gibberellins in the metabolic pathway, the response to a gibberellin biosynthesis inhibitor could be species-specific (Rademacher, 2000). It is possible therefore to explore gibberellin biosynthesis inhibitors other than PBZ in the future, so as to identify a better growth retardant for gentians.

The influence of GA_3 on promotion of axillary bud outgrowth in 'Diva' was noted previously (Chapter 6), as with shoot emergence in potato (Suttle, 2004) and growth of root buds in *E. esula* (Horvath, 1999). This response potentially suggests that GA_3 stimulates development of already existing meristems and, therefore, when applied to plants without any pre-formed bud initials on the transition zone, the above-ground growth and emergence of axillary buds (i.e. secondary shoots) was promoted, and the crown bud formation process was inhibited. As an extension of this logic therefore, it was hypothesized that GA_3 can induce the development of already existing bud initials from the transition zone provided plants have crown buds already formed, but may have no direct effect on the initiation of new meristems *per se*. GA_3 therefore, may have a role in bud emergence rather than initiation, and was investigated in this regard in subsequent chapters (refer Chapters 8 & 9).

7.5.3 Influence of TDZ

While a greater response to TDZ was evident as early as the 4th week with the number (Figure 7.3B) and fresh weight of crown buds (Section 7.4.1.3), the influence on these variables was not maintained through to the 20th week (Figure 7.3C-D). As is also evident by the diameter of crown buds (Figure 7.5), similar to the findings reported previously (refer Chapter 6), TDZ negatively affected the quality of the crown buds (Figure 7.9E & Figure 7.10E). It could therefore be hypothesized that while TDZ promotes the formation of adventitious bud initials (Hosokawa et al., 1996; Martínez et al., 2008; Proctor et al., 1996; Sanago et al., 1995) in the transition zone (Chapter 5), it did not have a sustained effect on developing these initials into clusters of crown buds of high quality (Figure 7.4). As evident by the earlier timing of a response at the 4th week to TDZ compared to the other growth regulators however, the use of TDZ appears to be a good strategy for initiation of adventitious buds earlier. While in theory this provides sites for crown bud clusters to develop, future research should target determining the optimum dosage required, and the impact on subsequent development of crown buds within clusters.

The crown shoots which developed in plants treated with TDZ in the second growth cycle had fewer nodes and were shorter in length compared to those in other treatments, i.e. were of poor quality (Table 7.4), which is also consistent with the findings reported in other species (Murthy et al., 1998). This also supports the hypothesis that TDZ may be influential only on the initial stages of the crown bud formation process, i.e. initiation of crown bud initials, but not in the subsequent development of crown buds within the cluster. Hence even if bud initiation may be promoted, use of TDZ does not appear to give the qualitative output for floral shoot production. In terms of the limitations associated with the use of TDZ, it was additionally noted that compared to the previous study when TDZ was applied (refer Chapter 6), in the current study a greater effect on initiation and development of crown buds was not observed compared to that achieved with the other growth regulators (Figure 7.3C-D & Figure 7.4). Plants used during the current study however, were more developed compared to this previous study, and the potential response to TDZ could vary based on the stage of growth of plant material, as observed in *Pinus pinea* L. (Valdes et al., 2001). Considering the greater number of bud initials following application of TDZ (Figure 7.3B; Chapter 6), the potential exists for its use in the commercial production of gentians, however in order to resolve the differential response between the two experiments, the response to a range of

concentrations at different growth stages might be examined in any future experiments. Alternatively however, as discussed below (refer to Section 7.5.5), the potential benefits of TDZ on bud initials could be used to improve the quantity of crown buds in conjunction with other growth regulators like ethephon.

Plants treated with TDZ under the naturally occurring short photoperiodic regime, developed a greater number of root buds and root shoots in the first growth cycle (Figure 7.3, Figure 7.8, Figure 7.9 & Figure 7.10), which persisted into the second growth cycle (Section 7.4.1.7). In other plant species promotion of adventitious buds from roots occurs in conjunction with root growth after application of TDZ, as previously reported *in vitro* (Medina et al., 2009; Sanago et al., 1995) and *in vivo* (Proctor et al., 1996). Hence during the current study, it is assumed that following application of TDZ, the development of below-ground growth was further stimulated via the enhanced partitioning of photo-assimilates towards root buds under short photoperiods (refer Section 7.5.1).

7.5.4 Influence of ethephon

The application of ethephon was the single most effective hormonal treatment promoting the crown bud formation process in 'Diva' (Figure 7.3C-D, Figure 7.4, Figure 7.5, Figure 7.9B, Figure 7.10B & Figure 7.13). While the influence of ethephon on increasing the quantity of crown buds in gentians has been reported previously (Chapter 6) and in preliminary technical reports (Morgan and Debenham, 2008; Sato et al., 1988), neither the timing and qualitative aspects in response to ethephon nor its influence in combination with other growth regulators, have previously been addressed. Hence the research reported here presents the promotive influence of ethephon on the crown bud formation process based on quantity, quality as well as timing, as a single application or in combination with PBZ or TDZ.

It is assumed that the application of ethephon results in the production of ethylene (Warner and Leopold, 1969). While an increase in the initiation and development of buds following exposure to ethylene has not frequently been reported in other species, naturally produced ethylene, from source materials *in-vitro*, has been associated with the formation of adventitious buds in *Lilium* spp. (Van Aartrijk et al., 1985). As evident by the increased number (Figure 7.3B) and fresh weight of crown buds (Section 7.4.1.3), the promotive influence of ethephon became evident under the long

photoperiodic regime as early as the 4th week after treatment application. This presumably occurred via the initiation of bud initials on the transition zone. It could therefore, be hypothesized that ethephon/ethylene also has a role in the initiation of crown bud initials, similar to that evident with TDZ. During the current study however, histological studies were not conducted in order to confirm the actual initiation of new bud initials. The use of histological investigations focussed on the transition zone in any future investigation, would distinguish the role of ethephon as well as TDZ in the initial stages of the crown bud formation process.

All the treatments which included ethephon resulted in a two times greater combined total of crown buds and crown shoots per cluster than in the Control or TDZ treatments (Figure 7.4C). Clearly therefore, in addition to initiation of bud initials, ethephon also increased development of buds within a cluster. The diameter of crown buds at the 20th week was also greater in ethephon-treated plants, irrespective of the growing environment (Figure 7.5A-B), indicating qualitative effects in accordance with previous findings (Chapter 6). As noted in preliminary technical reports, between 1000 and 4000 ppm ethephon was applied at multiple times (i.e. between three and seven times) to achieve a 20% increase in the number of crown buds (Sato et al., 1988). In contrast, as evident in the current study, a single application of 864 ppm of ethephon was sufficient to promote the crown bud formation process quantitatively (i.e. 5-fold increase under long day regime) and qualitatively, indicating the potential to use ethephon at lower concentrations and less frequently. Future research may, therefore, benefit from evaluating different concentrations and frequencies of ethephon, so as to identify an optimum dosage to achieve an adequate quantity of crown buds.

As discussed above, ethephon promoted the crown bud formation process, compared to the other treatments. Compared to treatments with TDZ alone, PBZ or, TDZ followed by ethephon however, a single application of ethephon did not enhance growth of storage roots, development of buds from roots within the short photoperiodic regime (Figure 7.3 & Figure 7.8), or the combined dry weight of primary and secondary shoots (Figure 7.11). From this it is inferred that the influence of ethephon on the crown bud formation process cannot be explained via partitioning of photo-assimilates to below-ground growth. As an alternative therefore, as also proposed with *L. speciosum* (Van Aartrijk et al., 1985), ethylene may have promoted cell division at potential sites for initiation of adventitious buds promoting the initial stages of the crown bud formation

process. Supporting this, the roles of exogenous ethylene as a stimulator of sucrose transport in *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. (Dusotoit-Coucaud et al., 2009) and in mobilization of storage starch in *Sesbania virgata* (Cav.) Pers. (Tonini, 2010) was noted. As evident by a greater diameter in the transition zone in response to ethephon (Section 7.4.1.5), it is also possible that these resources are directed to development of phloem and xylem tissues in the transition zone (Yamamoto et al., 1987; Yamamoto and Kozlowski, 1987), due to increased activity of the cambium (Junghans et al., 2004). Thus ethephon could have a role in development of the transition zone of 'Diva', which is presumed to be a prerequisite for the initiation of crown buds (Chapter 5). The observation of a greater number of buds and shoots within the cluster with application of ethephon however, could be attributed to the growth and development following release of axillary buds from paradormancy within the cluster (Hillman and Yeang, 1979), leading to growth of individual crown buds. It may be possible that development of the transition zone, bud initials and, buds within clusters, were stimulated irrespective of the photoperiodic regime via ethephon, unlike with the other plant growth regulators and their combinations utilized. As a result therefore, development of bud clusters from roots did not occur with a single application of ethephon, as compared to these other growth regulator treatments.

Application of ethephon with or without other growth regulators, promoted the crown bud formation process (Figure 7.6C), as well as the emergence of crown shoots, as early as the 12th week following treatment application (Figure 7.6A-B). It could be concluded therefore, that ethephon is not only capable of promoting early initiation of crown buds, but also promoting their emergence by shortening/avoiding the period of dormancy. The response could be, however, dosage dependent, as in potato where short-term ethylene treatment terminated tuber dormancy leading to shoot emergence, while continuous treatment was capable of inhibiting shoot emergence (Rylski et al., 1974). Presence of both negative (Daniels-Lake et al., 2005) and positive influences (Pruski et al., 2006) on shoot emergence with ethylene in potato, emphasises the potential significance of the dosage and developmental stage at the time of application (Prange et al., 1998; Suttle, 2004). As also observed in other species (Ruttink et al., 2007; Sumitomo et al., 2008), it was hypothesised at the beginning of this study, that the promotion of the number of crown buds by ethephon may have been attributed to its effect on dormancy. Ethylene signalling and biosynthesis has been reported to induce dormancy in response to short photoperiod in *Populus* spp (Ruttink et al., 2007) and

Betula pendula Roth (Ruonala et al., 2006). Further to that, exogenous application of ethylene (1000 mg/l) caused dormancy in *C. morifolium* (Sumitomo et al., 2008). In contrast, the increased emergence of shoots (Figure 7.6) in response to ethephon, irrespective of photoperiodic regime, infers that rather than induction of dormancy, ethylene may also be involved in breaking endodormancy. The role of ethephon/ethylene in shoot emergence, could also be an indication of the release from ecodormancy as also reported in *E. esula* (Horvath et al., 2006) or paradormancy as reported in *Phaseolus vulgaris* L. (Hillman and Yeang, 1979; Yeang and Hillman, 1984). While its application to the current study cannot be determined, ethylene could be involved in both entry and exit from dormancy (Suttle, 2003). Thus based on these contrasting reports on the role of ethylene, multiple roles in bud development as well as at the start of shoot emergence relative to dormancy (endo, eco or para) are inconclusive. Despite this, ethephon was the most effective single growth regulator promoting the crown bud formation process. With the limited previously published articles on the role of ethylene in initiation and development of dormant buds in herbaceous perennials and endodormancy, it will be important to study these roles in the future.

Due to early emergence of shoots in all treatments involving ethephon, by the time of data collection at the 20th week, the majority of shoots could be confirmed as being floral. Coupled with the influence on timing and quantity of crown buds and shoots, irrespective of the photoperiodic regime, plants treated with ethephon also had crown shoots of greater quality. This was evident by the increased dry weight, shoot length, number of nodes and diameter at the 20th week (Figure 7.7). In contrast to these observations, negative effects on the quality of floral shoots were reported in other herbaceous perennials with increasing ethephon dosage (Hayashi et al., 2001; Kristensen and Adriansen, 1988). It would appear however, that these qualitative effects observed at the 20th week following ethephon treatment with 'Diva' were primarily due to the earlier shoot emergence, as no treatment differences in shoot quality were evident when assessed after shoots within all treatments had eventually been harvested (Table 7.4). Hence what if any concentration and/or frequency of application of ethephon may be optimum for better quality shoots in 'Diva', still requires further research.

As evident in Experiment Three, application of ethephon led to a greater number of crown buds and/or crown shoots as compared with application of the ethylene

antagonist 1-MCP, or 1-MCP followed by GA_3 (Figure 7.13). This therefore provides further supportive evidence confirming the direct involvement of ethylene in the crown bud formation process. Similar to detecting responses to ethylene, the sensitivity of 1-MCP by cultivar, developmental stage, frequency of application (Blankenship and Dole, 2003), and temperature, has previously been noted (Chope et al., 2007). Hence combined with the high variability between individual plants, the failure to observe statistically different effects relative to plants within the Control treatment, could be attributed to one or several of these factors. While ethephon and 1-MCP had opposite effects on emergence of crown buds in 'Diva', as also noted in potato (Pruski et al., 2006), inhibition of shoot emergence by exogenous ethylene and promotion by 1-MCP, has also been reported in onion (Bufler, 2008) and potato (Suttle, 1998). It would appear therefore that the response to ethylene and its antagonists, are likely to be highly sensitive to genotype, dosage, frequency and developmental stage. The variation in response could be due to the changes in number of receptors and the affinity of receptors present within tissues (Davies, 1995; Firn, 1986) to ethylene, and this needs to be addressed relative to gentians. Further to that, due to limited plant availability during the current study, both ethephon and 1-MCP have not been applied in a complete factorial of treatment applications, which would have provided further information on the role of ethylene. With numerous potential hypotheses to be evaluated, how ethylene is involved in the initiation and development of buds through to shoot emergence deserves further study, and this treatment combination may therefore assist.

7.5.5 Interactions between the plant growth regulators

Application of PBZ followed by ethephon under the long photoperiodic regime, led to a greater quantity of crown buds and/or crown shoots than in any of the non-ethephon treatments (Figure 7.3C & Figure 7.9D), including GA_3 followed by 1-MCP (Figure 7.13). Since the number of clusters produced within this treatment was also greater than occurred following ethephon, it is suggested that PBZ may have the ability to promote the formation of bud initials beyond that achieved by ethephon (Figure 7.4A).

Since emergence of crown shoots was not promoted by the application of PBZ alone, the greater number of shoots when PBZ was applied followed by ethephon (Figure 7.6B & C) is likely to have been solely due to the ethephon component of this treatment (Section 7.5.4). Similar to when PBZ followed by ethephon was applied, under the long photoperiodic regime plants treated with TDZ followed by ethephon also had a greater

number of clusters as well as crown buds and crown shoots per cluster (Figure 7.3C, Figure 7.4A and Figure 7.9F). The ability of TDZ to produce bud initials, coupled with the effect of ethephon on initiation, development, and emergence of crown buds, may have contributed to the greater success of this treatment. Additionally however, enhanced ethylene production following application of TDZ has been noted in other plants (Hutchinson et al., 1997; Mutui et al., 2007; Suttle, 1986). Hence the enhanced success of this treatment combination could have resulted from a promotive effect on ethylene production, when supplemented with TDZ. Alternatively it could be hypothesized that while bud initials could occur under many conditions (either TDZ and/or ethephon within this treatment), differentiation of some of these adventitious buds into actual crown bud clusters may require special environmental stimuli, such as a shortening photoperiod, and ethylene may also have a role as discussed elsewhere (refer to 7.5.4). Hence, ethylene appears to be involved in multiple steps within the crown bud formation process, whereas PBZ and TDZ may act only on a few steps, presumably needing cross talk with ethylene in order to complete the process.

Compared with the other treatments, the qualitative influence on shoots was also greater with application of PBZ followed by ethephon, as evident in both the first (Figure 7.7) and second growth cycles (Table 7.4). Although application TDZ followed by ethephon resulted in a similar number of crown buds to that of PBZ followed by ethephon, the quality of shoots however was not greater (Table 7.4). Even under the naturally occurring short photoperiodic regime, where the influence of plant growth regulators was not prominent, the application of PBZ followed by ethephon led to development of shoots of similar quality to that achieved within the Control treatment (Table 7.4). Out of all the treatments therefore, this combination of PBZ followed by ethephon can be considered to produce the best quality floral shoots. Endogenous GA synthesis could vary based on the photoperiod, and was reported to be inhibited in the short photoperiod (Ruttink et al., 2007) and promoted at the long photoperiod (Olsen et al., 1997). PBZ applied to potato under non-inductive conditions for tuberization (i.e. long day), delayed physiological maturity, extended dormancy and improved tuber quality by diverting assimilates to tubers (Tekalign and Hammes, 2004). Relating this to the current study with ‘Diva’ therefore, it could be hypothesized that PBZ may similarly delay the premature emergence of crown shoots on the storage organ, which is normally promoted by ethephon (refer to Section 7.5.4), allowing the plants to acquire greater photo-assimilates and, as a consequence, may enable better shoot productivity and

survival in the next growth cycle. Enlargement of the rhizome was an indication of induction of dormancy in some herbaceous perennials (Masuda et al., 2006) and, in the current study, confirmation of a similar effect could be the greater diameter of the transition zone noted in plants treated PBZ (Section 7.4.1.5; Figure 7.9C & Figure 7.10C). The success of the treatment comprising PBZ followed by ethephon under the long photoperiodic regime, may have been attributed to mimicking the natural phenomenon of decreased GA activity coupled with the increase of ethylene involvement in formation of dormant buds under a short photoperiod (Horvath et al., 2006). Hence as indicated by the present study, PBZ supplemented with ethephon led to development of crown buds in plants with a sustained influence through to the next growth cycle. Considering the potential practical implications of a sequential application of PBZ with ethephon to achieve a greater number of crown buds of higher quality, future studies endeavouring to determine optimum dosages, timing of application and, potential impact in the subsequent growth cycles, deserves further study.

Despite application of ethephon, in combination with either PBZ or TDZ, being found to promote the crown bud formation process in 'Diva', these growth regulator combinations have not previously been noted in promoting formation of buds in gentians, nor other plant species. It will therefore be of interest to study the interactions of these growth regulators further, so as to understand the possible mechanism(s) of their interaction. While the application of ethephon with PBZ or TDZ enabled production of the greatest number of crown buds and/or crown shoots, the effect was apparent only under the long photoperiodic regime. In contrast, under the naturally occurring short photoperiodic regime, these treatments failed to develop a greater number of crown buds and/or crown shoots, as compared with plants in the Control treatment (Figure 7.4B). As discussed in the following sections (refer to Section 7.5.6), this indicates a level of the interaction with the photoperiodic regime.

7.5.6 Interaction between the photoperiodic regime and plant growth regulators

During the current study, a differential response to plant growth regulators based on the photoperiodic regime was evident for the majority of variables (Figure 7.3C-D, Figure 7.4A-B, Figure 7.5, Figure 7.6, Figure 7.9 & Figure 7.10), and this influence was summarised in a schematic diagram (Figure 7.14).

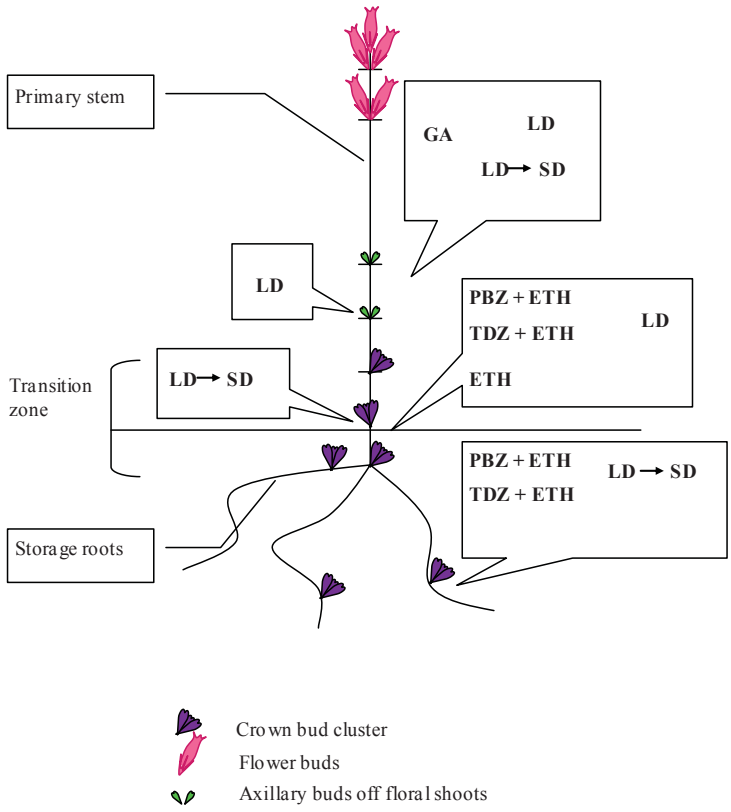

Figure 7.14 Schematic diagram based on Chapters 6 and 7, illustrating potential origin of buds (shoot, transition zone or root) and potential strategies for promotion using photoperiod of the growing environment and/or application of growth regulators. Treatment application abbreviations as per Table 7.1.

With the short photoperiodic regime being promotive for the crown bud formation process, the influence of growth regulators on this process was mostly apparent under the long photoperiodic regime, which by itself was inhibitive. Hence, the treatments which promoted the crown bud formation process (i.e. TDZ followed by ethephon, PBZ followed by ethephon) when compared with the Control treatment under the long photoperiodic regime, either did not differ or were less effective than the Control treatment when applied under the naturally occurring short photoperiodic regime (Figure 7.3D, Figure 7.4B, Figure 7.5B & Figure 7.6B). Under the short photoperiodic regime however, instead of crown buds originating from the transition zone, most of these growth regulator treatments resulted in a greater number of bud clusters or buds and/or shoots from roots (Figure 7.3D, Figure 7.8B & Figure 7.10D-F).

As discussed above (refer Section 7.5.2), the short photoperiodic regime may promote partitioning of photo-assimilates to the transition zone, and the long photoperiodic regime to above-ground growth (Figure 7.11C-D & Figure 7.12). Hence, if the crown bud formation process was promoted on transfer to the short photoperiodic regime indirectly, due to partitioning of photo-assimilates towards the transition zone, it could equally be hypothesized that under the naturally inhibitive long photoperiodic regime the growth regulators which were effective, may have done so by influencing partitioning of assimilates to the transition zone directly or indirectly (Marcelis, 1996). When plant growth regulators were applied following transfer to the short photoperiodic regime however, this translocation process was already being stimulated to preferentially direct assimilates towards the transition zone. Hence it could be hypothesized that the application of growth regulators under these naturally stimulatory conditions, diverts assimilates towards the initiation and development of buds on the roots instead of the transition zone (Figure 7.3D, Figure 7.8B, Figure 7.10 & Figure 7.14). It could be hypothesized that shoots are a sink and the site for organogenesis under the long photoperiodic regime, but application of growth regulators which are capable of diverting assimilates downward within the plant, enable the transition zone to become the primary sink and the site for organogenesis (Figure 7.14). When these same growth regulators are applied under the short photoperiodic regime, which is naturally inductive, the storage roots become the primary sink and, therefore, the site for organogenesis. Sink strength can be defined as the ability of an organ to attract assimilates (Marcelis, 1996) and, as previously discussed, the potential ability to develop bud initials via growth regulators like TDZ or ethephon (Section 7.5.3 and 7.5.4) may make a particular organ a stronger sink. This differential response to exogenous growth regulators could also be due to variation in sensitivity of a particular tissue (Firn, 1986) due to photoperiodic changes. Determining if and what particular internal mechanisms related to the partitioning of photo-assimilates and hormonal sensitivity determine the origin of initiation and development of buds (i.e. axillary buds off existing shoots (Chapter 6), crown buds, or root buds), in response to these external signals in gentians (Figure 14), will be of interest to study further. As utilized with kiwifruit (Lai et al., 1989) and roses (Mor and Halevy, 1979), use of a ^{14}C-labelled transfer technique, would potentially enable the identification of partitioning of the actual photo-assimilates from source to sink.

The increased diameter of the transition zone below the lowest node, potentially indicates the partitioning of photo-assimilates to the transition zone. This was noted as early as the 4th week within plants treated with growth regulators, as compared to within the Control treatment, although it was not evident later due to the interaction with the photoperiodic regime. This non-significance of the size of the transition zone later in the growth cycle, and relative to the number of crown buds and shoots evident at the 20th week, could be attributed to the assimilates being utilized for the development of crown buds and crown shoots instead of the transition zone itself. As previously noted by the presence of secondary thickening of the stem (Chapter 5), there were histological changes within the transition zone in clonally propagated plants when development of the transition zone and bud initials take place. As explored for other plant species (Micheli et al., 2000; Rinne and van der Schoot, 2003), in order to understand if, which, and how, any of these histological changes within the transition zone actually relate to the crown bud formation process in response to the photoperiodic regime or application of growth regulators, requires further research. This type of research will enable understanding of the more detailed steps within the multistep process of crown bud formation described within this thesis, such as cell division, differentiation, organ initiation and development (Albrecht and Lehmann, 1991). Such changes at a cellular level in organogenesis will also aid in developing an understanding of whether the photoperiod and plant growth regulators act on similar or different pathways. This will enable identifying specific requirements in each step. For example, as also identified with the changes of individual bud clusters and buds within the clusters during the current study, the requirement for the initiation of adventitious buds (bud initials) and axillary buds (crown buds within the cluster) could vary. In future therefore, it will be of interest to study how the physiological mechanisms of organogenesis associated with plant growth regulators and/or photoperiodic regimes, determine the type (axillary or adventitious) and origin of buds (stem, transition zone or roots) in gentians.

7.5.7 Practical horticultural implications

The significant interaction between the photoperiodic regime and growth regulators suggests a difference in hormonal sensitivity in plants based on the environmental stimuli. Investigation of optimum dosage and time of application therefore, need to be determined prior to commercial application.

Promotion of the crown bud formation process under the short photoperiodic regime highlights the need to consider the potential timing of establishment of vegetative propagules within the annual calendar of events. Hence if plants were established in late summer/autumn, when the natural progression towards shortening photoperiod exists, it will be possible to establish plants with crown buds for the next growth cycle without the need for additional treatments.

The promotive effect of some growth regulators under the long photoperioic regime, suggests the potential use of these growth regulators if young plants from tissue culture are being deflasked and established under long or increasing photoperiods (e.g. spring through summer). Hence, with the application of exogenous growth regulators, it is possible to propagate plants successfully earlier in the growing season, enabling earlier field establishment and, therefore, earlier cut flower production. On the other hand, the inability to display a similar level of influence of growth regulators under the short photoperiodic regime, indicates that the dosage may need to be modified in order to promote the crown bud formation process under the short photoperiodic regime and, therefore, different dosages under a range of photoperiods need to be investigated in future. There were however no detrimental effects of applying ethephon on the crown bud formation process, even when applied under the naturally occurring short photoperiodic regime, as compared with the other promotive hormonal treatments like TDZ, TDZ followed by ethephon and, PBZ followed by ethephon.

If treatment with ethephon is to be recommended however, the resultant stimulation of emergence of crown buds needs to be investigated further. This need primarily arises because the early emergence of crown shoots may or may not be desirable in commercial production. In addition, since the effect of ethephon on the number of bud clusters and quality of subsequent shoots was improved when applied with other growth regulators (e.g. PBZ and TDZ) under the long photoperiodic regime, then further research may establish the optimum dosage and timing of application. If the objective is to establish a well-developed transition zone with high quality buds, the hormonal combinations (PBZ followed by ethephon and TDZ followed by ethephon) could be successfully applied under the non-inductive long photoperiodic regimes, such as in spring through to summer.

Formation of crown buds from roots, as observed with applications of some growth regulators under the short photoperiodic regime, is not considered desirable in

commercial cut flower production. It could be used however in development of a successful propagation strategy, as these root bud clusters have separate developed root systems. If a root system is already established, as propagules they may require a shorter time to establish in the field. If the objective is plant multiplication therefore, the hormonal combination of either PBZ followed by ethephon or TDZ followed by ethephon could even be applied under the short photoperiodic regime, e.g. starting from late summer.

The potential benefits of the current investigations to the industry noted above are for application *in vivo*. In contrast however, in future it would be worth examining these treatment combinations which promote the crown bud formation process *in vitro.* As also implemented in other species (Ascough et al., 2008), it could be possible to promote the crown bud formation process earlier in the growth cycle, so that by the time of deflasking plants may consist of a transition zone with initials for crown buds or crown bud clusters. Potentially this should lead to successful regrowth in the subsequent growth cycle, and reduce plant death currently experienced during crop establishment.

7.6 Conclusions

During the current study it was confirmed that the naturally occurring short photoperiodic regime promoted the crown bud formation process qualitatively (diameter of buds and characteristics of shoots), quantitatively (number of crown bud clusters and, buds and shoots within a cluster), as well as early in the growth cycle. In contrast, this was not promoted when continuously grown under the long photoperiodic regime. Application of ethephon was the most effective growth regulator as a single application in increasing the number of crown buds earlier in the growth cycle. The application of PBZ followed by ethephon was however, the most effective treatment combination, when qualitative effects such as shoot length and number of floral nodes were also considered. The inability of application of other combinations of growth regulators to result in a similar level of influence on the crown bud formation process under the short photoperiodic regime, was hypothesized to be due to assimilates being utilized for the development of root buds, and changes in hormonal sensitivity. While a number of hypotheses have been proposed for the mechanism of action of the short photoperiodic regime or the exogenous growth regulators in promoting the crown bud formation process, validation of these hypotheses as to whether it is a direct or indirect effect of hormone-mediated assimilate partitioning and, if so, how it controls organogenesis,

warrants further investigation. While the ability to produce crown bud clusters in multiple origins has been reported in previous experiments (Chapter 5), the present study revealed that it is also possible to manipulate the origin of initiation of crown bud clusters (i.e. transition zone or roots). With further studies to determine the optimum dosage and frequency of these plant growth regulators, and critical photoperiodic conditions, it may be possible to achieve the required quality and quantity of crown buds at the required time, either on the transition zone or roots. This will not be undertaken during the current thesis, as subsequent chapters (Chapter 8 and 9) focus on factors influencing the emergence of these crown buds onto shoots. The influence of some environmental factors and exogenous application of plant growth regulators on the crown bud formation process in the second growth cycle, however, were assessed based on number of crown bud clusters and buds within the clusters within these chapters.

Chapter 8 Shoot emergence and development; Effect of chilling, GA_3, PBZ and developmental stage

8.1 Abstract

Experiments were carried out with the gentian cultivar 'Diva', in order to identify chilling and/or GA_3 responses at three different growth stages, i.e. plants with crown buds unemerged, with shoots recently emerged, or with shoots emerged and developed. At all stages of development examined, application of GA_3 (100 ppm) resulted in increased emergence of crown buds as shoots, leading to development of more shoots of harvestable quality compared to the control treatment. A similar influence on emergence of shoots was observed after exposure to chilling, but only when plants with unemerged crown buds were used. Plants with unemerged crown buds exhibited a gradual increase in shoot emergence (23 ± 1 to 197 ± 41%) as the duration of chilling (5º C) increased from 0, 2, 7 to 42 days. When GA_3 was applied to plants with buds that were recently emerged, or emerged and developed as shoots, the duration to harvest maturity did not vary among treatments, but a reduced spread in time to harvest maturity was observed. In contrast however, either chilling or GA_3 could reduce the time and spread of harvest maturity if applied prior to shoot emergence. Influence on qualitative variables and plant survival also varied based on developmental stage at which treatments were applied.

8.2 Introduction

Primary-crown buds which have over-wintered, give rise to the floral shoots harvested in gentians. Manipulation of their growth and development may, therefore, lead to changes in the yield, quality and timing of flower production. In previous experiments, while all floral shoots emerged from preformed primary-crown buds, not all crown buds present at the beginning of a growing season emerged as shoots (Chapter 3). As hypothesized within Chapter 3, if initiated at different times during the previous season, different crown buds would have experienced different environmental conditions during the crown bud formation process through to emergence in the next growth cycle. If for any population of buds, the crown bud formation process was spread over a wide duration, it was hypothesised that these buds may have undergone different physiological changes in terms of juvenility, dormancy and flower induction. If so, it

may be possible that shoots arising from these buds could emerge and perform differently during the subsequent growing season. It was further hypothesized that the spread of timing for individual shoots reaching harvest maturity could be controlled by first identifying and understanding the physiological basis of growth and development of these crown buds (Chapter 2 & 3) from initiation through to anthesis. In particular, understanding the mechanism(s) of dormancy (induction, maintenance and release) of these buds would help in developing strategies for control of timing harvest maturity. As previous chapters (Chapters 6 & 7) dealt with factors influencing the crown bud formation process in their first growth cycle, both the current and subsequent chapter (Chapter 9) deal with factors contributing to their emergence and subsequent development as shoots through to anthesis.

The potential influence of timing of shoot emergence on timing of flower harvest maturity, of late maturing cultivars like 'Starlet', was noted in an earlier experiment (Chapter 2). Hence it was envisaged that identification of the factors that influence shoot emergence, and their subsequent development, may enable manipulation of duration to harvest maturity. Shoot emergence, development and flowering may only occur in some temperate perennials when requirements for prior exposure to cold temperature have been satisfied, i.e. vernalization and/or breaking endodormancy (Iversen and Weiler, 1994). Depending on species, the requirement for vernalization can be qualitative/obligate (i.e. plants remain vegetative without cold exposure) or quantitative/facultative (i.e. cold hastens flower induction and initiation). Although a requirement for vernalization and/or chilling to break endodormancy for the new gentian hybrids have not been reported previously, plants of 'Spotlight' appear to have an obligate requirement for chilling (Chapter 3). In contrast however, cultivars like '03/04-114' (Appendix IV) and 'Diva' (Chapter 7) emerged and developed floral shoots without chilling, indicating the potential for a lack of any, or at most a facultative requirement for chilling. If low temperature is required for vernalization of gentians, it could influence flower induction in shoots and subsequently timing of harvest maturity. In a review article, delayed anthesis when forced in a protected environment was also reported with historical cultivars of gentians (Ohkawa, 1983), however this could illustrate a potential requirement of low temperature for vernalization, a delay in breaking endodormancy, or an influence of cultivation at supra-optimal temperatures. As observed in other species (Anderson et al., 2005; Sung and Amasino, 2005) even if endodormancy is broken by a limited number of chill units, any vernalization

requirement may require further exposure to low temperature before emergence. If the gentian cultivar 'Diva' has a similar differential response to chill units, the use of different durations of chilling as a treatment may enable identification of the existence of endodormancy and/or vernalization.

Presently there are no consistent recommendations for chill units in gentians, as it varies based on the information source; gentian *"crowns"* should be kept at 0 ºC for a "*long time*" for late flowering cultivars, and for a "*short time*" for early flowering cultivars (Ohkawa, 1983) and, according to industry-orientated information from New Zealand (NZ), the chilling requirement is met by "*temperatures below 5 ºC for 50 days"* (Anonymous, 2004). Chill unit recommendations for Japan are less likely to be applicable to NZ because areas of cultivation in NZ have a milder winter, where mean monthly minimum air temperatures can range from -2 to 9 ºC (Anonymous, 2011c). Due to this lack of consistent and reliable information, during the current study 5 ºC was used to supply chilling, as recommended for initial studies with geophytes of temperate origins (Dole, 2003).

Variation in temperature among different regions of cultivation, as occurs in NZ, leads to variation in chill unit accumulation over time. Inadequate accumulation of chilling can result in a delay in bud break, decrease in percentage of buds broken, and slow or weak shoot growth, in a range of plant species (Harvey and Nowierski, 1988; Ku et al., 2007; Lang et al., 1987). Extrapolating upon these concepts it was, therefore, hypothesized and explored within the current chapter that, by utilizing populations of plants with either partial or extended durations of chilling, it would be possible to identify the effects of adequate and inadequate chilling on shoot emergence and development in 'Diva'.

Exogenous application of GA_3 is known to replace, or partially replace, the requirement for chilling in many plants. GA_3 was effective in fully replacing the chilling requirement in *Gladiolus* × *Hortorum* (Tonecki, 1980) or partially in *Liatris spicata* (L.) Wild. (Zieslin and Geller, 1983). In a summary of unpublished technical reports, the application of GA_3 (100 ppm) to gentians resulted in shoot emergence in dormant plants without flowering, but GA_3 combined with chilling resulted in both shoot emergence and flowering (Ohkawa, 1983). From these reports it could be hypothesized that the low temperature requirement for vernalization could not be replaced by GA_3, but GA_3 was capable of replacing the low temperature requirement for endodormancy. These data

however are based on technical reports with no statistical validation and pertain to historical cultivars. The role therefore, of GA_3 in substitution of chilling, needs to be investigated with application to the current new cultivars. Given its ease of use within industry, within the current experiment the potential influence of GA_3 to substitute for chilling was investigated on plants differing in the partial or extended durations of chilling to identify any synergistic effects.

Despite the requirement for chilling (Chapter 3), shoots emerged (Chapter 4) and/or developed floral shoots (Appendix IV), when crown buds were relieved of paradormancy (i.e. via clipping) and were not endodormant. It was hypothesized that these treatments (chilling or GA_3) could result in a greater response in breaking endodormancy or fulfilling any vernalization requirement, when plants were fully or partially endodormant. To extend upon this further, and as examined within the current study, it was hypothesized that the stage of plant development at treatment application, may determine the qualitative and/or quantitative effects of these external stimuli.

During the current study, the chilling requirement for the gentian cultivar 'Diva' was investigated, as it was the primary cultivar used within this thesis (Chapters 2, 5, 6, 7), and because it is a key new cultivar for the NZ gentian industry. Given the observed difference in temperature response between gentian cultivars (Chapters 2, 4 & Appendix IV), ideally each cultivar should be assessed for its chilling requirement for commercial purposes. Given the limited resources of time, in the current study only one cultivar was examined.

Based on the findings related to timing of the crown bud formation process from earlier experiments (Chapters 6 & 7), within the experiments presented in the current chapter, two additional features of the plant material used were further developed:

- Firstly, treatments applied in the previous growth cycle can influence growth and development during the subsequent growth cycle (Appendix IV).
- Secondly, previous investigations on the crown bud formation process primarily focused on plants in their first growth cycle, devoid of either a developed transition zone or crown buds (Chapter 6 & 7).

In light of both these new insights, in the current experiment it was envisaged that it would be important to focus on treatment effects on the crown bud formation process

subsequent to the first growth cycle. Hence, the treatments applied at different developmental stages were assessed for their influence both in terms of the development of shoots during the current growth cycle and, the crown bud formation process for the second growth cycle.

The broad horticultural goal of the present investigation was to manipulate shoot emergence and development via chilling and GA_3, so as to subsequently enable enhanced productivity and scheduled timing of harvest maturity. In commercial reality these dormancy breaking treatments would ideally be applied earlier in the growing season, potentially in winter or spring, prior to shoot emergence. In the current experiment therefore, chilling and/or GA_3 was applied at the beginning of the growth cycle. It is additionally recognized that cold temperatures can be experienced not only in winter but also at the beginning of the growth cycle in spring. Due to the potentially non-obligatory requirement for chilling in cultivars like 'Diva' (Chapters 6 & 7), crown buds/shoots could be at different stages of development at a given time, i.e. from unemerged crown buds through to developing shoots, and would be under paradormancy, endodormancy, or both (Faust et al., 1997), at the beginning of the growth cycle. Within the current experiment therefore, the response to chilling and/or GA_3 was investigated by applying these treatments to plants with buds and shoots at different stages of development. As such, within the current chapter a series of three experiments were undertaken, with each utilizing one of three different stages of growth, i.e. plants with either unemerged crown buds only, shoots recently emerged, or emerged and developed shoots. In doing so, the specific objectives were to quantify the influence of;

- chilling on emergence and development of existing crown buds and crown shoots (paradormant and/or endodormant), and the crown bud formation process,
- GA_3 with or without chilling on emergence and development of existing crown buds (paradormant and/or endodormant) and crown shoots, and the crown bud formation process.

8.3 Materials and methods

8.3.1 General management of plants

Plants of *Gentiana triflora* × *scabra* 'Diva' were propagated by tissue culture at The New Zealand Institute for Plant and Food Research (Palmerston North, New Zealand; 40°37'S 175°60'E), deflasked and managed as per Section 6.3.1. During this first growth cycle plants were grown in a greenhouse, from either 9th or 14th April 2008 (heated at 15 ºC, ventilated at 20 ºC), so as to avoid accumulation of chill units, under a long photoperiod (LD; 2 h night-break lighting at 4.585 μmol s^{-1} m^{-2} from 23:00 HR to 01:00 HR each day during the natural short day length experienced in autumn through to spring). A liquid feed of half strength Peters General Purpose® foliar fertilizer (20N–8.7P–16.6K; Scott's Australia, NSW) was supplied once a week, while in cell trays.

During the first growth cycle, approximately 2 months after deflask, when a root-plug was established (21st May 2008), plants were repotted into black polythene bags (1.7 L) using the medium described in Section 6.3.1. Throughout the three experiments, plants were irrigated using a drained capillary system that supplied between 50 and 60 ml of water per plant per day, supplemented with one overhead watering per week. During chilling treatments, plants were hand-watered at a frequency dependant on plant demand.

At the beginning of their second growth cycle, approximately 8 months from deflask, plants were selected for each of the three experiments based on the degree of development of crown buds and/or crown shoots, i.e. plants with either unemerged buds only, shoots recently emerged, or with shoots emerged and developed (Figure 8.1). Within each of the three developmental stages, the selected plants typically comprised 1-2 crown bud clusters. Plants with unemerged buds had between 2 and 5 visible crown buds per plant. Plants with recently emerged shoots had between 1 and 4 visible crown buds and 1 to 3 recently emerged shoots per plant. Plants with shoots emerged and developed had between 1 and 4 visible crown buds, 1 to 2 recently emerged shoots, and 1 to 3 developed shoots (16 ± 1 cm) per plant. At this time, plants also carried between 1 and 3 floral shoots (past the point of anthesis), which had arisen during the first growth cycle. Based on the arrangement of buds within a cluster (Figure 5.22), one of the unemerged buds would be an apical bud capable of producing more buds during the experiment, and the other crown buds would be axillary and capable of developing into flowering shoots.

Figure 8.1. Representative samples of plants of 'Diva' at three developmental stages used for: (A) Experiment One, plants with buds unemerged, (B) Experiment Two, plants with buds recently emerged, (C) Experiment Three, plants with emerged and developed shoots.

8.3.2 Treatment application

For all three experiments, chilling treatments (5 ºC) were provided by placing plants in a cool-store with a 16 h photoperiod (4.89 µmol s^{-1} m^{-2} at plant height provided by incandescent bulbs). Treatment application commenced approximately 8 months after deflask, at the beginning of the second growth cycle (17th November 2008), with durations of chilling of 0, 2, 7 or 42 days, depending upon the experiment (Figure 8.2).

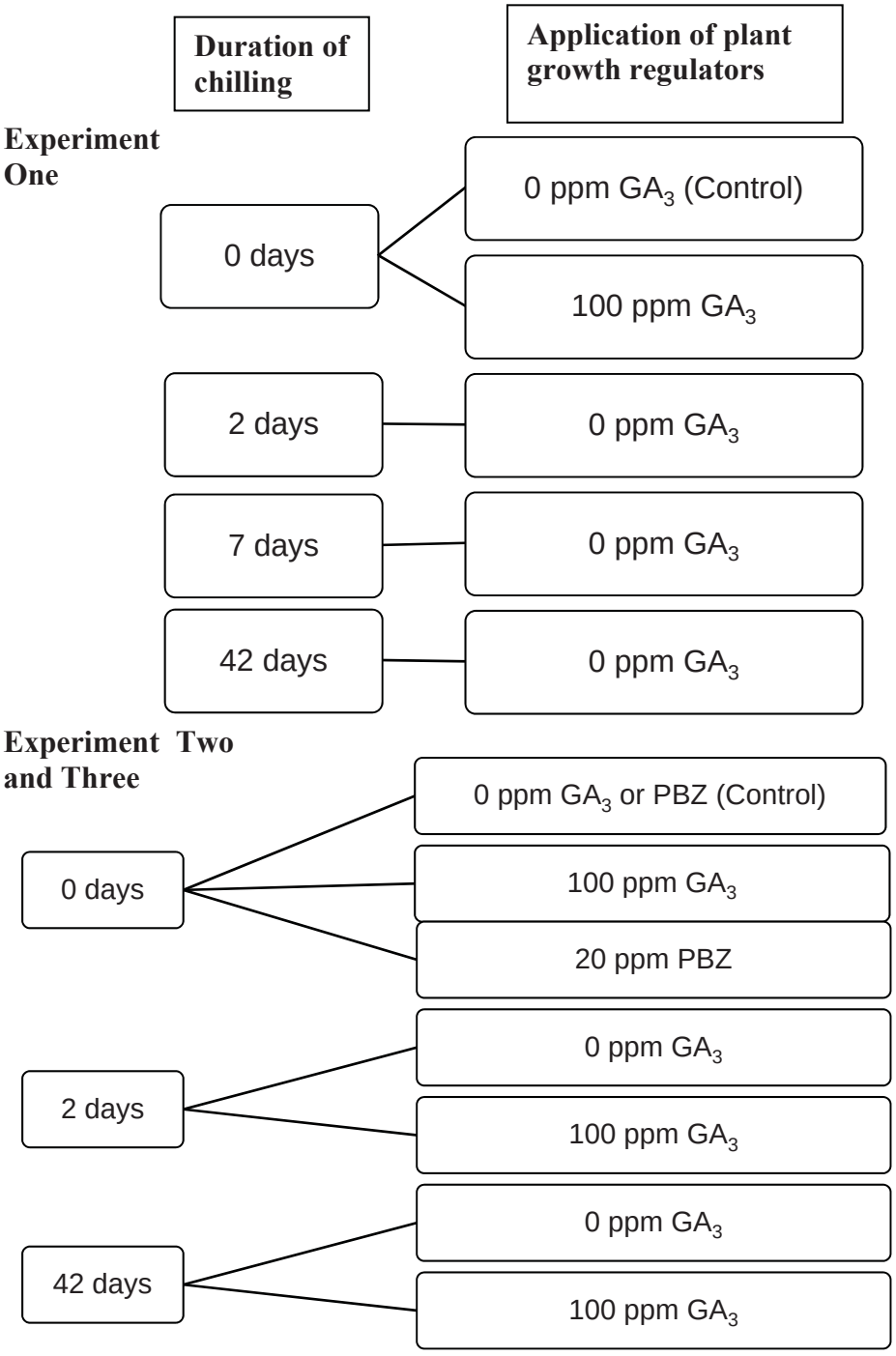

Figure 8.2. Arrangement of treatment combinations within Experiments One, Two and Three.

GA_3 was applied to the foliage, exposed crown and the surface of the growing medium, as a spray to run-off (≈ 70 ml per plant) at either 100 ppm (+GA) or 0 ppm (-GA; Control). GA_3 was prepared by being dissolved in 10 ml 95% ethanol, and made up to the required volume with distilled water together with 0.05% Tween 20 (Sigma CAS No. 9005-64-5) as a surfactant. The control treatment (-GA) comprised water, ethanol

and Tween 20. So as to improve foliar absorption, GA_3 treatments were applied three days after plants were transferred from the cool-store to the heated greenhouse.

PBZ (commercial preparation of Pay back™; 250 g L^{-1} paclobutrazol; Nufarm Limited, Auckland, NZ) was applied at either 0 ppm or 20 ppm at the commencement of Experiments Two and Three (7th November 2008; Figure 8.2). This was applied as a spray to the foliage, exposed crown and the surface of the growing medium, to run-off (≈ 50 ml per plant) with 0.05% Tween 20 (Sigma CAS No. 9005-64-5) as a surfactant.

8.3.3 Experiment One; chilling or GA_3 when crown buds unemerged

Due to the limitations in availability of an adequate number of plants at the appropriate stage of development, Experiment One was limited to provision of a range of durations of chilling, with application of GA_3 only in the absence of chilling (Figure 8.2). Plants were subjected to chilling treatments comprising 5 ºC for 0, 2, 7 or 42 days. For plants in the treatment with 0 days of chilling, GA_3 was applied at either 100 ppm (+GA) or 0 ppm (-GA; Control). Hence there were 5 treatments in total, with 5 to 6 individual plant replicates per treatment.

Approximately 10 months after the start of treatment application, at the end of the second growth cycle (25th September 2009), all existing crown shoots were clipped off and plants in all treatments were subjected to chilling for three weeks (under the same conditions as detailed in Section 8.3.2). Subsequent shoot emergence was monitored at the beginning of the third growth cycle. Due to plant death at this time, the number of individual plant replicates per treatment available for data collection during the third growth cycle varied between 2 and 6.

8.3.4 Experiment Two; chilling, GA_3, or PBZ when shoots recently emerged

Plants with recently emerged shoots in their second growth cycle were selected and subjected to treatments of chilling and/or GA_3 or PBZ, as indicated in Figure 8.2. Plants within each chilling treatment (5 ºC for 0, 2 or 42 days) were also treated with GA_3 at either 0 ppm (-GA; Control) or 100 ppm (+GA) as per Section 8.3.2. PBZ was applied to plants (refer Section 8.3.2) within 0 days of chilling treatment at either 0 ppm or 100 ppm. Hence there were 7 treatments in total (Figure 8.2), comprising either 7 or 8 individual plant replicates per treatment combination.

Approximately 9½ months after treatment application commenced, at the beginning of the third growth cycle (7th September 2009), all shoots were clipped off and the resulting growth during the third growth cycle was monitored following a further period of chilling (i.e. three weeks at 5 °C, as per Section 8.3.2). Due to plant death at this time, the number of individual plant replicates available for data collection during the third growth cycle varied between 2 and 6.

8.3.5 Experiment Three; chilling, GA_3 or PBZ when shoots emerged and developed

Shoots that were already emerged and developed within replicate plants were marked prior to treatment application. Plants were subjected to treatment combinations of chilling, GA_3 or PBZ as indicated in Figure 8.2 and, as described in Section 8.3.4. The number of replicates ranged between 9 and 14 individual plants per treatment combination.

Approximately 6 months after treatments were first applied, at the beginning of the third growth cycle (28th May 2009), all shoots were clipped off (seven replicates per treatment) and the resulting growth during the third growth cycle was monitored following a further period of chilling (i.e. three weeks at 5 °C as per Section 8.3.2). Seven individual plant replicates per treatment were used for data collection during the third growth cycle.

8.3.6 Variables recorded

In all three experiments the following data were recorded prior to or within the second growth cycle;

- number of unemerged crown buds prior to treatment application,
- at flower harvest maturity for crown shoots emerging from the transition zone during the second growth cycle (i.e. other than floral shoots of the first growth cycle);
    - duration from commencement of the experiment to flower harvest maturity,
    - shoot length,
    - number of nodes and,
    - number of axillaries in flower on shoots arising from primary-crown buds.

At the end of the second growth cycle, the following was recorded;

- number of visible crown buds and,
- crown shoots emerged, i.e. for regrowth in the third growth cycle and,
    - the developmental status of each (i.e. floral/vegetative).

During the third growth cycle, the following was recorded:

- number of crown shoots emerged at weekly intervals.

Within Experiment One, the following additional data were recorded;

- number of crown shoots emerged after treatment application at weekly intervals until maximum shoot emergence, i.e. 14 weeks from first treatment application (27th February 2009),
- shoot length and,
- number of nodes on the emerged crown shoots (as at 27th February 2009).

Within Experiment Two and Three, the following additional data were recorded before application of treatments;

- number of crown shoots emerged but not developed (shoots emerging from the transition zone, other than existing floral shoots from the first growth cycle),
- number of crown shoots which had emerged and developed, and their
    - shoot length and,
    - number of nodes.

Within Experiment Two and Three, for individual crown shoots present at the beginning of the experiment, the following data were recorded at harvest maturity;

- duration from commencement of the experiment to harvest maturity,
- shoot length,
- number of nodes and,
- number of axillaries in flower.

8.3.7 Variables calculated

For Experiment One, the number of crown shoots that emerged and reached harvest maturity was calculated as the proportion of the number of visible crown buds that were present within a plant at the time of first treatment application. This number may exceed 100% as new crown buds were initiated during the course of the experiment. For Experiment Two and Three, the number of crown shoots that reached harvest maturity was calculated as a proportion of the combined number of visible crown buds and

crown shoots either emerged or developed within a plant at the time of first treatment application. Within these two experiments, the number of crown shoots that emerged following treatment application (i.e. excluding shoots present at the time of treatment application) was also assessed as a proportion of the number of visible crown buds (unemerged) present at the time of first treatment application. When the proportions exceed 100%, the origin of this additional shoot emergence was considered to be from the crown buds which were present within the apical bud of the cluster (axillary buds). While the growth and development of this apical bud into multiple crown buds/shoots following the treatment application was not monitored during the current experiment, it was investigated subsequently (Chapter 9).

In order to evaluate any carry-over effect of treatments, for the third growth cycle the number of crown shoots emerging following clipping and chilling was calculated as the proportion of the combined number of crown buds and crown shoots present at the beginning of the third growth cycle.

8.3.8 Experimental design and statistical analysis

All experiments were arranged as a Completely Randomized Design. Data pertaining to Experiment One and Two were analysed using the General Linear Models (GLM) procedure in Statistical Analysis System (SAS) version 9.2 (SAS Institute, Cary, NC, USA). If data were not normally distributed then either a square root or log transformation was carried out to improve the normality. Means were separated using either Duncan's New Multiple Range Test (DNMRT) or Scheffe test, depending on the number of replicates available for each variable.

Within Experiment Three, the variables recorded at harvest, relative to crown shoots already present at the time of treatment application, were analysed using the analysis of covariance procedure in SAS, i.e. for each individual shoot. Shoot length at the commencement of treatment application was considered as a covariate for both the variables, duration to harvest maturity and shoot length at harvest. The number of nodes at the commencement of treatment application was used as a covariate for both the variables, number of nodes at harvest, and number of nodes with floral axillaries at harvest. Means were separated using LSMEANS at the 0.05% level, but the means presented were weighted means. Since the crown shoots that emerged and developed into floral shoots were more than those which were already developed at the start of the experiment, the combined number of crown shoots was also analysed. These combined

data, and the rest of the data from Experiment Three, were analysed using the GLM procedure in SAS. Means were separated using either DNMRT or Scheffe test, based on the number of replicates used for each variable.

Box and whisker plots (Sigma Plot version 10, Systat Software Inc., San Jose, CA, USA) were used to quantitatively describe the distribution of duration from the date the last treatment was applied through to harvest maturity. This data utilised that derived from the multiple shoots across all replicates collectively. The 80% spread was calculated based on the number of days to harvest maturity between the 10^{th} and 90^{th} percentiles of the box and whisker plot. Within Experiment Three, 80% spread was also assessed separately for shoots that were present at the time of treatment application. Due to the presence of a limited number of the shoots, which were recently emerged within a plant at the time of treatment application within Experiment Two, 80% spread in time to harvest maturity was not presented for those shoots.

8.4 Results

8.4.1 Overview

As detailed in the following sections, the response to chilling and/or GA_3 varied according to the developmental stage at which treatments were applied, i.e. differing between Experiment One, Two and Three. During the second growth cycle, shoots that were already emerged or developed at the beginning of treatment application, as well as some of the unemerged crown buds which subsequently emerged, reached harvest maturity in variable numbers dependent on the treatment. The majority of shoots which emerged were floral, with few remaining vegetative irrespective of the treatment application. During the course of the experiments new crown buds appeared, developed and some emerged as crown shoots in variable numbers dependent on treatment application. These new crown buds formed the basis for shoots which developed in the third growth cycle. As an additional variable, the combined total of crown buds and crown shoots were, therefore, frequently used as a measure of the crown bud formation process. As presented in the following sections, treatment effects were evident on shoot quality, quantity and timing of harvest maturity during the second growth cycle, the crown bud formation process and, emergence of crown buds in the third growth cycle.

8.4.2 Experiment One; chilling or GA_3 when crown buds unemerged

When expressed as a proportion of crown buds present at the beginning of the treatment application, there was a gradual increase in shoot emergence with increasing duration of

chilling, as evident by the 2 to 8-fold increase in shoot emergence with the duration of chilling from 2 to 42 days ($P < 0.05$; Figure 8.3A). GA_3 was capable of stimulating shoot emergence similar to that achieved by 42 days of chilling. As a result, the number of floral shoots harvested per plant was greater ($P < 0.05$) following application of GA_3 (5 ± 1), as compared to treatments receiving either 0 days (1 ± 0) or 2 days of chilling (2 ± 0). Although not significantly different ($P = 0.1$), the number of floral shoots harvested following 42 days of chilling was 3 times greater than that achieved with 0 days of chilling (i.e. from 1 to 3 shoots).

Application of either chilling or GA_3 to plants with unemerged crown buds reduced the duration to harvest maturity ($P < 0.0001$; Figure 8.3B), with plants receiving either 7 or 42 days chilling, or GA_3, taking between 28 and 49 days less to harvest than treatments with only 0 or 2 days chilling. The 80% spread in timing of flower harvest maturity was reduced with application of chilling, and with application of GA_3 (Figure 8.5A). Even with the greater number of shoots produced following 42 days of chilling, the 80% spread in timing was 83 and 20 days less than observed following either 2 days of chilling or GA_3, respectively. Treatment with no chilling achieved a spread of 15 days, however there were only three shoots for all the replicates in this treatment.

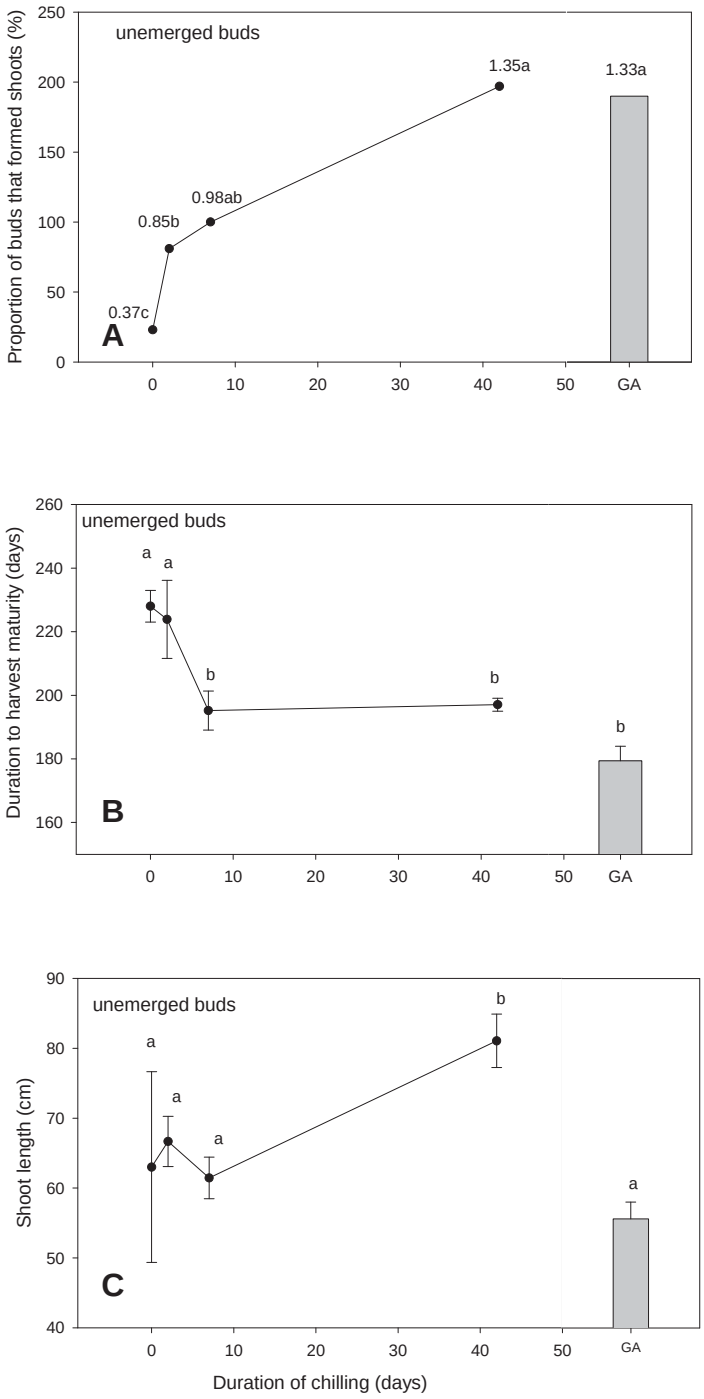

Figure 8.3. (A) Proportion of shoots emerged and reaching harvest maturity as compared with unemerged crown buds present at the time of first treatment application, (B) duration to harvest maturity for each shoot and, (C) shoot length at harvest, following increasing duration of chilling at 5 °C or application of GA_3 to plants of 'Diva' with unemerged buds (Experiment One). For each variable, means with different letters were significantly different. Within (A) mean separation is based on the log transformed means. Vertical lines represent ± SEM. Mean separation by DNMRT at $P < 0.05$.

In terms of shoot quality at harvest maturity, shoots arising following 42 days of chilling were approximately 20 cm longer than those in all other treatments ($P < 0.0001$; Figure 8.3C). Although the total number of nodes at harvest did not vary significantly ($P > 0.05$), compared to when GA_3 was applied, there were 3 more nodes with floral axillaries following 0, 2 and 42 days of chilling ($P < 0.01$; Figure 8.4).

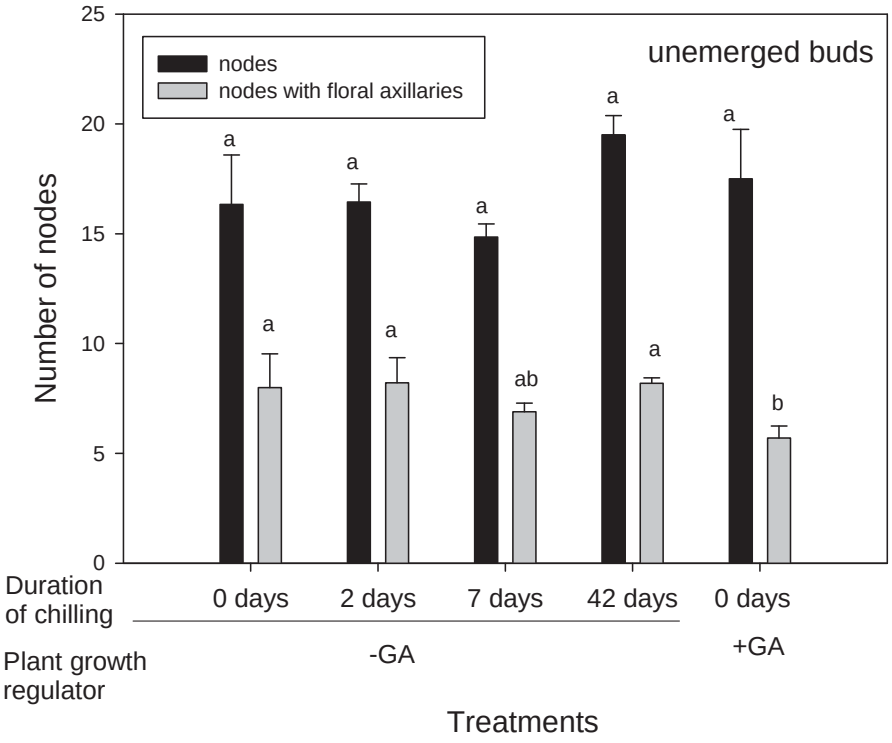

Figure 8.4. Number of nodes and number of nodes with floral axillaries at harvest following increasing duration of chilling at 5 °C or application of GA_3 to plants of 'Diva' with unemerged buds (Experiment One). For each variable means indicated by different letters were significantly different. Vertical lines represent SEM. Mean separation by DNMRT at $P < 0.05$.

8.4.2.1 Influence in the third growth cycle

When the number of plants surviving at the beginning of the third growth cycle was expressed as a proportion of plants present at the commencement of treatment application, there was a gradual increase in survival from 40% to 100% with increasing duration of chilling from 0 to 42 days. Plants treated with GA_3 achieved a similar level of survival to plants receiving 42 days of chilling.

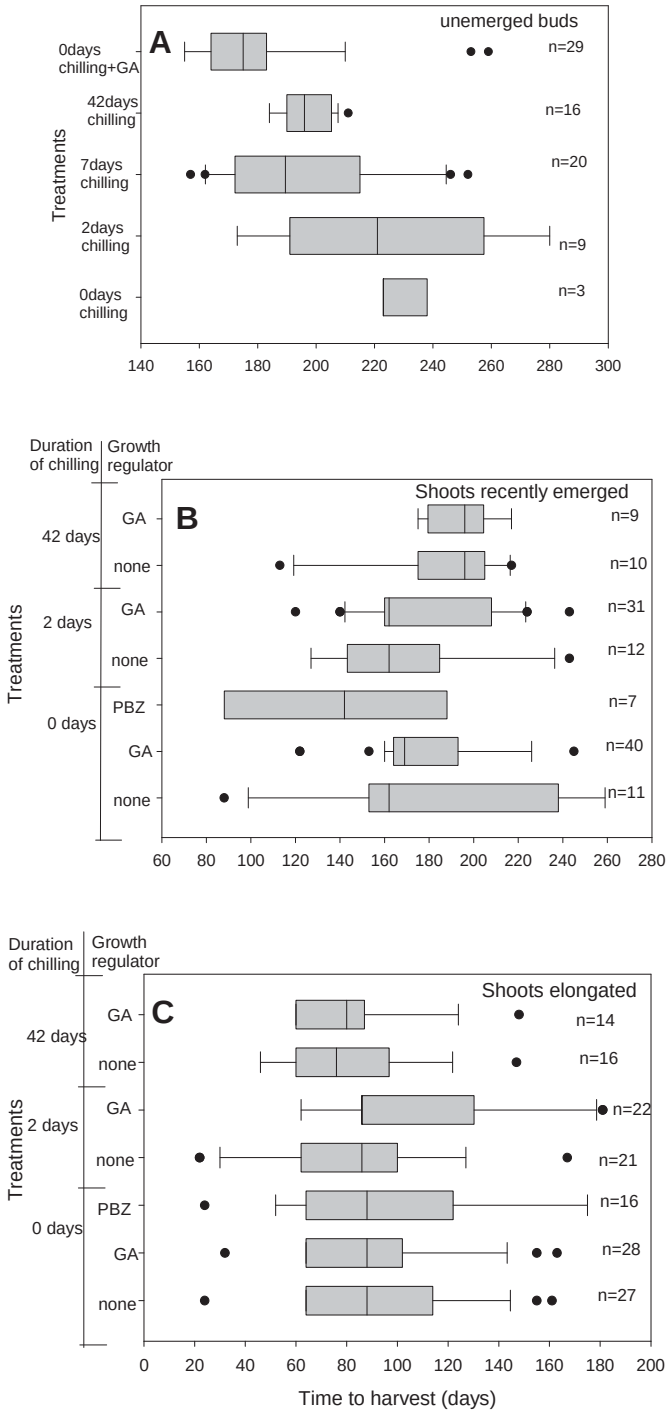

Figure 8.5. Spread in time to harvest maturity of shoots following treatment application to plants of 'Diva' at three developmental stages comprising; (A) unemerged buds (Experiment One), (B) shoots recently emerged (Experiment Two), (C) elongated shoots (Experiment Three). The number of shoots contributing to the distribution is indicated to the right of each treatment's box plot. Solid lines in the centre of each box indicate the median. Boundaries of the box indicate the 25th and 75th percentiles, whiskers indicate 10th and 90th percentiles and, solid dots indicate one or more individuals as outliers beyond these percentile limits.

At the end of the second growth cycle the number of crown buds (7 ± 2) or crown shoots (1 ± 0) per plant, or the combined total of crown buds and crown shoots within a cluster (6 ± 1), was not influenced ($P > 0.05$) by either chilling or GA_3. When quantified 3 weeks into the third growth cycle, the proportion of crown shoots that had emerged, as compared to the number of crown buds present within a plant at the end of the second growth cycle, did not vary among treatments ($P > 0.05$), with an average of 50% ± 8% across all treatments.

8.4.3 Experiment Two; chilling, GA_3, or PBZ when shoots recently emerged

When expressed as a proportion of the combined total of crown buds and crown shoots present at the commencement of treatment application, the shoots that reached harvest maturity increased with the application of GA_3 ($P < 0.0001$; Figure 8.6A). This was evident in treatments receiving GA_3 after either 0 or 2 days of chilling exposure, achieving between 2 and 3 times greater emergence compared to all other treatments. No influence on the proportion of shoots which reached harvest maturity was evident following application of PBZ. In contrast to Experiment One, at this stage of development exposure to chilling did not increase shoot emergence. In the presence of GA_3, exposure to the longest period of chilling halved the proportion of shoot emergence with no or limited chilling.

When the shoots emerging, i.e. excluding those present at the time of treatment application, was expressed as a proportion to the unemerged crown buds at the time of treatment application, a similar trend of treatment differences to that of the proportion of shoots that reached harvest maturity was evident ($P < 0.0001$). Even with application of GA_3, chilling reduced shoot emergence from 480% with 0 days of chilling to 150% with 42 days of chilling. As evident by values of shoot emergence greater than 100% within treatments with GA_3, crown buds which were not visible at the commencement of the experiment, emerged as shoots.

Treatment differences for the number of floral shoots harvested, followed a similar trend of treatment effects to that of the proportion of shoots that reached harvest maturity ($P < 0.0001$), hence figures or tables are not presented. Application of GA_3 after 0 or 2 days of chilling produced between 2 and 4 more floral shoots per plant than the rest of the treatments. Plants treated with GA_3 following 0 days of chilling had four times greater floral shoots (7 ± 1) than those treated with PBZ (1.5 ± 1).

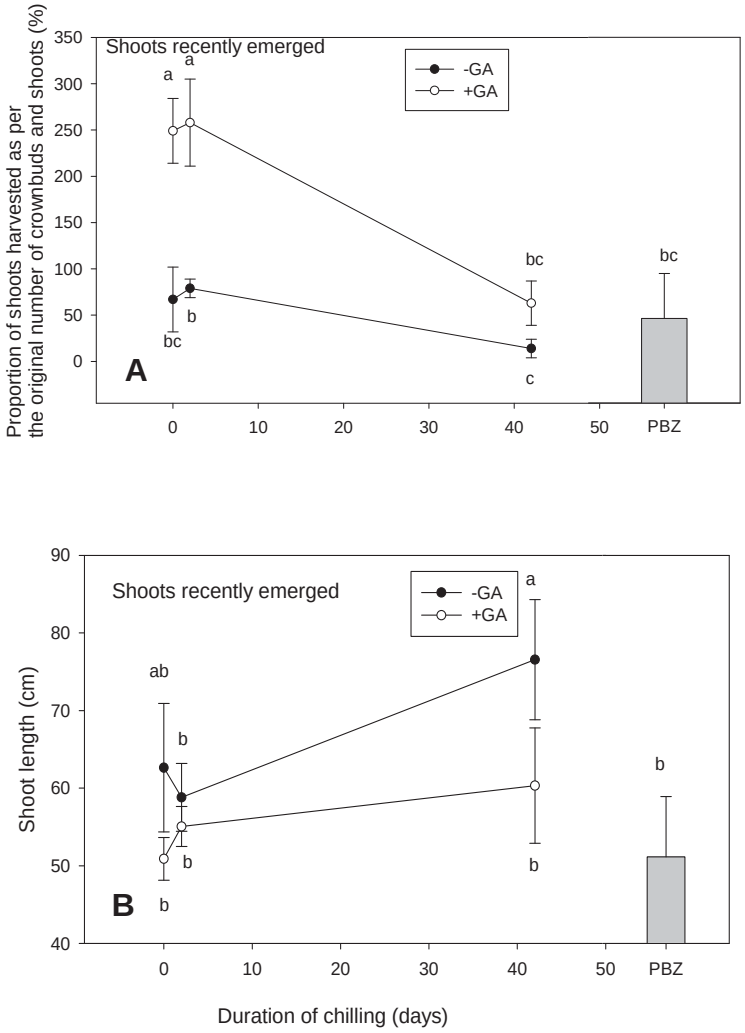

Figure 8.6. (A) Proportion of shoots reaching harvest maturity as compared with the combined number of unemerged crown buds and crown shoots present at the time of first treatment application, and (B) shoot length at harvest of shoots developed following increasing duration of chilling at 5 °C and/or application of GA_3 or PBZ to plants of 'Diva' with shoots recently emerged (Experiment Two). For each variable means indicated by different letters were significantly different. Vertical lines represent ± SEM. Mean separation by DNMRT at $P < 0.05$.

In terms of shoot quality, shoot length at harvest was primarily influenced by the duration of chilling, as evident by those treated with 42 days of chilling being between 16 and 26 cm longer than those from all other treatments, except the Control ($P < 0.01$; Figure 8.6B). The inability to detect a significant difference between 0 and 42 days of chilling is likely to have been attributable to the high variability between plants, and an inadequate number of replicates. In contrast to chilling, application of GA_3 tended to reduce shoot length at harvest and, after 42 days of chilling, resulted in a reduction by 16 cm ($P < 0.01$). No treatment influence was evident with the number of nodes on shoots at harvest ($P > 0.05$; Figure 8.7). In contrast however, provided plants were not

applied with GA_3, there were 5 to 8 more nodes with floral axillaries following 42 days of chilling, compared to the rest of the treatments ($P < 0.0001$). Treatment differences for the variables related to shoot quality were similar when analysed separately for shoots recently emerged and, therefore, data were not presented.

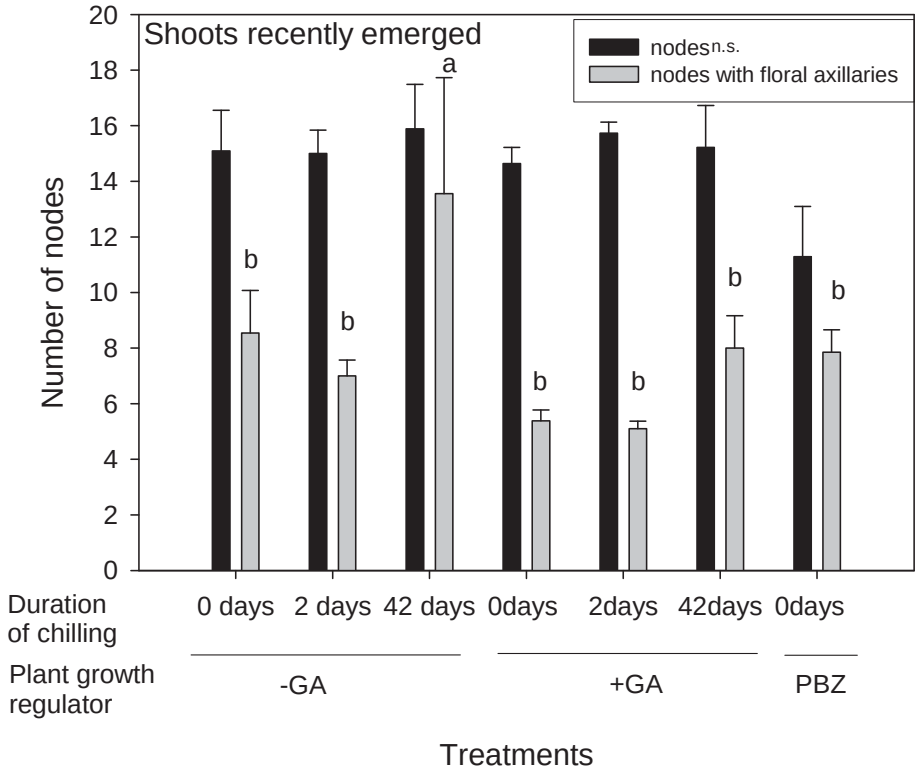

Figure 8.7. Number of nodes and axillaries with flowers, at harvest of shoots developed following increasing duration of chilling at 5 °C and/or application of GA_3 or PBZ to plants of 'Diva' with shoots recently emerged (Experiment Two). For each variable means indicated by different letters were significantly different. Vertical lines represent SEM. Mean separation by DNMRT at $P < 0.05$.

The duration to harvest was not affected when chilling, GA_3 or PBZ was applied to plants with buds recently emerged ($P > 0.05$), with an average for all treatments of 177 ± 10 days. Within Experiment Two, due to poor plant survival following 42 days chilling, the variable number of shoots produced per plant resulted in wide variability in the data for the 80% spread in time to harvest maturity. Compared to when no chilling was applied, the 80% spread in time to harvest maturity of shoots was however, reduced by 43 and 55 days following 2 days and 42 days of chilling, respectively (Figure 8.5B). Application of GA_3 reduced the 80% spread, irrespective of exposure to chilling. In treatments receiving no chilling, the 80% spread was reduced to half following

application of GA_3. Hence, this reduction of spread (Figure 8.5B) was observed, despite the increased shoot number (7 ± 1 per plant; Figure 8.6A).

8.4.3.1 Influence in the third growth cycle

At the end of the second growth cycle all plants receiving 0 days or 2 days exposure to chilling, both with and without application of GA_3 survived. By contrast, exposure to 42 days of chilling led to only 25% plant survival, which was increased to 50% when GA_3 was applied. Survival was 80% for plants treated with PBZ.

Across all chilling durations, by the end of second growth cycle the application of GA_3 had reduced the combined total of crown buds and crown shoots per cluster by between 2 to 4 ($P < 0.001$; Figure 8.8A). In contrast, application of PBZ resulted in the greatest combined number of crown buds and shoots per cluster, achieving 3 more than in the Control treatment (0 days of chilling).

In the absence of GA_3, the duration of chilling had no influence on the number of crown buds and crown shoots per cluster (Figure 8.8A). When expressed on a per plant basis, the combined total number of crown buds and crown shoots varied between treatments in a similar pattern to that when expressed on a per cluster basis ($P < 0.001$) and, therefore, data were not presented.

When expressed as a proportion of the combined total of crown buds and crown shoots present at the end of second growth cycle, shoot emergence at the beginning of the third growth cycle was greater in all treatments which had received GA_3 in the second growth cycle ($P < 0.01$; Figure 8.8B). GA_3 combined with 0 or 2 days of chilling resulted in between 3 and 5 times greater shoot emergence as compared with the rest of the treatments. In contrast, no influence of either chilling or PBZ was evident on shoot emergence in the third growth cycle. Hence GA_3 hastened shoot emergence, but reduced or delayed the appearance of crown buds. The number of crown shoots that emerged however did not vary ($P > 0.05$) between treatments and, therefore, GA_3 had no influence on quantity of buds.

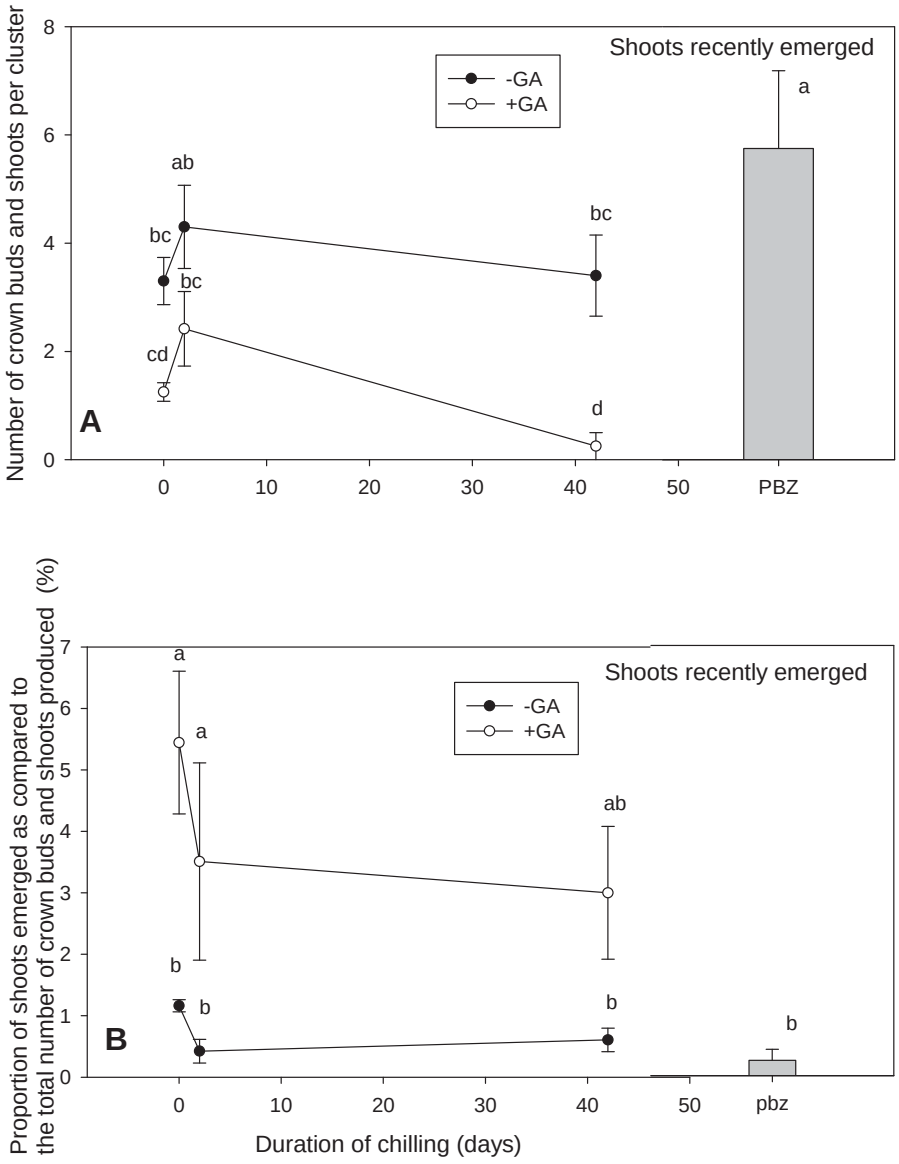

Figure 8.8. During the third growth cycle, following increasing duration of chilling at 5 °C and/or application of GA_3 or PBZ to plants of 'Diva' with shoots recently emerged (Experiment Two); (A) combined number of crown buds and shoots per cluster and, (B) the proportion of shoots emerged as compared to the combined number of crown buds and crown shoots at the beginning of third growth cycle. For each variable means indicated by different letters were significantly different. Vertical lines represent ± SEM. Mean separation by DNMRT at $P < 0.05$.

8.4.4 Experiment Three; chilling, GA_3 or PBZ when shoots emerged and developed

When expressed as a proportion of the combined total of crown buds and crown shoots present at the commencement of treatment application, the combined total of all the shoots that were present or emerged and reached harvest maturity, was not influenced by chilling in the absence of GA_3 ($P < 0.0001$; Figure 8.9A). In contrast, application of

GA_3 increased the emergence of new shoots, with application following no chilling or limited chilling achieving more than double the shoot emergence as compared to all other treatments. The application of GA_3 was, however, incapable of increasing the number of shoots emerging if plants were subjected to 42 days of chilling. Application of PBZ did not result in a change in emergence of new shoots. Irrespective of whether the crown shoots which were already emerged at the time of treatment application were or were not included in the calculated proportion, the pattern of treatment differences was the same, therefore separate data were not presented.

The treatment response for the number of harvested shoots produced per plant varied similarly to that for the proportion of shoot emergence ($P < 0.0001$), with treatment application of GA_3 with either 0 (5 ± 1) or 2 days (4 ± 1) of chilling achieving the greatest number of shoots, which was 3 times greater than in the rest of the treatments (1 ± 0). As such, the unemerged crown buds emerged and reached harvest maturity only within the treatments comprised of GA_3 with either 0 or 2 days of chilling.

Of the shoots that were already emerged and developing at the commencement of the experiment, the duration to harvest maturity was not influenced by chilling, GA_3 or PBZ ($P > 0.05$), averaging 81 ± 5 days (covariate adjusted mean). The covariate, i.e. initial height of the shoot at the commencement of treatment application, was significant for determining the duration to harvest maturity ($P < 0.001$). Even when the duration to flower harvest maturity was determined for all floral shoots, i.e. irrespective of whether emerged at the commencement of the experiment, a similar treatment influence was evident averaging 89 ± 7 days.

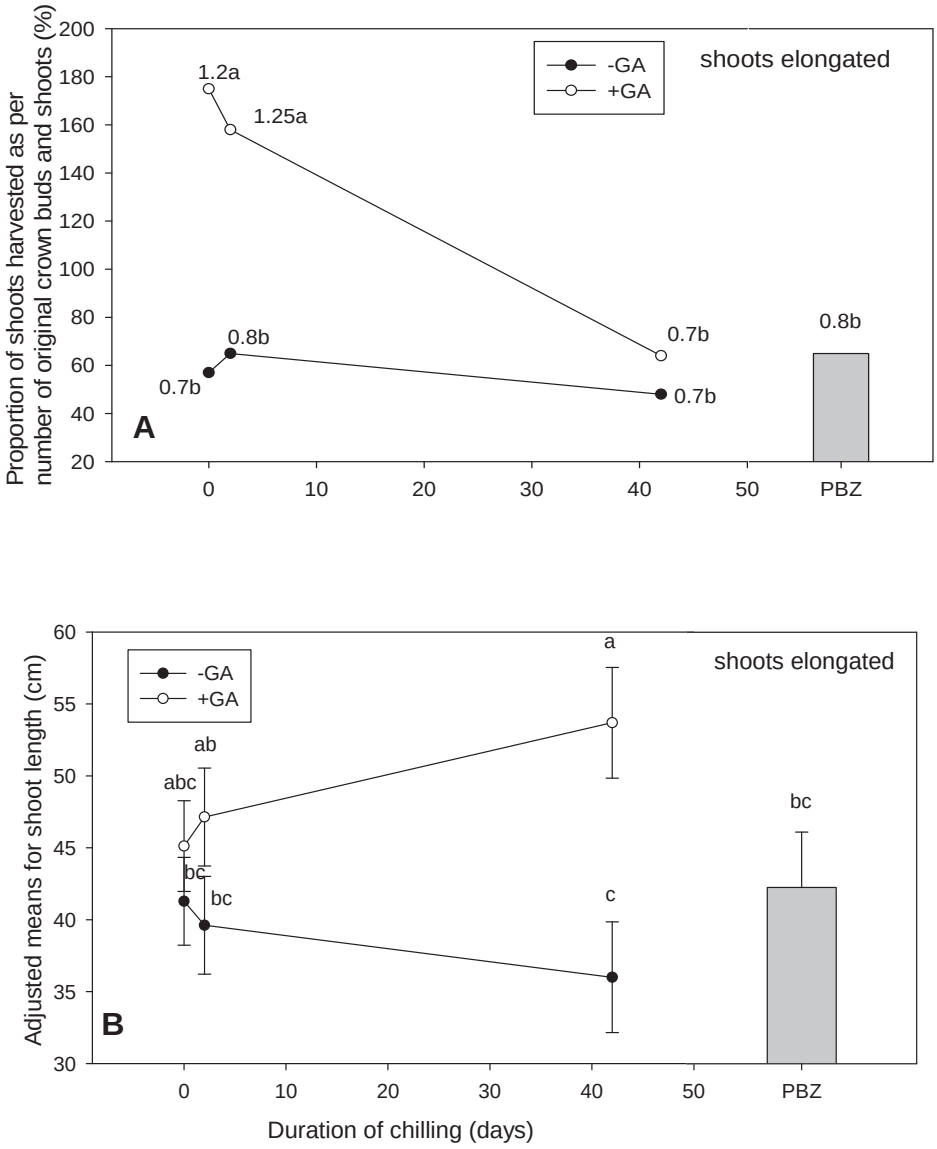

Figure 8.9. (A) Proportion of shoots reaching harvest maturity as compared with combined number of unemerged crown buds and crown shoots present at the time of first treatment application and, (B) shoot length at harvest of shoots (present at the beginning of the treatment application; adjusted means are presented) following increasing duration of chilling at 5 °C and/or application of GA_3 to plants of 'Diva' with elongated shoots (Experiment Three). Within (A) mean separation is based on the log transformed means. For each variable means indicated by different letters were significantly different. Vertical lines represent ± SEM. Mean separation by DNMRT at $P < 0.05$

The 80% spread in duration to flower harvest maturity was not inconsistent (Figure 8.5C). The narrowest 80% spread, which was 17 days less than that following 0 days of chilling (Control treatment), was observed in plants which received 42 days of chilling. Although the spread was narrow, the number of shoots also was less (14) in treatments subjected to 42 days of chilling compared to the rest of the treatments. The 80% spread

was narrower in the plants treated with GA_3 by 43 days, as compared to PBZ; however treatment with GA_3 was similar to the Control treatment. The 80% spread of flower harvest maturity of shoots which were already emerged and developed by the commencement of treatment application, was also examined and, within these shoots, treatment with GA_3 (38 days) presented the narrowest 80% spread (data not shown), which was 20 days less than for the Control treatment.

The covariate, i.e. initial height of the shoot at the commencement of treatment application, was significant for determining the length of the harvested shoot ($P < 0.0001$). The covariate adjusted shoot length was increased by GA_3, however plants treated with GA_3 following 42 days of chilling produced shoots with the greatest length, being approximately 20 cm longer than those harvested from non-GA_3-treated plants ($P < 0.05$; Figure 8.9B). Application of PBZ did not influence the covariate adjusted shoot length. There was no evidence of an influence of chilling, GA_3 or PBZ ($P > 0.05$) for the covariate adjusted number of nodes (10 ± 2; Adjusted mean) or the number of nodes with floral axillaries (5 ± 1; Adjusted mean). The covariate number of nodes at the commencement of treatment application was significant for these two parameters ($P < 0.0001$ and $P < 0.001$, respectively).

Data for shoot productivity and quality at floral shoot harvest during the second growth cycle were also analysed, irrespective of whether or not the harvested shoot had already emerged at the commencement of the experiment. When analysed in this manner the duration to harvest maturity (89 ± 7 days) or number of nodes (10 ± 1) was not influenced by treatments ($P > 0.05$). Greater shoot lengths were observed in plants treated with GA_3, irrespective of whether chilling was applied ($P < 0.01$). Application of GA_3 with 2 days (54.5 ± 4.4 cm) or 42 days chilling (54.5 ± 5.8 cm) resulted in longer shoots than plants receiving no chilling (41 ± 2.6 cm) or PBZ (45 ± 3.7 cm). Exposure to chilling did not influence the shoot length, with plants receiving 2 (41 ± 3.6 cm) or 42 days of chilling (35 ± 3 cm), producing shoots with a similar length to those harvested following no chilling.

8.4.4.1 Influence in the third growth cycle

When treatments were applied to plants with shoots emerged and developed, the survival rates were not greatly influenced by the treatments, with all treatments ranging between 70% and 85% survival. At the end of the second growth cycle, the combined

number of crown buds and crown shoots was generally less in plants treated with GA_3 in the previous growth cycle, compared to those without GA_3 ($P < 0.0001$; Figure 8.10). This influence of GA_3 was most evident with plants also exposed to chilling for 2 days, which developed 4 fewer crown buds and crown shoots if also treated with GA_3. Application of PBZ increased the number of crown buds and crown shoots 2-4 times compared to GA_3 applied with 2 or 42 days of chilling; however this was not different as compared with 0 days of chilling.

Crown bud clusters which developed within GA_3-treated plants displayed elongation of the cluster-stem to which individual crown buds/shoots were attached, being similar to that observed previously (Chapter 5). The number of crown shoots emerged expressed as a proportion of the combined crown buds and crown shoots present at the beginning of third growth cycle, was influenced only by exposure to chilling for 42 days followed by application of GA_3, as compared to the rest of the treatments (215 ± 88%; $P < 0.01$). Influence of the application of chilling or GA_3 separately however, was not evident, with the rest of the treatments achieving a similar proportion of emergence (23 ± 14%). The number of crown shoots emerged at the beginning of the third growth cycle however did not vary ($P > 0.05$).

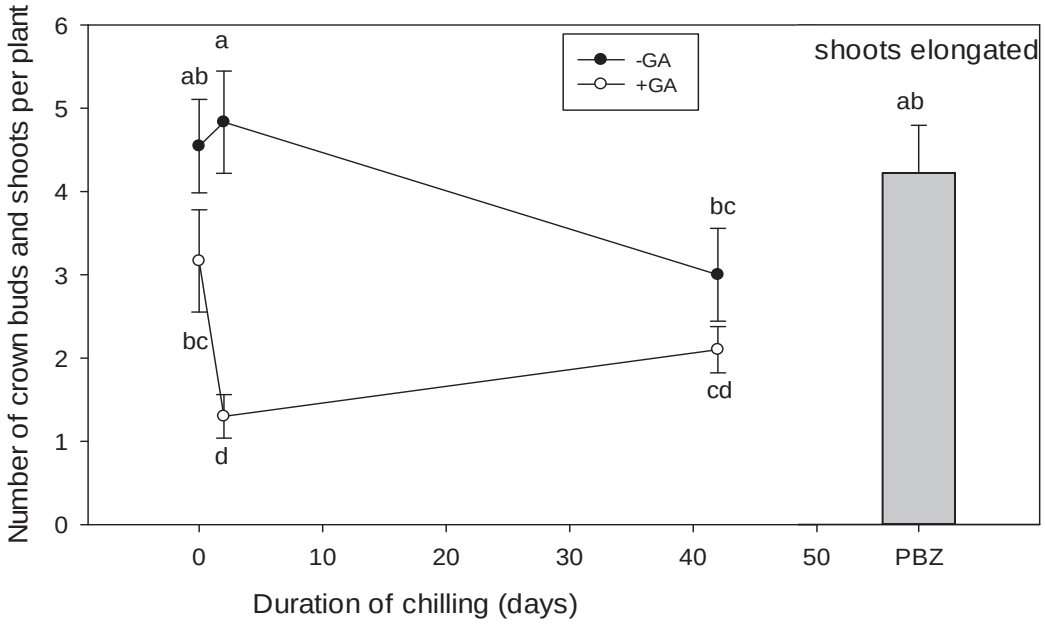

Figure 8.10. The combined number of crown buds and shoots per plant, during the third growth cycle, following increasing duration of chilling at 5 °C and/or application of GA_3 or PBZ to plants of 'Diva' with elongated shoots (Experiment Three). For each variable means indicated by different letters were significantly different. Vertical lines represent ± SEM. Mean separation by DNMRT at $P < 0.05$

8.5 Discussion

8.5.1 Possible roles of chilling and gibberellin in dormancy and flowering

8.5.1.1 Endodormancy

Within the current experiments, the change in response to chilling was dependent on the developmental stage (Figure 8.3A, Figure 8.6A and Figure 8.9A), and supports the hypothesis that changes in sensitivity to dormancy breaking treatments occur with degree and type of dormancy present (i.e. para, endo and/or eco; (Faust et al., 1997)). This differential response could be due to changes in the sensitivity of the cells, tissues or plant organs to external environmental stimuli or plant growth regulators (Bradford and Trewavas, 1994). As noted in other geophytes, plants must be physiologically capable of perceiving the chilling treatment (Dole, 2003; Whitman et al., 1996), therefore the developmental stage prior to emergence was responsive to chilling as compared with the latter two stages. The increased response to chilling prior to emergence is interpreted as indicating the crown buds were showing signs of being endodormant. Following the start of shoot emergence, crown buds could be primarily paradormant, but minimally endodormant and, therefore, non-responsive to chilling. As an increased duration of chilling gradually increased the proportion of shoot emergence (Figure 8.3A), the chilling requirement in 'Diva' was consistent with being a facultative response, as reported in other species (Gracie et al., 2000; Søgaard et al., 2008; Wall et al., 2008). Compared to 'Spotlight' (Chapter 3), the chilling requirement in 'Diva' is therefore considered to be mild, as shoot emergence was evident in the absence of chilling (Figure 8.3A).

GA_3 substituted for chilling in breaking endodormancy prior to emergence, as evident by the percentage of shoot emergence (200%) being similar to the treatment with the longest duration of chilling (Figure 8.3A). As evident in other plant species, it could be hypothesized that the stimulation of crown buds to emerge by chilling is associated with an increase in endogenous GA (Chao et al., 2007), and exogenous application of GA_3 may be capable of achieving increased GA levels within tissues, even without chilling. It was concluded therefore, that even if adequate chilling was not perceived, a single application of GA_3 at 100 ppm, is sufficient to substitute for the chilling requirement to break endodormancy when applied prior to emergence in 'Diva'. Due to limitations of plant availability, PBZ was not utilized as a treatment prior to emergence and, therefore, it was not possible to identify the influence of this GA antagonist on shoot emergence at

this developmental stage. Future investigations may, therefore, also utilize this treatment in conjunction with GA_3, so as to provide further insight into the role of GA in endodormancy in gentian.

The type of dormancy is indicative of the ability to survive under adverse environmental conditions and, endodormancy in particular helps survival during winter (Anderson et al., 2010; Anderson et al., 2001). The presence of specific proteins required for cold tolerance of crown buds have been noted in *G. triflora*, with their lowest concentration recorded immediately before shoot emergence (i.e. non-endodormant) as compared with the period prior to (i.e. endodormant) and after that (i.e. gradually becoming endodormant) (Takahashi et al., 2006). It is possible therefore, that these cold tolerance proteins could also be associated with the plants of 'Diva' with some buds recently emerged, i.e. no longer fully endodormant, lacking the ability to tolerate cold (75% plant death). Comparatively, plants with shoots emerged and developed could be more tolerant to cold, as crown buds start to accumulate proteins required for cold tolerance following shoot emergence in spring through to summer (Takahashi et al., 2006). Alternatively it is also possible that plants of 'Diva' were at a physiologically active stage at the beginning of shoot emergence (Experiment Two), with stored carbohydrates already mobilized, and hence were more susceptible to any damage from cold (Friend et al., 2011; Jouve et al., 2007). The negative effect of cold at this developmental stage was not able to be totally recovered by GA_3, as evident by the 50% survival rate following the longest period of chilling combined with GA_3 (Experiment Two).

In contrast to the plants with buds recently emerged (Experiment Two), prior to emergence, when plants within the current experiment were endodormant, plant survival also decreased with reduced exposure to chilling; as also found with the cultivar 'Spotlight' (Chapter 3). Despite the fact there is no influence of chilling on quantitative aspects of the crown bud formation process (Chapter 3, 6 & 7), there appears to be an influence on overall plant survival. In the current experiment, GA_3 was also capable of substituting for that effect as well. Since the plants were endodormant at the developmental stage prior to emergence, plants were no longer damaged by chilling injury, therefore, chilling or GA_3 may have increased the vigor of the plants. Any association of chilling or GA_3 prior to emergence in triggering production of specific proteins responsible for cold tolerance (Takahashi et al., 2006) will be of interest for further study. Additionally however, the present study utilized a single cultivar and only

a single replication of percentage plant survival. Future research might, therefore, also benefit from a re-examination of these contrasting roles of chilling in plant survival with developmental stages, and whether it varies between cultivars, while utilizing greater number of replicates.

8.5.1.2 Paradormancy

Once the predominance of endodormancy is diminished, via chilling and/or GA_3, paradormancy remains within the cluster due to differences in age and/or the hierarchical arrangement of buds within the cluster (Chapter 5). In *Ulmus* spp (Ghelardini et al., 2010) and *Rubus idaeus* L. (Mazzitelli et al., 2007), following application of chilling it was found that paradormancy mainly prevents shoot emergence when the comparative level of endodormancy of individual buds was reduced. It is possible for paradormancy to exist together with endodormancy in gentians, however during the current study the differences in response to chilling or GA_3 between individual buds was not addressed. To identify the potential co-existence of paradormancy and endodormancy in 'Diva', the differential response of buds within the cluster to chilling or GA_3 needs to be investigated further (refer Chapter 9).

GA_3 promoted shoot emergence at all developmental stages examined (Figure 8.3A, Figure 8.6A & Figure 8.9A). At the developmental stage prior to the start of emergence (Experiment One), GA_3 substituted the chilling requirement (to break endodormancy) for shoot emergence, but not when emergence had started (Experiments Two and Three), because plants were no longer endodormant. It could be hypothesized that once shoot emergence had started, GA_3 stimulated emergence of crown buds which were under paradormancy derived from the shoots already developing (Chapter 4 & Appendix IV) and, presumably, also from the rest of the buds within the cluster. Breaking of paradormancy of axillary buds of floral shoots, when GA_3 was applied to plants without any crown buds, has previously been noted with 'Diva' (Chapter 6). In addition to endodormancy, GA_3 therefore, can also break paradormancy of buds in the gentian cultivar 'Diva', as also noted in other species (Chao et al., 2007; Horvath et al., 2002).

As compared to GA_3, PBZ however had no influence on shoot emergence within either of the two developmental stages investigated and, therefore, no increase in paradormancy was observed (6A & 9A). The Control treatment however displayed poor

shoot emergence, therefore any additional imposition of paradormancy may not have been detected.

As discussed above, GA_3 was capable of breaking potentially all or at least two types of dormancy (i.e. endo and para), albeit the mode of action may vary. During dormancy, primordia initiation ceases when the meristem is inactivated (Rohde and Bhalerao, 2007) following cessation of cell division in the sup-apical tissue, as noted in poplar *(Populus tremula x Populus alba)* (Ruttink et al., 2007). GA_3 promoted differentiation of axillary buds by induction of cell division in sub-apical tissues of axillary buds in both poplar (Hytönen et al., 2009), and *Salix pentandra* L. (Hansen et al., 1999) during dormancy break. As buds within the cluster of 'Diva' are also axillary, potentially GA_3 broke paradormancy of the crown buds via increased rates of cell division within them. As evident during the current study however, GA_3 stimulated their emergence irrespective of whether they were primarily endodormant or paradormant. In the future, determining whether both forms of dormancy are broken due to a similar mechanism of action, could be established via histological investigation of crown buds following application of GA_3 or chilling at different developmental stages, corresponding to types and degree of dormancy.

8.5.1.3 Flowering and shoot development

The influence of chilling varied based on the development stage, as evident by the duration to flower harvest maturity and, the variables related to floral shoot development during the current study. When applied prior to shoot emergence, exposure to chilling reduced the duration to harvest maturity (Figure 3B) as also noted in other plants (Christiaens et al., 2012; Granhus et al., 2009; Ruiz et al., 2007). There is however, no obligate vernalization requirement in 'Diva', as flowering could occur without chilling. In some species exposure to chilling reduced the heat units required to reach developmental stages (Huang et al., 1999; Ruiz et al., 2007) and, therefore, shoot development could be faster with increased chilling, leading to a reduced duration to flower. It is also possible that increased mobilization of stored assimilates following chilling (Gonzalez-Rossia et al., 2008) facilitates this response. Along with a greater number of shoots and early harvest maturity, shoot length was also greater with exposure to chilling prior to emergence (Figure 8.3A & Figure 8.4). When chilling was applied after shoot emergence had started however, the duration to harvest and shoot emergence was unaffected, (Figure 8.6A & Figure 8.9A), but shoot length and number

of nodes at harvest were increased. Hence it could be hypothesized that shoot emergence and shoot development are separately controlled by chilling or low temperature. Although shoot development may be promoted by chilling, exposing already emerged shoots to low temperature in the growing environment may reduce their rate of development (Chapter 2), as evident by the lack of any influence on duration to harvest maturity during developmental stages following when shoot emergence had started. As the optimum temperature, and the other parameters determining the temperature response of specific growth functions, could vary with developmental stage (Dole, 2003; Funnell, 2008), it will be important to develop this understanding with gentians in the future, in order to enable increased floral productivity.

When applied prior to emergence, GA_3 increased shoot emergence and reduced the duration to harvest maturity, however it was not capable of substituting for the increased shoot length via chilling (Figure 8.3C) and the number of nodes with flowers, as found in *Tulipa gesneriana* L. (tulip) (Van Bragt and Van Ast, 1976), *Brassica napus* (Dahanayake and Galwey, 1999), and *Liatris* (Wanjao and Waithaka, 1983). As discussed above, chilling may stimulate the activity of endogenous GAs within plants (Chao et al., 2007), resulting in emergence of buds that are endodormant. Exogenous GA_3 could, therefore, substitute for this requirement for enabling shoot emergence. The shoot elongation and increase in number of floral nodes however, may be induced by chilling separately, without involvement of GA (Ogasawara et al., 2001), and is, therefore, not able to be replaced/supplemented by exogenous GA_3. The hypothesis that GA_3 was not involved in shoot elongation of 'Diva' was also supported by the fact that the antigibberellin PBZ did not cause any reduction in shoot length. To further investigate any interaction between chilling and GA_3 on shoot elongation, it will be important use treatment combinations with both chilling and PBZ.

Within plants with buds recently emerged, or emerged and developed, GA_3 caused an increased shoot length when applied following chilling (Figure 8.6B & Figure 8.9B), as also reported previously in other species (Al-Khassawneh et al., 2006; Christiaens et al., 2012; Paroussi et al., 2002; Shoub and De Hertogh, 1974). In contrast to the current study however, with these other species the duration to harvest maturity was also reduced. This was attributed to these species receiving chilling prior to emergence and application of GA_3. In the current study however, chilling was applied after shoot

emergence had started, i.e. Experiment Two and Three. It is possible therefore, that GA_3 can stimulate shoot development even when applied prior to emergence following exposure to chilling, as noted in tulip (Fukuda et al., 1994; Rebers et al., 1994). In tulip, endogenous GA in shoots increased in response to chilling after planting, but not prior to planting (Rebers et al., 1995). If applicable to 'Diva', it could be hypothesized that there is another trigger that leads to GA synthesis in shoots in response to chilling and, therefore, exogenous GA could induce this response only when shoot elongation had at least partially started. During the current study, due to limitations of availability of planting material, GA_3 was not applied following exposure to chilling in plants with unemerged buds. In order to get a better idea of the role(s) of GA_3 and chilling in shoot emergence, duration to harvest maturity and stem development, future studies will need to utilise application of GA_3 to plants with both partial and saturating durations of chilling, with unemerged buds.

'Diva' did not have an obligate vernalization requirement, as flowering was observed without chilling. It was, therefore, not possible to distinguish between a requirement of chilling for breaking dormancy and induction of flowering in 'Diva'. In contrast to the results reported in the current study however, the exogenous application of GA_3 to historical cultivars of *G. triflora* was capable of only partially substituting for the chilling requirement, as plants did not flower in the absence of chilling (Ohkawa, 1983). Hence pronounced effects of GA_3 was observed in these historical cultivars only in conjunction with chilling, being also similar to that reported with some other species (Ogasawara et al., 2001; Oka et al., 2001; Rudnicki et al., 1976; Saniewski et al., 1977; Sumitomo et al., 2009). Since flowering was observed without chilling in 'Diva', it is possible that this cultivar does not have a requirement for vernalization. Additionally, during the current study, chilling and GA_3 were not used in combination on plants with unemerged buds (i.e. fully endodormant) hence, in future research, any differences in flowering in response to partial or prolonged chilling, in combination with GA_3, needs to be determined. Instead of using a cultivar like 'Diva', which has a facultative requirement of chilling, by using a cultivar like 'Spotlight', which shows an obligate requirement for chilling (Chapter 3), it may be possible to examine the requirement of chilling and GA_3 in flowering (if any) more effectively.

8.5.2 Crown bud formation process

When applied prior to emergence, neither the application of chilling nor GA_3 influenced the crown bud formation process during the second growth cycle or emergence of these crown buds as shoots at the beginning of the third growth cycle. This was in direct contrast to the reduced appearance of additional crown buds evident when GA_3 was applied at the two later developmental stages of shoot emergence, i.e. Experiment Two and Three (Figure 8.8A & Figure 8.10). When treated prior to shoot emergence in the previous growth cycle, therefore, chilling or GA_3 have no carry-over effect into the next growth cycle.

When applied to plants following the commencement of shoot emergence (Figure 8.8 & Figure 8.10), the longest duration of chilling reduced the crown bud formation process quantitatively only in plants with shoots already emerged and developed, i.e. Experiment Three. The number of crown buds within treatments delivering partial chilling however, were similar to that achieved by the Control treatment. Similar to previous experiments therefore, it is concluded that exposure to cold temperature had little or no influence on the crown bud formation process (Chapters 3, 6 and 7). Since emergence of these crown buds was also not influenced by chilling in the subsequent growth cycle (Figure 8.8 & Figure 8.10), any carry-over effect of chilling when applied during these two growth stages was minimal.

As evident by double the number of new crown buds and crown shoots, as well as crown buds per cluster (Figure 8.8), the crown bud formation process was promoted in the second growth cycle by PBZ, when applied at the developmental stage immediately following shoot emergence (i.e. Experiment Two). This indicates that although treatment differences were not evident in many of the other variables describing growth, the dosage of 20 ppm is sufficient to be both absorbed and metabolized in 'Diva'. In contrast to PBZ, all plants treated with GA_3 resulted in a lower number of crown buds, irrespective of whether chilling was received or not. Hence the involvement of inhibition of GA in promoting the appearance of crown buds, was confirmed during the current study. When applied to plants in the first growth cycle however, PBZ at 20 ppm did not influence the number of crown buds (Chapters 6 & 7). In contrast to the plants in these previous studies, those used in the current study were in their second growth cycle and, therefore, already had an established transition zone with bud initials. It is possible that inhibition of synthesis of GA, such as that achieved with PBZ, is effective

in the later stages of the crown bud formation process, i.e. development of individual buds within a cluster, or development of dormancy (Molmann et al., 2005; Olsen et al., 1997; Tekalign and Hammes, 2004), but not in the early stages of development of the transition zone and bud initials, such as in the first growth cycle following deflasking. As in the current study, development of bud clusters on the transition zone following application of PBZ was also observed in *Fragaria ananassa* Duchesne (Nishizawa, 1993). Together with the previous observations of PBZ resulting in crown buds and shoots of greater quality (Chapter 7), and the delayed appearance of crown buds following application of GA_3, it is hypothesized that PBZ may not directly influence the adventitious initiation of crown buds/bud initials, but instead acts on existing axillary meristems, leading to development of individual crown buds. It is possible that this is in coordination with promotion of endodormancy, as previously noted with potato (Tekalign and Hammes, 2004), and as also noted in the current study by reduced shoot emergence in the third growth cycle (Figure 8.8). Although the appearance of buds was less compared to PBZ, following the application of GA_3 a greater percentage of shoot emergence was observed in the next (i.e. third) growth cycle, when GA_3 was applied to plants following shoot emergence had started, i.e. Experiment Two. Hence GA_3 could possibly delay the development of crown buds at the point before they are visually apparent, although they may have already been initiated within the cluster. In line with this, it is possible that GA_3-treated plants divert more resources for development of the apical bud (MacDonald and Little, 2006), the stem of the bud cluster (Experiment Three) and, above ground growth (Chapter 6), causing a delay in the appearance of new crown buds. To test this/these hypotheses, future experiments investigating the crown bud formation process in response to exogenous GA_3 in combination with partitioning of assimilates between tissues could be undertaken.

8.5.3 Potential commercial applications

Depending upon the developmental stage of the plant, application of chilling and/or GA_3 can be used to influence floral productivity qualitatively, quantitatively and/or influence timing of harvest maturity. As evident in the current study, even though 'Diva' has a facultative requirement for chilling, the longest duration of chilling increased floral productivity by 8-fold, when applied prior to emergence, along with a reduced duration to flower harvest maturity by 49 days. Even with a 3 times greater number of floral shoots (Figure 8.3A), and as reported in other species (Padhye and

Cameron, 2008), the 80% spread in duration to harvest maturity decreased with increased exposure to chilling prior to emergence (Figure 8.5A). Hence from a horticultural perspective, exposure of 'Diva' to adequate chilling is not only important for optimising floral productivity, but is also essential for narrowing down the spread of harvest maturity. When grown in a temperate climate like NZ, this would be important in a cultivar like 'Diva', with its facultative requirement for chilling, because crown buds could potentially emerge with only partial chilling, but for optimal yield and a narrow spread in flower harvest, this partial chilling may not be sufficient.

Prior to shoot emergence, GA_3 was capable of replacing the effect of the longest duration of chilling for both shoot emergence (Figure 8.9A) and the duration to harvest maturity, but did not increase the quality of the resulting floral shoots, as with chilling (Figure 8.3C & Figure 8.4). Since the quality of shoots is subjective to minimum grade standards, commercial growers may need to determine whether the benefits of application of GA_3 outweigh the reduced profits from reduced quality. Application of GA_3 however, also reduced the 80% spread in timing of harvest (Figure 8.5). Application of GA_3 is, therefore, not only capable of substituting for chilling in attaining a greater number of floral stems and reduced duration to harvest maturity, if sufficient chilling is not received, but could be applied so as to narrow the 80% spread in time to harvest maturity. If a reduction of spread by 83 days is considered valuable in 'Diva', then supplementation of partial chilling with additional chilling is required. If provision of chilling is not economical, 63 days of reduction could be achieved by supplementation with GA_3 prior to emergence. Such manipulations of spread will better enable growers to achieve a targeted number of floral shoots within a narrow period of time, with less labour cost.

While chilling improved floral productivity and timing, when applied prior to shoot emergence, in commercial reality however, effective chilling temperatures of 5 °C may be received at any time between prior to and after shoot emergence. Subjecting the plants to long-term chilling once shoot emergence had commenced, reduced floral shoot production quantitatively (Figure 8.6A & Figure 8.9A), but the quality of shoots was increased by exposure to chilling at any stage prior to the beginning of shoot development (Figure 8.3C, Figure 8.4, Figure 8.6B, Figure 8.7 & Figure 8.9B). This positive effect of the longest duration of chilling on shoot quality cannot be justified commercially however, because of poor shoot emergence and plant death (75%). Hence

if plants have not received sufficient chilling prior to shoot emergence, plants may not respond to further chilling after emergence has started. In fact when plants have shoots that are recently emerged, chilling exposure is more detrimental for the survival of the plant than any other developmental stage examined. In natural growing conditions, this could occur in spring, hence plant death observed in mature plants in commercial fields (Ed Morgan, Pers Com.; Takashi Hikage, Pers Com.) could be at least partially due to plants getting exposed to cold in spring after shoot emergence has started, i.e. after partially completing their chilling requirement for endodormancy. Hence, in addition to identifying factors influencing productivity of floral shoots and their timing, the current study provides information regarding factors potentially contributing to survival of gentians and, therefore, a potential tool for those needing to decide what climatic zones might best suit cultivars like 'Diva'. The current study however, only provides information relative to the cultivar 'Diva'. Given the differences in response to temperature observed within the cultivars used for experiments within this thesis (Chapters 2 & 3), and as reported in other plant species (Gariglio et al., 2006), it will be important to quantify the chilling requirement and the corresponding plant responses in other new cultivars of gentians, for the purpose of commercial application.

In contrast to chilling, application of GA_3 can be used to increase the number of floral shoots irrespective of the developmental stage. A reduction in the duration to harvest could only be achieved if GA_3 was applied prior to emergence, but the 80% spread could be narrowed by GA_3 at any stage of development (Figure 8.5), even halving the spread when applied to plants with shoots starting to emerge. Additionally, if plants have not received enough chilling, due to mild winter conditions which prevail in some regions of NZ, GA_3 could be used to supplement this requirement even after shoot emergence has started. If shoot emergence has already started, however GA_3 cannot reverse any damage caused by exposure to cold. If plants experience decreased shoot emergence due to partial chilling however, GA_3 can be applied at any developmental stage to get a greater number of harvestable shoots without any effect on shoot quality and still achieve reduced spread. If the chill unit requirement for optimum flower production is understood for a given cultivar, growers will be able to decide whether chilling has been adequate to naturally satisfy the chilling requirement on any particular date in winter/spring and, therefore be able to decide whether GA_3 is required. In a commercial horticultural situation therefore, a decision support system would enable the

grower to utilize application of chilling or GA_3 based on the primary requirement of quantity, quality or timing of floral shoots for the target market.

8.6 Conclusion

As evident during the current study, in 'Diva' crown buds develop endodormancy with a facultative requirement of chilling for shoot emergence. If encountered following the start of shoot emergence (i.e. when crown buds are paradormant and/or non-endodormant or partially endodormant), exposure to chilling could cause plant death with no influence on shoot emergence, however chilling prior to shoot emergence (fully endodormant) improved production of floral shoots qualitatively and quantitatively. GA_3 could substitute chilling prior to emergence with the same quantitative effects. Following emergence, when paradormancy predominates, GA_3 improved flower productivity qualitatively and quantitatively, even when plants were partially damaged by chilling injury. This study therefore, provides potentially valuable information to improve the production of floral shoots either based on the stage of development, duration of exposure to chilling or GA_3 and, based on commercial requirements (i.e. quality, quantity or timing of flower harvest). Further to that, the current study confirmed that inadequate chilling prior to emergence could lead to a wider spread in timing of flower harvest in 'Diva'. While exposure to chilling following emergence did not narrow the spread, GA_3 could substitute for the requirement of chilling for a narrower spread in flower harvest maturity at any developmental stage. Hence the current study provides an insight to the factors influencing the spread in flower harvest, and the potential manipulative strategies relative to shoot emergence in 'Diva'.

Chapter 9 Shoot emergence and development; Differential response of buds within the cluster

9.1 Abstract

The comparative growth potential of buds within the cluster was investigated in the cultivar 'Diva', in response to treatments that break dormancy (chilling, gibberellic acid (GA_3), clipping). The developmental end points of the bud clusters, and crown buds within the cluster, were evaluated by tracking their development over two growth cycles. Paradormancy from the previous season's floral shoots was not evident at the stage of development of the plants utilized. The hierarchical relationship of crown buds within the cluster however, led to a differential response to application of chilling or GA_3. The buds positioned at the proximal end (positions 1-3) displayed a similar duration to reach shoot emergence or harvest maturity. In contrast, a positive correlation from proximal to distal ends of the cluster was evident for those buds located at the distal end (position 4 and greater), resulting in an increase in duration to harvest maturity of 14 days with each increment in hierarchical position. For the qualitative characteristics of shoot length and number of nodes, slight negative correlations with the position of the bud from the proximal to distal end were evident, within plants treated with GA_3, wherein a 7 cm decrease in shoot length or reduction of 1-2 nodes was observed per incremental hierarchical position. The results thus greatly increase the understanding of why there is a spread in time to harvest maturity, and quality, of floral shoots within a single plant.

9.2 Introduction

As presented in Chapter 8, the response to chilling and/or gibberellic acid (GA_3) in 'Diva' varied with the developmental stage of the plant at the time of treatment application. At all stages of development examined, application of GA_3 (100 ppm) resulted in increased emergence of crown buds as shoots, leading to development of a greater number of shoots of harvestable quality. A similar influence on emergence of shoots was observed after exposure to chilling, but only when applied at the stage of development with unemerged crown buds present. Plants with unemerged crown buds exhibited increased shoot emergence as the duration of chilling exposure (5 ºC) increased from 0 to 42 days, with GA_3 being capable of replacing the effect of 42 days of chilling. With more shoots emerging than crown buds visible at the beginning of the

experiment, it was not clear from this preceding series of experiments where these additional new shoots had emerged from. It was considered possible that the additional shoots had arisen from either the existing bud cluster or, from a new crown bud cluster that developed as the experiment progressed (refer Chapter 8). As explored further within the current experiment, the origin of these additional shoots therefore, remained to be investigated. As hypothesized in Chapters 5 & 8 however, due to the arrangement of buds within the cluster, paradormancy could exist within the cluster, leading to differences in response to dormancy-breaking treatments. If so, this could also influence the time for a shoot to reach developmental end points such as emergence and flower harvest maturity and, therefore, affect the spread in duration to harvest maturity for a plant (refer Section 1.3.1). To develop an understanding of where the additional shoots arise from, and the developmental hierarchy within crown bud clusters, in the current experiment the correlation between these developmental end points and the position of buds within the cluster was investigated.

Some gentian cultivars like 'Spotlight' have an obligatory requirement for chilling to break endodormancy of crown buds (Chapter 3), whereas 'Diva' had a facultative response (Chapter 8). In these previous experiments, plants receiving chilling and/or GA_3 already had floral shoots (i.e. past anthesis) at the time of treatment application. Given this situation, the ability to detect evidence of breaking of endodormancy in response to chilling, may have been confounded by the influence of paradormancy from the primary shoot. In order to address the influence of chilling and GA_3 on endodormancy, and to eliminate the influence of paradormancy, it was proposed that in the current experiment the existing shoots would be removed, i.e. clipped off. Although how it applies to gentians is not understood, it is possible that crown buds also experience ecodormancy, wherein the growing conditions do not permit growth (Horvath et al., 2003). If gentians are under cold-induced ecodormancy, this can be broken by warm temperature (Lang et al., 1987) and, given the warm growing environment used during the current study, it was considered that ecodormancy would be absent.

In previous experiments, the importance of identification of the point of attachment of new crown buds was highlighted (Chapter 5). It was unclear however, whether new crown bud clusters are formed or if activation of existing clusters results in new crown buds and, therefore, forms the basis for floral shoots in the subsequent growth cycle. In

order to identify the potential locations for development of new crown buds, continuous monitoring of changes within the transition zone for two growth cycles was required, as investigated during the current study.

The overall aim of the current study was to develop an understanding of the potential changes within bud clusters when dormancy was broken and, what if any influence this has on the timing and location of initiation of the next growth cycle's crown buds. For the current experiment, the specific objectives were to;

1. identify the comparative growth potential of crown buds within the cluster when treated with GA_3 or chilling,
2. quantify the effect of chilling or GA_3 on breaking endodormancy in the absence of, paradormancy from existing shoots or, ecodormancy from the growing environment,
3. identify the point of attachment of new crown buds.

9.3 Materials and methods

9.3.1 General management of plants

Plants of *Gentiana triflora* × *scabra* 'Diva' were propagated and managed as per Section 8.3.1 Unless stated otherwise, throughout the experiment plants were grown in a greenhouse (heated at 15 ºC, ventilated at 20 ºC) under a long photoperiod (LD; comprising 2 h night break lighting at 4.585 μmol s^{-1} m^{-2} from 23:00 HR to 01:00 HR each day), with tracking of the development of individual crown buds and shoots within clusters over two growth cycles (refer Section 9.3.3).

At the commencement of the experiment, plants with one or two primary shoots past anthesis, and one or two crown bud clusters (3-7 unemerged crown buds per cluster), were selected at the end of their second growth cycle (3rd September 2009; Figure 9.2A).

9.3.2 Treatment application

Due to the unavailability of a large enough population of uniform plants, it was not possible to carry out a factorial arrangement of treatments. Despite this limitation however there were four treatments where plants either had their existing primary

shoots clipped off or not and, in the plants that were clipped, they were either treated or not treated with GA_3 or chilling (Figure 9.1).

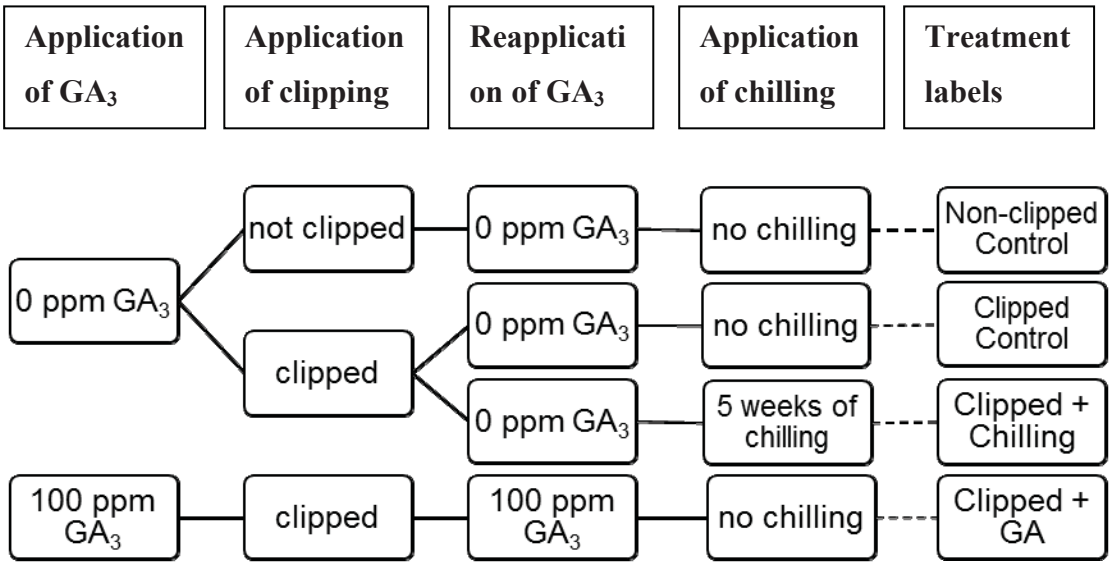

Figure 9.1 Schematic representation illustrating the sequence and composition of treatments applied.

At the commencement of the experiment (3rd September 2009), prior to treatment application with clipping, GA_3 was applied onto the foliage, exposed crown and the surface of the growing medium as a spray to run-off (≈ 70 ml per plant) at either 100 ppm (+GA) or 0 ppm (-GA), as described in Section 8.3.2. Three days following the application of GA_3, all existing shoots were either clipped off (Clipped) at their base (Figure 9.1), leaving no remaining nodes (6th September 2009) or, plants were not clipped and the same GA_3 treatments (0 ppm or 100 ppm) reapplied (Figure 9.1).

For the treatment comprised of Clipped + Chilling (Figure 9.1), the plants that were both clipped and treated with GA_3 at 0 ppm, were transferred to cold storage (5 ºC) immediately following clipping (16 h photoperiod of 4.89 μmol s^{-1} m^{-2} at plant height provided by incandescent bulbs) for 30 days. During this same period, plants in all other treatments remained in the heated greenhouse. Following completion of chilling, plants were transferred to the heated greenhouse, and all plants were grown under the same conditions through two subsequent growth cycles.

9.3.3 Procedure for tracking buds

Growth and development of crown buds were tracked as detailed in Section 3.3.2. Data were collected prior to treatment application, at weekly intervals for 7 weeks following application of the last treatment and, at 6 weekly intervals thereafter until two growth cycles had been completed.

Within a cluster, to track changes in the number of crown buds and their development over time, at each date of data collection a diagram illustrating the crown bud clusters of each plant was made. Throughout the period of data collection individual clusters, the order of appearance of crown buds and, shoots within a cluster, were identified and tracked (Figure 9.2). So as to identify the hierarchical position of crown buds and shoots within a cluster, each were numbered in ascending order from the proximal end of the cluster (i.e. from the base of the cluster) towards the distal end.

A crown bud was defined as having emerged as a crown shoot when it was more than 2.5 cm in height above the point of attachment at its base. Floral shoots were considered to have reached commercial maturity, when the top-most flower bud was not open but had developed colour (Eason et al., 2004).

9.3.4 Variables recorded and calculated

The variables recorded prior to the treatment application included the position of individual crown buds within a cluster, and the position of the previous season's senesced shoots. Following treatment application, at each date of data collection the variables recorded included; the date of appearance of new crown buds, their hierarchical position, and date of shoot emergence. In order to evaluate treatment effects on shoot emergence, seven weeks after the last treatment application, shoot length and the number of nodes visible on all crown shoots were recorded.

During the first growth cycle, for each shoot at flower harvest maturity, the following was recorded; date of harvest, shoot length, number of nodes, and number of nodes with floral axillaries. In order to evaluate shoot emergence during the first growth cycle, the number of harvested crown shoots was expressed as a proportion of the number of crown buds visible at the beginning of the first growth cycle.

Figure 9.2 A representative plant of 'Diva' with a single bud cluster at differing stages of development during the first growth cycle within the Clipped + GA treatment, illustrating the methodology of identifying the hierarchical position of crown buds; (A), prior to treatment application (3rd September 2009) indicating positions (1-4) of crown buds within the cluster from oldest (1) to the most recent bud which is oblong in shape (4; red circle), (B), as at 19th October 2009 following shoot emergence, indicating newly visible crown buds (positions 5 & 6; red circle) which arose from within the previously most recent bud (4) and, (C), as at December 2009, indicating emergence of shoots from crown buds that were not visible prior to treatment application (red arrow at position 5).

9.3.5 Experimental design and statistical analysis

The experiment was arranged as a Completely Randomised Design. There were four treatments; Non-clipped Control, Clipped Control, Clipped + Chilling, and Clipped + GA_3, with 4 individual plant replicates. Due to poor plant survival beyond the first growth cycle, within the Non-clipped Control treatment, data were only available for this treatment from the first growth cycle following treatment application.

Data were analysed using the General Linear Models procedure of Statistical Analysis System (SAS) software version 9.13 (SAS Institute, Cary, N.C., USA). If data were not normally distributed, Square root or Log transformation was carried out to improve the normality. Mean comparisons were conducted using Duncan's New Multiple Range Test (DNMRT).

Relationships between the position of the crown bud and the variables related to the duration to reach any developmental stage or, quality variables of shoots, were evaluated using Regression Analysis (Sigma Plot version 10, Systat Software Inc, San Jose, CA, USA). Correlations were determined utilizing all the crown shoots which emerged within all individual plant replicates. Box and whisker plots (Sigma Plot) were used to describe the treatment effects on the timing of emergence of shoots from different hierarchical positions of crown buds, and flower harvest maturity. The median duration to achieve a developmental stage or position of the crown bud was used to describe any difference between the treatments.

9.4 Results

9.4.1 Shoot emergence and development - first growth cycle

When expressed as a proportion of the crown buds present at the commencement of treatment application, compared with the Clipped Control there was an 80% increase in shoot emergence with application of GA_3 ($P < 0.0001$; Table 9.1). Although not significantly different ($P > 0.05$), emergence was two times greater within the Clipped + Chilling than for the plants in the Clipped Control treatment. Non-clipped and Clipped Control treatments were also not significantly different ($P > 0.05$), but the emergence was greater in the Clipped Control treatment. As compared to the Clipped Control, the duration to shoot emergence was 23 days shorter following application of either GA_3 or chilling, subsequent to clipping ($P < 0.0001$; Table 9.1), and both Clipped + Chilling and Clipped + GA_3 presented a similar duration. No influence of clipping was evident in terms of the duration to shoot emergence, as no difference was observed between the Clipped Control and Non-clipped Control treatments.

Seven weeks following the last treatment application, compared to the crown shoots in the Clipped Control treatment, shoot length, and the number of nodes recorded, was up to 5 times and 2 times greater, respectively, in the treatments which also received Chilling or GA_3 ($P < 0.01$; Table 9.2). At harvest maturity however, the shoot length was 15-30 cm longer within treatments receiving Clipped + Chilling than in either the Clipped Control or Clipped + GA_3 treatments ($P < 0.001$). The total number of nodes on floral shoots at harvest varied marginally, with the greatest number of nodes evident within the Clipped + Chilling treatment, being 3 nodes more than in shoots within the Clipped + GA_3 treatment ($P = 0.08$). Treatment effects were not evident for the number of nodes which were vegetative at harvest, with an average of 14 ± 2 ($P > 0.05$). In contrast, the number of nodes with floral axillaries was greater for shoots arising from the Clipped + Chilling treatment, being 2 nodes more than those from the Clipped Control, and 6 more than shoots within the Clipped + GA_3 treatment ($P < 0.0001$). Application of GA_3 resulted in the shortest duration to harvest maturity, being 20 days less than for those in the Clipped Control treatment ($P = 0.05$).

The spread in time to harvest maturity was 23 days greater in the Clipped Control treatments than Clipped + GA_3 and Clipped + Chilling (Figure 9.3). Even with 4 times greater shoots, 50% of shoots were harvested within 160 days of the last treatment application within the Clipped + GA_3 treatment, and this median date was between 20

and 23 days earlier than all other treatments. Of the 80% harvest, 65% was achieved within 18 days in Clipped + GA_3 treatment, with no outliers evident, 15% of late flowering shoots extended the harvest period by 10 days, i.e. from 168 to178 days.

Table 9.1 Proportion of, and duration to, shoots emerging from crown buds of 'Diva' in the first growth cycle following treatment application.

Treatment	Proportion of buds that formed shoots (%)[Z, Y]	Duration to emergence (days)[Y, X]
Non-clipped Control	8 ± 8 c	80 ± 56 (4.0 a)
Clipped Control	22 ± 8 bc	38 ± 3 (3.6 a)
Clipped+GA	108 ± 8 a	15 ± 2 (2.8 b)
Clipped+Chilling	42 ± 4 b	15 ± 0 (2.7 b)

[Z] as a percentage of the number of crown buds at day 0.

[Y] Means (± standard error) followed by different letters were significantly different at $P < 0.0001$ for each variable using DNMRT groupings.

[X] Log transformed values are given within parenthesis.

Table 9.2 Quality and timing characteristics of shoots from plants of 'Diva' at two developmental end-points in the first growth cycle following the last treatment application.

Variable	Clipped + Chilling	Clipped + GA	Clipped Control [x]	Statistical significance
Shoot length (cm) 7 weeks after last treatment application[z]	22.5 ± 6 [a]	25.1 ± 2 [a]	5.3 ± 2 [b]	$P < 0.01$
Number of nodes 7 weeks after last treatment application	13 ± 1 [a]	13 ± 1 [a]	7.7 ± 2 [b]	$P < 0.01$
Shoot length at harvest (cm)	93.5 ± 3 [a]	62.5 ± 3 [b]	76.7 ± 9 [b]	$P < 0.001$
Number of nodes at harvest	25.5 ± 1	22 ± 1	24 ± 0.6	$P = 0.08$
Number of vegetative nodes	14 ± 2	13 ± 1	12 ± 1	n.s.
Number of nodes with floral axillaries at harvest	13.5 ± 1 [a]	7.3 ± 1 [c]	11 ± 1 [b]	$P< 0.0001$
Duration to harvest maturity (days)	184 ± 6	165 ± 4	186 ± 14	$P = 0.05$

[z] Means (± standard error) followed by different letters were significantly different at the probability indicated for each variable using DNMRT groupings. n.s.= not significant

[x] Data for Non-clipped Control was not included due to poor plant survival

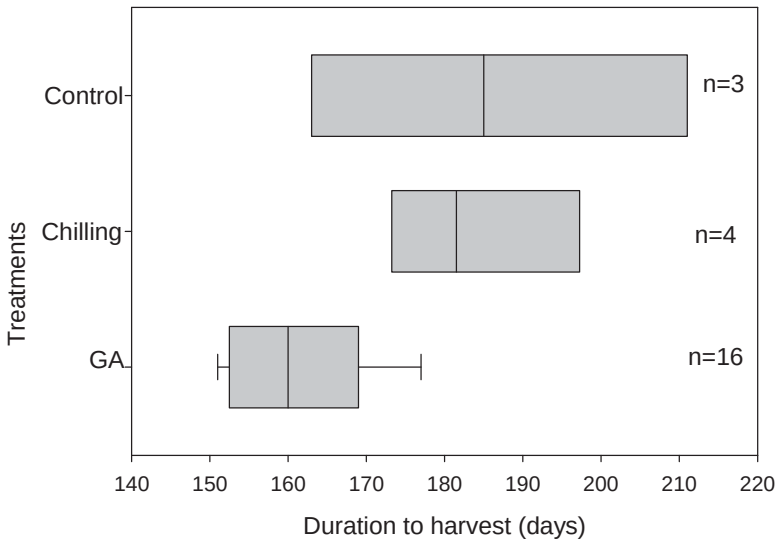

Figure 9.3 Distribution of duration to harvest from the last treatment application for crown shoots on plants of 'Diva' during the growth cycle immediately following treatment application. Solid lines within each box indicate the median. Boundaries of the box indicate 25th and 75th percentiles, and whiskers (if present) indicate 10th and 90th percentiles. n=number of shoots

9.4.2 Influence of hierarchical position of the bud

During the first growth cycle following treatment application, the median value for the hierarchical bud positions within a cluster which emerged as shoots increased from Position 2 for the Non-Clipped Control, to Position 5 for the Clipped Control (Figure 9.4). In contrast, for plants that were clipped, those also treated with GA_3 resulted in the median hierarchical position being reduced from Position 5 for the Clipped Control to Position 3, with those in the Clipped + Chilling treatment reduced to a median value of Position 2.5.

Due to so few shoots being evident in most treatments, the regression correlations were significant only within the Clipped + GA_3 treatment, which had the greatest number of shoots. Results for all treatments were, however, presented in order to get a general overview of the relationships evident within each treatment. Within the Clipped + GA_3 treatment, the relationship between the hierarchical position of the crown buds and the duration to shoot emergence, was positive but depended upon the hierarchical position of a bud. Within this treatment, crown buds located at the proximal end, up to position 3, had a similar duration to emergence of 10 ± 1 days (Figure 9.5A). In contrast, crown buds that were located at the distal end within the cluster (i.e. from position 4 onwards) progressively increased in their duration to emergence, with an additional 14 days in

duration per increment in hierarchical position. No relationship between the hierarchical position and duration to emergence was evident within either the Clipped Control or Clipped + Chilling treatments, averaging 38 ± 3 and 15 ± 0 days, respectively.

Within the Clipped + GA_3 treatment, crown buds located at the proximal end, i.e. positions 1-4, presented a similar duration to flower harvest maturity, with an average of 157 ± 1 days (Figure 9.5B). Within the remaining buds towards the distal end (from position 4 onwards) however, duration to harvest increased by 10 days with each increment in hierarchical position. Within the limited number of shoots emerged within the Clipped + Chilling treatment, buds positioned at the proximal end took a similar duration of 175 ± 3 days, whereas the buds at the distal end presented an increase in duration of 14 days with each increment in hierarchical position ($R^2 = 0.76$; $P > 0.05$). Such a relationship between the hierarchical position of the bud and duration to harvest maturity was not evident within the Clipped Control treatment, averaging 186 ± 14 days. Within the Clipped + GA_3 treatment, the relationship between the duration to emergence and duration to harvest, displayed a similar pattern to what was observed between the hierarchical position and duration to harvest. Buds that had emerged within 15 days following treatment application displayed a similar duration to harvest maturity, whereas buds which emerged afterwards ($R^2 = 0.51$; $P < 0.001$) took 2 days longer to emerge for each increment in hierarchical position.

In the plants treated with GA_3, a slightly negative correlation was evident between the quality variables of both shoot length (Figure 9.6A) and number of nodes (Figure 9.6B), and the hierarchical position of the buds progressing from the proximal to distal end ($P < 0.01$). Buds at positions 1 and 2 however, displayed relatively similar shoot lengths of 71 ± 3 cm. The rest of the buds, from position 3 to 6 (i.e. the maximum position evaluated), displayed a 7 cm decrease in shoot length per incremental hierarchical position. The total number of nodes on the shoots however, decreased by 1-2 nodes with each increment in hierarchical position. Shoots derived from both the Clipped + Chilling (n = 4) and Clipped Control (n = 3) treatments had positive correlations with the hierarchical position of the bud for the number of nodes ($R^2 = 0.8$ and $R^2 = 0.7$ respectively) however, with so few shoots contributing, this may not warrant further interpretation. As noted in the Clipped + GA_3 treatment, the correlation between the number of nodes which were vegetative and the hierarchical position of the bud, was negative with a reduction of 2 nodes with the each increment in hierarchical position

($R^2 = 0.61$; $P < 0.05$; Figure 9.7). There was no correlation evident however, between the number of nodes which were floral and the hierarchical position of the bud ($R^2 = 0.13$; $P < 0.05$).

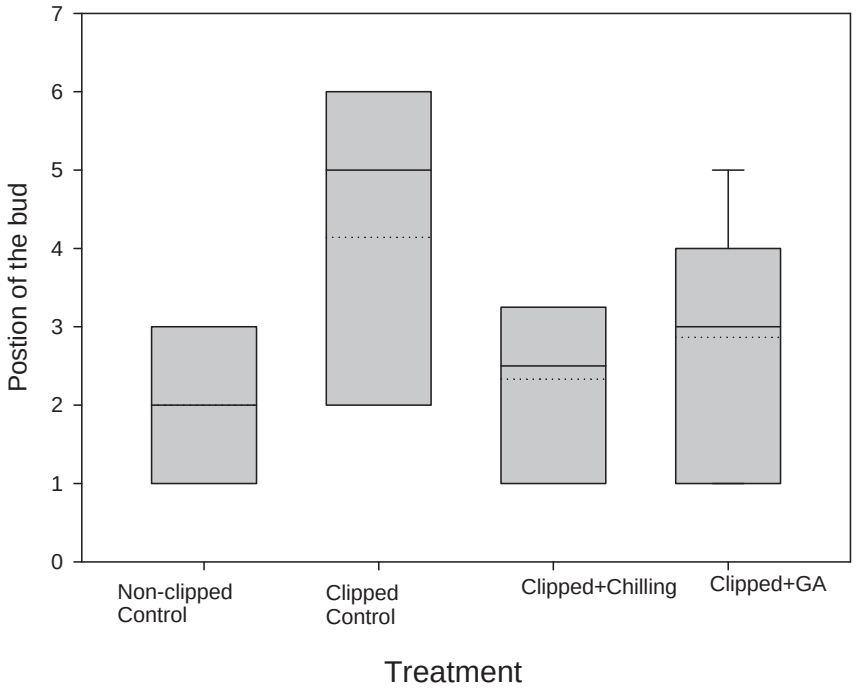

Figure 9.4 Spread in the hierarchical position of the bud within a bud cluster of 'Diva' which emerged during the growth cycle immediately following treatment application. Position of buds was numbered from proximal to distal end of the cluster. The number of shoots represented within each treatment were; Non-clipped Control (n = 3), Clipped Control (n = 7), Clipped + Chilling (n = 6), GA (n=15). Solid and dashed horizontal lines in the centre of each box indicate median and mean, respectively. Boundaries of box indicate 25th and 75th percentiles, and whiskers (if present) indicate 10th and 90th percentiles.

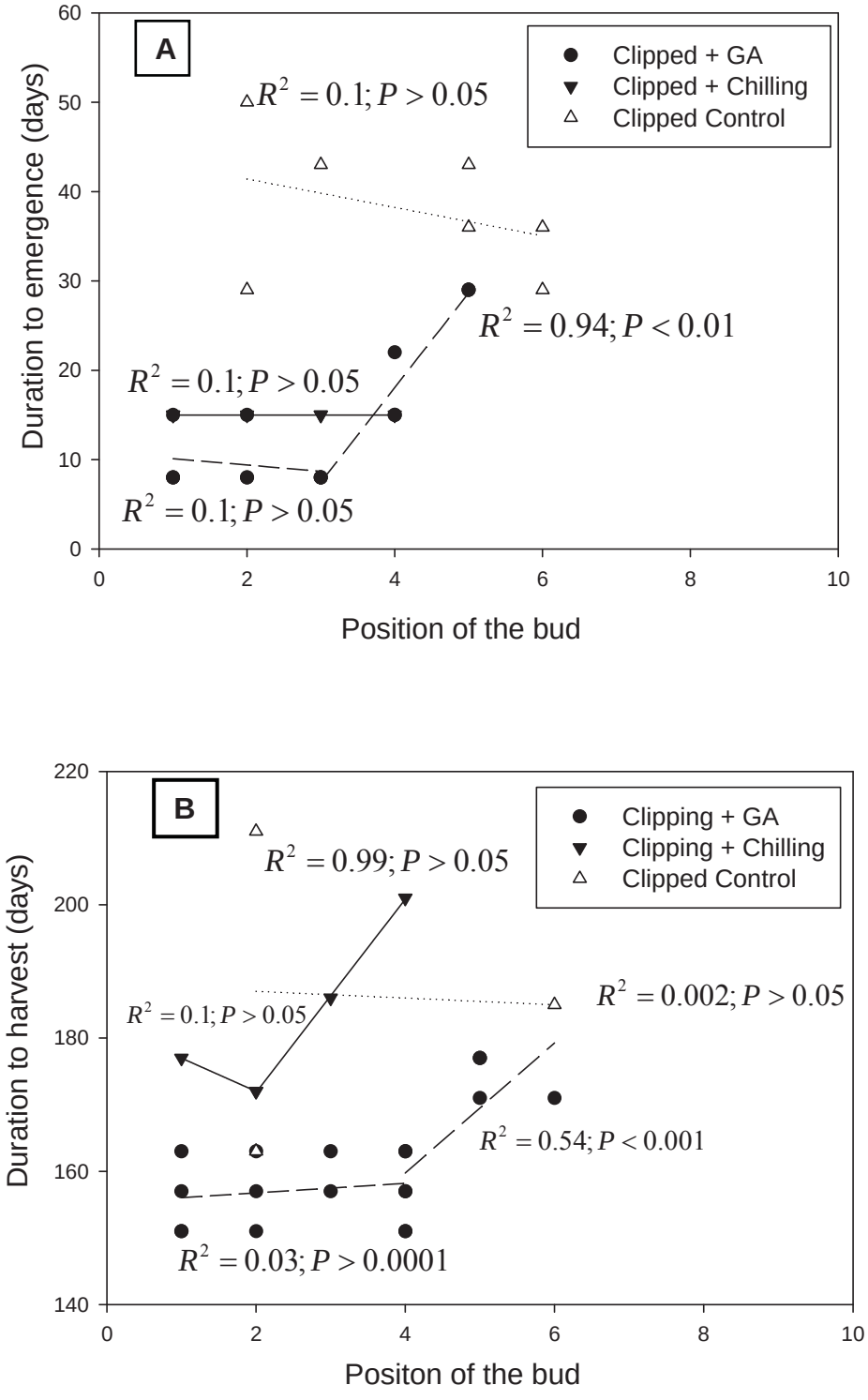

Figure 9.5 Relationship between the hierarchical position of the bud within the cluster, from proximal to distal end of the cluster and; (A), Duration to emergence and, (B), Duration to harvest maturity, among treatments of 'Diva' in the first growth cycle following treatment application.

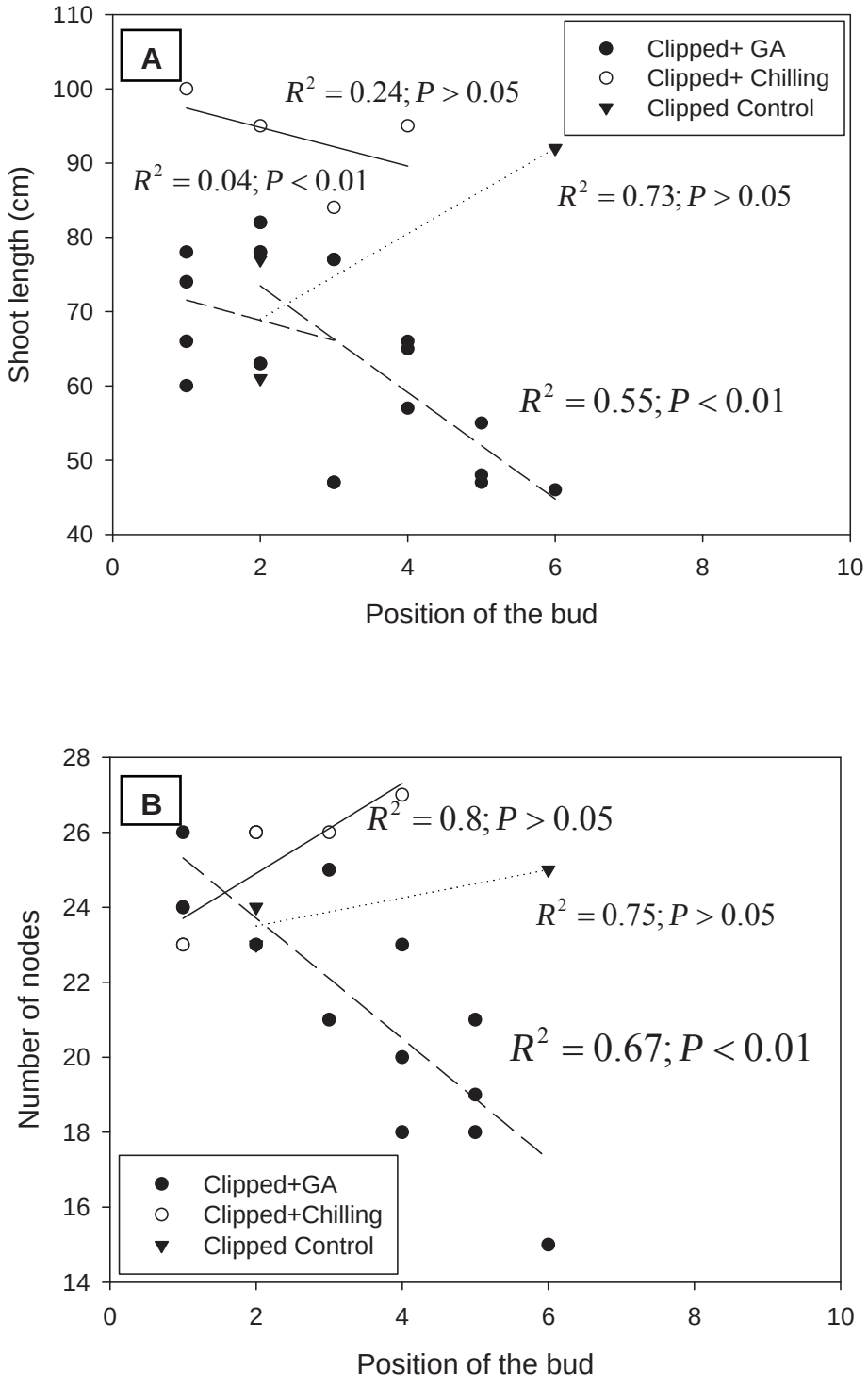

Figure 9.6 Relationship between the hierarchical position of the bud from proximal to distal end of the cluster and; (A) shoot length and, (B), number of nodes at harvest, among treatments applied to plants of 'Diva' in the first growth cycle following treatment application.

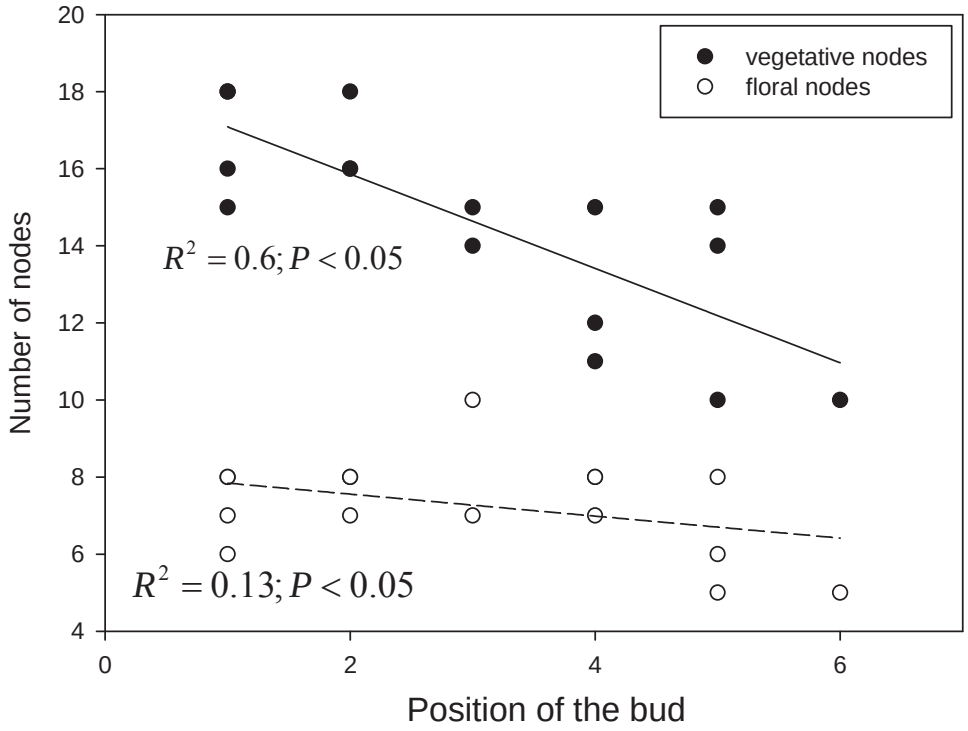

Figure 9.7 Relationship between the hierarchical position of the bud from proximal to distal end of the cluster and number of vegetative and floral nodes at harvest of 'Diva' treated with GA_3 in the first growth cycle following treatment application.

9.4.3 Shoot emergence - second growth cycle.

Due to low plant survival in most treatments, only plants within the Clipped + GA_3 treatment were used for presentation of data in the second growth cycle. Of the emerged shoots the duration to bud appearance, and duration to emergence of these buds as shoots, was not strongly correlated in these plants (R^2 = 0.35; P > 0.05). The median date for appearance of all crown buds in this treatment was 11/2/2010 however, for those buds that also subsequently emerged as shoots during this second growth cycle, the median date of appearance was 18/4/2010 (Figure 9.8). In contrast to observations in the first growth cycle, during the second growth cycle the median hierarchical position of buds that emerged as shoots within the cluster was Position 6, and no strong correlation was evident between the hierarchical bud position and duration to emergence (R^2 = 0.11; P > 0.05).

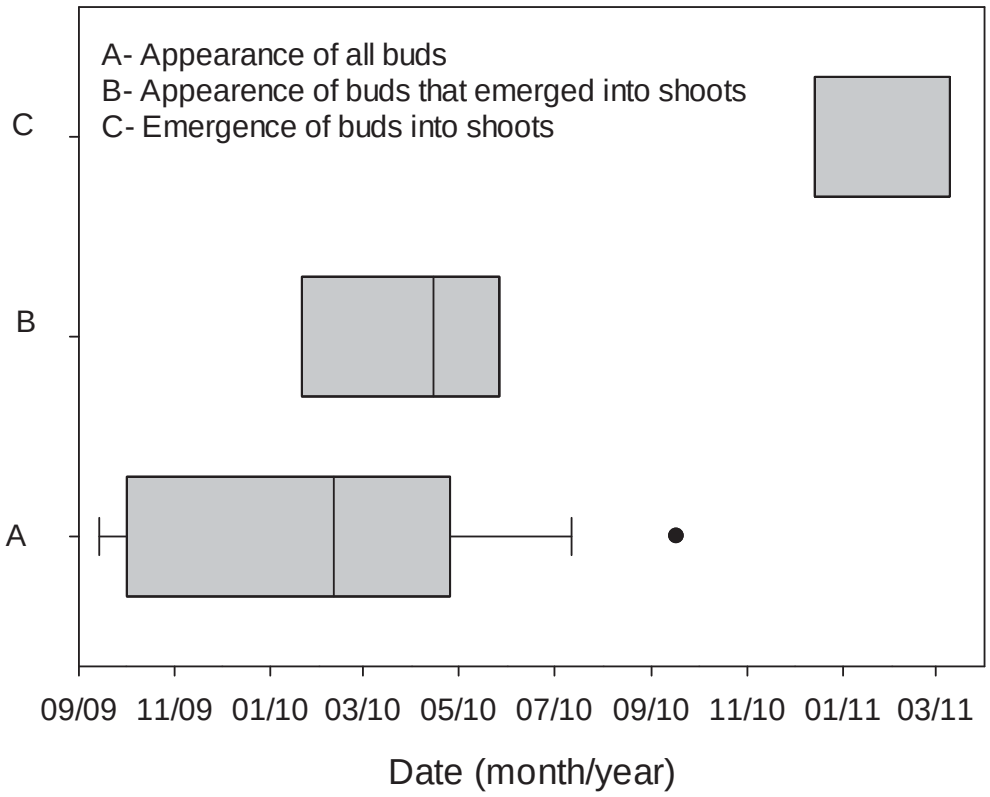

Figure 9.8 Distribution of dates of appearance of crown buds and/or their emergence as shoots, for plants of 'Diva' treated with GA_3 in the first growth cycle; (A), date of appearance of all crown buds in the first growth cycle, (B), date of appearance of crown buds that emerged into shoots in the second growth cycle following treatment application and, (C), the date of emergence of crown shoots in the second growth cycle following treatment application. Solid lines within each box indicates median. Boundaries of the box indicate the 25th and 75th percentiles, whiskers (if present) the 10th and 90th percentiles and, solid dots any outliers.

9.4.4 Morphological and developmental changes over two growth cycles

Within the crown bud clusters, the most apical crown bud that was visually apparent was typically oblong in shape during most of the growth cycle. This bud was enveloped by scale leaves, and contained a number of developing axillary crown buds (refer to Figure 5.14). Within a cluster, new crown buds, one bud at a time, progressively became physically separated from this most apical crown bud, revealing a spiral pattern and thereby determining its hierarchical bud position. Within the cluster the most mature buds were located at the proximal end, with the least mature at the distal end. The size of the new crown buds appearing from alongside the apical bud were comparatively larger in size than the most recently visible apical bud, hence at times this apical crown bud was typically positioned to one side of the cluster, until the next crown bud developed (Figure 9.2A).

Following treatment application, while the majority of crown shoots emerged from the crown buds already visible at the beginning of the experiment, treatment with GA_3 induced shoot emergence from crown buds which had not yet been released from the most apical crown bud (e.g. bud Position 4, Figure 9.2A). When the appearance of new crown buds occurred later in the first growth cycle following treatment, most of these buds originated by reactivation of the most apical bud of the already existing cluster. Only two plants within the current experiment developed new crown bud clusters from different points of attachment on the transition zone.

9.5 Discussion

9.5.1 Developmental potential and bud hierarchy

The current study provided further insight to the previously acquired knowledge of morphological features of crown buds and clusters presented within Chapters 3 and 5. The hierarchical arrangement of the crown buds from the proximal to distal end of the cluster, defines the timing of development of buds from the earliest to latest within the cluster and, therefore, their level of maturity. Within a cluster, application of GA_3 resulted in all crown buds that were visible, as well as some crown buds that were not yet visibly apparent within the cluster, to emerge (Figure 9.2; Table 9.1). This result therefore, confirmed the findings reported within the previous study, wherein application of GA_3 resulted in more than 100% of visible buds emerging as shoots (Chapter 8). These previously non-visible crown buds, which emerged as shoots within the same growth cycle as their appearance, were derived from the most apical bud of the existing clusters, and not from new crown bud clusters. The apical meristem of the cluster appears to remain vegetative, while axillary buds develop as floral shoots and, therefore, the apical bud can be defined as having an indeterminate growth pattern.

Buds that were developed earlier within the cluster (proximal) have similar durations in reaching shoot emergence or harvest maturity, and were faster than buds developed later (distal; Figure 9.5). This result is in accordance with correlations observed between the duration to emergence and harvest maturity in three gentian cultivars, including ‘Diva’ (refer Chapter 2). In addition to the duration to reach a stage of development, qualitative characteristics of shoots (shoot length and number of nodes) also progressively increased with the position of the bud from the proximal to the distal end of the cluster. Thus, based on their hierarchical position and/or maturity, there was a

differential response to dormancy breaking treatments between the crown buds within the cluster. In *Rosa hyb*rida L. (rose) (Le Bris et al., 1998; Marcelis-Van Acker, 1994), this differential response was a reflection of the level of development of a bud, i.e. maturity. Similarly within the current study, the reduction in qualitative characteristics, i.e. shoot length and node number, with increments in the hierarchical position, may also be indicative of the level of development achieved by the time of treatment application. Since the number of vegetative nodes remained similar among treatments, irrespective of dormancy breaking treatment, it appears floral initiation commenced following achievement of a defined degree of development (leaf number) as found in some plants, e.g. maize and banana (Sachs, 1999). Within the Clipped + GA_3 treatment however, there was a reduction in the number of vegetative nodes with increments in hierarchical position, as also reported in rose (Marcelis-Van Acker, 1994). Shoot emergence may have occurred in response to the application of GA_3, irrespective of their degree of development, i.e. number of vegetative primordia. An increased number of floral nodes within shoots harvested from the Clipped + Chilling treatment infers that floral initiation continued for a longer period within this treatment. Hence both types of dormancy breaking treatment, as well as the hierarchical position, contribute to the number of nodes which are vegetative and floral in gentians. While the above interpretations of the differential response of buds within the cluster is based on the differing maturity of buds, an alternate hypothesis focussing on the existence of paradormancy was also explored (refer Section 9.5.2).

Increased shoot emergence in response to the application of GA_3 or chilling was an indication of the existence of endodormancy in 'Diva' (Table 9.1), which also substantiates that observed previously (Chapter 8). While, compared to the Clipped Control treatment, application of GA_3 narrowed the spread in time to flower harvest maturity by half, the distribution of flower harvest maturity was skewed towards later in the main harvest period (i.e. 80% spread; Figure 9.3). The 15% of the shoots in the 75^{th} to 90^{th} percentile contributed one third of the overall spread, and originated from shoots emerged from crown buds at Position 4 or later. It can be concluded therefore, that the late emerging shoots that contributed to the spread in harvest maturity in the field following exposure to chilling (Chapter 2), have emerged from buds positioned at the distal end of the cluster. The position of the bud therefore makes a significant contribution to the spread in harvest maturity, despite buds being relieved from endodormancy.

9.5.2 Presence of paradormancy and differential responses within the cluster

As evident by a similar proportion of crown buds emerging as shoots in both the Clipped Control and Non-clipped Control treatments (Table 9.1) at the start of the experiment, paradormancy from the existing floral shoots was not the factor limiting emergence of crown buds. Although not evident at the developmental stage of clipping during the current experiment (i.e. when shoots were past anthesis), when floral shoots were clipped earlier in the growth cycle (i.e. December, when shoots were developed, but had not reached anthesis), more than 100% shoot emergence was observed from the unemerged crown buds (Chapter 4 & Appendix IV). Similar to the findings within the current study therefore, clipping of floral shoots later in the growth cycle (i.e. past anthesis), led to little or no shoot emergence (Chapter 4 & Appendix IV). As also hypothesized within Chapter 4, the comparative level and/or dominance of paradormancy from existing floral shoots on unemerged crown buds therefore, appears to vary within the growth cycle. From the point of view of the hierarchical arrangement of buds/shoots within a cluster, clipping of shoots removes paradormancy by crown shoots located at the proximal end of a cluster, i.e. not the apical dominance as also described in *Asparagus officinalis* L. (Ku et al., 2005). The period of paradormancy from these developing shoots could be expressed most strongly, and/or predominantly, over the rest of the buds within the cluster, during late spring through to late summer (Chapter 4 & Appendix IV), however not during late autumn to winter. The inability to observe significant differences in the proportion of buds emerging as shoots between the Clipped Control and Non-clipped Control treatments, despite an almost 3-fold difference (Table 9.1), could also be due to an inadequate number of replicates utilised during the current study and, therefore, would benefit from being reinvestigated in the future.

A differential response from crown buds within the cluster, which was either negative or positive dependent upon the variable (i.e. durations or shoot length or number of nodes), was however evident, based on the hierarchical position from the apical bud within the treatments of chilling or GA_3 (Figure 9.5, Figure 9.6 and Figure 9.7). Since endodormancy was broken by these treatments, late emergence of buds at the distal end was hypothesized to be due to existence of paradormancy within the cluster. The response further away from the apex was greater in both timing (Figure 9.5) and quality (shoot length and number of nodes) effects on the resulting shoots (Figure 9.6); a response also reported in rose (Marcelis-Van Acker, 1994). As crown buds are axillary

buds, the most apical bud of the cluster could inhibit the outgrowth of the rest of the axillary buds of the cluster as reported in other species (Cline and Deppong, 1999; Müller and Leyser, 2011). It can be concluded therefore, that in addition to the paradormancy exerted by any developing floral shoots, crown buds are also under the paradormancy exerted by the apical bud of the cluster.

Within bud clusters subjected to the Clipped Control (no GA_3 or chilling) treatment, a successive emergence pattern from proximal to distal ends of the cluster was not evident, as shoots emerged from only a few crown buds in the mid-range of the hierarchical positions (Figure 9.4 & Figure 9.5A). As a result, in bud clusters within the Clipped-Control treatment, no correlation between the hierarchical position of a bud and the duration to reach developmental end-points, or changes in qualitative characteristics were evident (Figure 9.5). No treatment effect on shoot emergence was detected in the second growth cycle following application of GA_3 in the first growth cycle (Chapter 8). This lack of any obvious hierarchical relationship in shoot emergence was, therefore, observed in the second growth cycle, even on plants within the Clipped + GA_3 treatment. This difference between growth cycles in the bud positions which emerged, was expressed as bud Position 3 being the median hierarchical position which emerged in the first growth cycle, as compared with Position 6 in the second growth cycle, if not treated with GA_3 (Figure 9.4). Similar to previous studies (Chapter 3), the median date of appearance of these buds that emerged in the second growth cycle was later than that for all buds that appeared in the first growth cycle (Figure 9.8). Collectively, this evidence supports the hypothesis that buds that developed later in the season, i.e. positioned in the mid-range of the cluster stem, have more potential to emerge than buds that developed earlier or later. As previously postulated, if crown buds emerge primarily based on status of maturity (refer Section 9.5.1), or presence of apical dominance within the cluster, the buds located at the proximal end would have emerged first. In plants like *Rubus ideas* L. with both para and endodormancy co-existing, proximal buds were found to be more dormant compared to the distal buds (White et al., 1998), and this was proposed to be due to presence of inhibitory substances (Bredmose and Hansen, 1996). While it was not possible to determine the source of these inhibitory substances on crown buds at the proximal end during the present study, it was postulated that the source of paradormancy on the buds within the proximal positions, could possibly be from other axillary buds within the cluster (Zieslin and Halevy, 1976) and/or from the bud scales (Schneider, 1968). Inhibition from bud scales was not investigated in the

current study, but it was observed that the proportion of shoots emerging from a cluster was reduced if an individual cluster had greater numbers of crown buds, as with cultivar '03/04-114' (Appendix IV). It seems reasonable therefore, that a given crown bud could also be under paradormancy from other axillary buds within the cluster, in addition to that exerted by the apical bud, and any developing floral shoots.

In addition to alleviating endodormancy, the doubling of shoot emergence following application of GA_3 compared to chilling (Table 9.1), raises the question of whether GA_3 was also stimulating axillary crown buds within the cluster to emerge via alleviating paradormancy within the cluster. The ability of GA_3 to overcome paradormancy was previously noted when GA_3 was applied to plants of 'Diva' with no crown buds, resulting in outgrowth of axillary buds from floral shoots (Chapter 6). Additionally, when GA_3 was applied to plants with both developed shoots and unemerged crown buds (crown buds being paradormant, but not endodormant), these crown buds emerged as shoots (Chapter 8), potentially by breaking paradormancy (Ali and Fletcher, 1971; Horvath, 1999) exerted by the developing floral shoots. Based on the progressive response towards the apex of the cluster (Figure 9.5 & Figure 9.6), it could be hypothesized that for clipped plants, by application of GA_3, crown buds managed to overcome paradormancy imposed on buds at the proximal end of the cluster (i.e. from the rest of the axillary crown buds or bud scales), however not from the apical bud. The most mature buds within the cluster (Positions 1-3) however, emerged at the same time, potentially due to the lack of paradormancy over individual crown buds with increased distance from the apex, via reduced concentration of auxins (Cline, 1997) as discussed above. As found in *E. esula*, GA_3 partially overcame signals of paradormancy exerted by mature leaves, but not the apically derived signal (Horvath et al., 2003; Horvath et al., 2002). Based on what was found in gentians and other plant species, it was concluded for gentians therefore, that paradormancy exerted by the rest of the buds/bud scales and existing floral shoots, is mediated via GAs, but not the paradormancy from the apex.

Based on the evaluation of sources of paradormancy presented above, considering the full annual growth cycle, a crown bud could be under the influence of paradormancy by different organs, i.e. apical meristem of the cluster, other crown buds within the cluster, bud scales and, developing shoots. In order to completely remove the influence of paradormancy therefore, clipping of the developing shoots may not be sufficient, as it only relieves a single component of the sum total of paradormancy from different

sources which may be influencing an individual crown bud. Since the sources of paradormancy existing within the cluster were identified during the current study, future research will benefit from studying the degree of paradormancy existing within the cluster during the growth cycle. As a result, this will enable identification of potential strategies to manipulate paradormancy more effectively. Potentially, by determining a suitable methodology to remove the paradormancy exerted by the apical bud, which is embedded within the cluster, it will be possible to identify any paradormancy imposed by the rest of the axillary buds within a cluster.

As discussed above, the presence of paradormancy within the cluster influenced the emergence and subsequent growth and development of a particular crown bud in response to dormancy breaking treatments. As a result, even with endodormancy breaking treatments (GA_3 or chilling) a differential response was evident. Within the cluster, therefore, both paradormancy and endodormancy (Faust et al., 1997) seem to co-exist in 'Diva'. In contrast to endodormancy, paradormancy apparently exists throughout the growth cycle of 'Diva', although the primary organ responsible for the paradormancy varies.

9.5.3 Future directions

From a research perspective, the potential existence of paradormancy within the cluster (Section 9.5.2) limits the exact identification of endodormancy and paradormancy separately. Application of treatments to the intact bud cluster may not specifically reveal whether the differential response was due to paradormancy. As an alternative research strategy, utilising single isolated buds (e.g. *in vitro*) as an experimental unit, may provide a clear indication of shoot emergence in the absence of any paradormancy from the rest of the buds within the cluster ; (Dennis, 2003; Ghelardini et al., 2010; White et al., 1998). Removal of any bud scales within this experimental unit would reveal any inhibition from bud scales. Further to that, as hypothesized above (Section 9.5.1), differing maturity of buds could also be a reason for this differential response. If at the start of treatment application the number and level of development of buds at each position within the cluster were determined by dissection, experiments utilising single isolated buds would enable identification of the comparative growth potential of these buds devoid of paradormancy. Within these experiments, if any differences were encountered between crown buds, the influence of maturity status could be determined. Hence, to quantify levels of endodormancy and/or paradormancy within 'Diva', and to

determine any influence of maturity of crown buds, future experiments might benefit from comparing the response to chilling and/or GA_3 of both whole bud clusters and single isolated buds.

It has been suggested that, from an evolutionary perspective, the differential pattern of emergence of shoots may reduce competition for resources among individual shoots (Stafstrom, 1995). Factors such as exposure to light (Beveridge et al., 2003), which was determined by the spatial arrangement of buds (Ishii and Takeda, 1997) could naturally affect emergence of buds. For gentian plants like 'Diva', it is currently not understood therefore, whether the level of exposure of a crown bud to light, which will depend upon its hierarchical position within the cluster, may also have some influence over shoot emergence, e.g. causing only the buds in the mid-range within the cluster to emerge if not induced by dormancy breaking treatments (Figure 9.4). The potential involvement of exposure to light in controlling the spread in emergence therefore, requires further study and, with the use of single isolated buds, it will be possible to identify whether any differential exposure to light has an influence on shoot emergence.

In contrast to the previous study where chilling led to a proportion of shoot emergence similar to that achieved following application of GA_3 (Chapter 8), during the current study the proportion of emergence was 66% less when treated with chilling than with GA_3 (Table 9.1). Incomplete shoot emergence within the current study may be due to an inadequate number of chill units from the 5 weeks at 5 °C as compared to 6 weeks in the previous experiment. During the current study however, application of GA_3 (100 ppm) caused only 108% shoot emergence, as compared to 200% in the previous study (Chapter 8). As the plants in the current study were continuously exposed to long days and warm temperatures throughout three growth cycles, as compared with only the second growth cycle in the previous study, the non-attainment of a similar level of response between these two experiments may not be unexpected.

9.5.4 Position of new crown bud clusters

During the course of two growth cycles the apical bud of the cluster did not emerge and develop as a floral shoot, and the appearance of crown buds for the subsequent growth cycle most commonly occurred following reactivation of this apical bud within the existing cluster. In contrast, the development of new crown bud clusters on the transition zone was only noted infrequently. Thus the previous observation that the

location of most new buds arose associated with existing shoots (Chapter 3), can now be attributed to new crown buds being developed from within existing clusters, not new clusters. The current study however, was conducted under a long photoperiodic regime, which has been noted as being inhibitory to the crown bud formation process (Chapter 6 & 7). During the current experiment the initiation and development of any new crown bud clusters may, therefore, have been affected negatively. Since additionally there could also be a wide varietal difference in the number of crown buds and clusters (Chapter 5), the application of these findings to other gentian cultivars and growing conditions should be investigated.

9.5.5 Conclusion

Different sources and levels of paradormancy and endodormancy exerted on a crown bud vary based on the timing during the growth cycle, and the hierarchical position within the cluster. Not only the degree of paradormancy, but its components (from floral shoots, apical buds and, the other axillary crown buds) could change with duration of time within the growth cycle. Thus the developmental potential of a crown bud is determined by its hierarchical position within the cluster, and the stage in the growth cycle (which determines the external stimuli). The competence to respond to dormancy breaking treatments increased with decreasing position (maturity) of the bud, as evident by shortened days to emergence and anthesis in buds from the distal as compared to the proximal end following chilling and GA_3 treatments. The hierarchical position of the bud within the cluster therefore, influences the duration to achieve a particular developmental end-point, e.g. duration to harvest maturity, once endodormancy is broken. As a result, hierarchy of the buds within the cluster influence spread in timing of flower harvest maturity.

Chapter 10 General discussion

10.1 Introduction

The wide spread in time to harvest maturity of floral shoots, and the failure to develop an adequate number of crown buds during propagation, were perceived as problems for commercial production of cut flower gentians (Section 1.3). As explored within the current thesis, and discussed within Sections 10.2, 10.3 and 10.4 of this chapter, the plant and environment related factors that potentially influence these two problematic situations were examined.

At the commencement of the current study, the available literature relating to the growth and development of gentians was limited to one review article based on historical cultivars derived primarily from *G. triflora*, and without statistically validated data (Ohkawa, 1983). Information describing the types of buds, their anatomy, ontogeny and seasonal development in gentians was, therefore, developed (Chapters 2 - 5) to provide a basis for subsequent experiments (Chapters 6-9) aimed at identifying factors that may be influential at various developmental stages (Figure 1.7).

In this chapter, the stages within the developmental process (Figure 10.1) are discussed in relation to: the physiology of growth and development, influence of experimental treatments applied, commercial implications (timing, qualitative and quantitative effects) and, directions for future research (Sections 10.2 – 10.4).

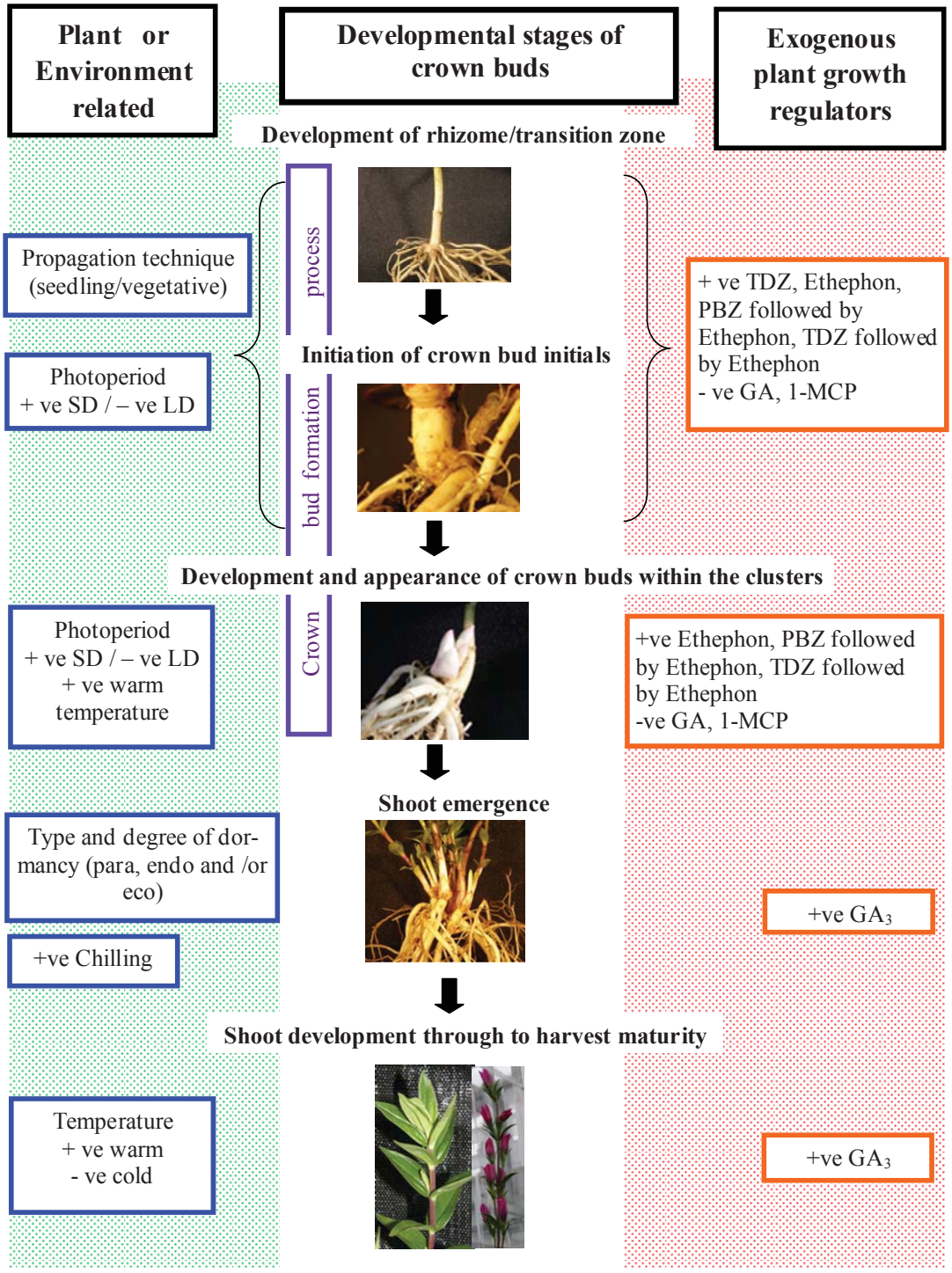

Figure 10.1 Determinants of the development of crown buds through to harvest maturity, their influence on the process, and potential manipulative strategies, for commercial cultivars of gentian. (+ve = promotes, -ve = inhibits or less promotive).

10.2 Crown bud formation process

Prior to the current research, the type (axillary/adventitious), origin, or even the existence of crown bud clusters, or multiple steps in the formation of crown buds, had not been reported in the literature. Following identification of this multiple step process, in the current thesis, the crown bud formation process became a central focus of this research (Figure 10.1). Since within any one experiment, data on all steps could not always be examined, the term "crown bud formation process" was utilised in an attempt to simplify reference to all steps collectively, without inferring any greater importance on one step over another.

10.2.1 Origin and ontogeny

This study determined that crown buds most commonly originate adventitiously from the transition zone, with clusters of buds subsequently derived from differentiation of axillary buds (Figure 5.22). Although the end product of interest for the horticulturist is the crown bud, its formation was identified as part of a multistep process; starting with the development of transition zone, initiation and development of adventitious bud initials, initiation of axillary buds from the axils of this adventitious bud, development of these axillary buds as individual crown buds (Figure 10.1; Section 5.5) and, possibly, acquisition of para and endodormancy (Section 9.5.2). In the first growth cycle however, in addition to the transition zone, it was acknowledged that bud clusters could also arise at nodes at the base of floral shoots or on storage roots, dependent upon either whether seedlings or *in vitro* propagated plants were used (Section 5.4.1.4 & 5.4.2.2), or the influence of photoperiod and/or exogenous plant growth regulators (Figure 7.14).

The crown bud formation process commences in the first growth cycle with the development of the transition zone, upon which bud initials for the crown bud clusters form. In terms of the crown bud formation process, following a period of dormancy, the second growth cycle begins with the apical bud of the existing clusters restarting development, with the continued initiation and development of axillary buds, leading to the appearance of new crown buds (Section 9.4.4). From the second growth cycle and beyond therefore, the crown bud formation process comprises initiation and development of axillary buds within existing clusters and, the development of new adventitious initials.

10.2.2 Factors controlling the crown bud formation process

A short photoperiodic regime was facultative in its promotion of crown bud formation, while exposure to different temperature regimes had no impact (Figure 6.2, Figure 7.3 & Figure 7.12). In contrast, the long photoperiodic regime promoted above-ground growth (Figure 6.2, Figure 7.11 & Figure 7.12), but not the crown bud formation process (Figure 6.2, Figure 7.11 & Figure 7.12). Under the long photoperiodic regime used in experiments, application of exogenous growth regulators, e.g. ethephon alone or in combination with other growth regulators, was able to achieve similar numbers of crown buds to that achieved under the short photoperiodic regime. In contrast, application of gibberellic acid (GA_3) inhibited the crown bud formation process, but promoted above-ground growth (Figure 6.2, Figure 6.4, Figure 6.5, Figure 7.3 & Figure 7.14). Application of exogenous growth regulators, which promoted the formation of crown buds on the transition zone under the long photoperiodic regime, led to development of meristems for bud initials from roots when applied under the short photoperiodic regime (Figure 7.3). As a result of this apparent interaction between the photoperiodic regime and exogenous growth regulators, it was hypothesized that the environmental stimuli (photoperiod) influences source-sink relationships within the plant, directly or indirectly, in association with endogenous hormones. In contrast to commonly reported interpretations of partitioning of assimilates between shoots and roots (Wilson, 1988), herbaceous perennials like gentians with a transition zone carrying meristems for regrowth in the next season (i.e. the resulting rhizome) have three organs involved in source and sink relationships, i.e. shoot, transition zone/rhizome and storage roots. During the current study, the mechanism by which growth regulators mediate the source-sink relationship leading to organogenesis was not investigated. It was not conclusive therefore, whether growth regulators stimulated accumulation of assimilates in a particular organ leading to formation of bud initials and clusters of crown buds, or whether bud initials were formed first within the organ, leading to an increase of sink strength (Marcelis, 1996) which caused assimilate partitioning. With future research, utilising histological evaluation of organs (shoot, transition zone and storage roots) and analysis of endogenous hormones, the physiological mechanisms that occur in response to external stimuli could be determined. This will also require analysis of assimilate partitioning within tissues potentially using a ^{14}C-labelled transfer technique (Lai et al., 1989; Mor and Halevy,

1979), so as to determine whether carbohydrate accumulation, or formation of bud initials, occurs first during the crown bud formation process.

As evident during the current study, a multitude of plant or environment factors could influence each step in the crown bud formation process differently (Figure 10.1). Initial steps such as formation of the transition zone and bud initials were promoted by thidiazuron (TDZ), ethephon or the short photoperiodic regime (Figure 7.3 & Figure 7.4). It is apparent that the final steps of the crown bud formation process, i.e. development and appearance of individual crown buds within the cluster, may be promoted by warm temperature (Figure 3.2 & Figure 3.3), short photoperiod (Figure 6.3, Figure 7.3 & Figure 7.12), ethephon (Figure 7.3 & Figure 7.4) and also by inhibiting GA activity (e.g. application of paclobutrazol (PBZ); Figure 8.8). With the understanding now achieved of the multiple steps and key factors influencing each step, analysis of endogenous growth regulators following transfer to different photoperiodic regimes (Horvath et al., 2006; Rohde and Bhalerao, 2007; Ruttink et al., 2007) could potentially improve the level of understanding of how growth regulators and their interactions mediate individual steps within the crown bud formation process.

During the current study the presence of endodormancy was noted (Section 3.5.4, 8.5.1.1 & 9.5.2), however what causes endodormancy was not identified as it was not the primary focus of the thesis. While chilling enabled breaking of endodormancy, the role of low temperature in the induction of endodormancy needs to be explored further, as plants growing in a greenhouse environment, and not exposed to low temperature had high mortality in 'Spotlight' (Chapter 3). The short photoperiodic regime and exogenous growth regulators (PBZ), that aided in the initiation and development of crown buds (Figure 7.14), are known to induce or increase the period of dormancy in some species (Masuda et al., 2006; Ruttink et al., 2007; Sumitomo et al., 2008; Tekalign and Hammes, 2004). Future studies examining the interactions between growth regulators and/or environment (photoperiod or cold temperature), and their impact on endodormancy will, therefore, potentially enhance understanding of whether the crown bud formation process and acquisition of an endodormant status are two separate or connected processes. If in this future research, instead of using a cultivar with a facultative requirement for cold to satisfy endodormancy, like 'Diva' (Section 8.5.1.1 & 9.5.2), a cultivar like 'Spotlight', which has an obligate requirement (Section 3.5.4), was used, it would enable a possible demarcation between the crown bud formation process

and endodormancy, as well as the requirement of chilling for induction or breaking of endodormancy.

10.3 Shoot emergence and development through to harvest maturity

10.3.1 Types of dormancy and factors influencing shoot emergence

Once they are formed, crown buds can develop into shoots at any time provided conditions for breaking dormancy (endo/eco/para) have been broken (Section 4.4.1, 8.4, 9.4.1 & Appendix IV), although this would normally occur in spring. As explored below, in gentians (Section 9.5.2 & 8.5.1) and other plant species (Faust et al., 1997; Horvath et al., 2003), the external stimuli needed to break dormancy depends upon the type(s) and degree of dormancy that a crown bud is subject to at a given time during the annual growth cycle (Figure 10.2).

Potential sources of paradormancy influencing a crown bud could be from the apex of the cluster, other crown buds within the cluster, and/or crown buds already emerged as shoots (Section 9.5.2). The fate of an individual crown bud however, depends on the position of that crown bud in the hierarchy at the beginning of a growth season, i.e. closer to apex or further away (as discussed later in this section). As illustrated in Figure 10.2, if a new crown bud is initiated in summer, it is immediately subject to paradormancy imposed primarily by any emerged shoots, i.e. shoots arising from crown buds in the same cluster that developed in the previous season (Table 4.1, Figure 4.2 & Appendix IV). As evident by the 100% shoot emergence of buds following clipping of previously emerged shoots, the inhibition of shoot outgrowth by other sources of para, endo or ecodormancy was negligible on new crown buds in summer/early autumn (Table 4.1). If stimulated to emerge in the same growth cycle in which they formed, these buds developed into floral shoots (Appendix IV) or rosettes (Figure 4.3).

If not stimulated to emerge during summer/autumn, a crown bud will gradually develop additional types of dormancy (Figure 10.2), i.e. endodormancy, additional forms of paradormancy due to other buds within the cluster (Sections 3.5.4, 4.5, 8.5.1 & 9.5.2), and may also be ecodormant (Section 3.5.3). Endodormancy can be broken by chilling, and the chilling requirement for shoot emergence can be substituted by exogenous GA_3 (Figure 8.3 & Table 9.1). During this period (i.e. late autumn to winter), as the floral shoots of the season start to senesce naturally, the dominant sources of paradormancy are from the apex of the cluster, and the buds within the cluster, as an individual cluster

consists of an increased number of crown buds by this stage (Section 9.5.2). Following release of endodormancy, the time taken for a crown bud to emerge and reach a developmental stage increased with increasing hierarchical position from the proximal to distal end of the cluster (Figure 9.5). This was hypothesized to be due to apical dominance, and/or the variable degree of maturity of buds along the cluster (Section 9.5.2). If dormancy breaking treatments were not applied, this hierarchical response was not evident, instead only crown buds located in the mid-range of hierarchical positions emerged (Figure 9.4 & Figure 9.8). This modified response was hypothesized to be due to the influence of paradormancy from both the apex and the other buds within the cluster (Section 9.5.2). In contrast to crown buds located closer to the apex or further away therefore, crown buds located at the mid-range in the hierarchy within a cluster, have the potential to emerge under any circumstances (Figure 3.6 & Figure 9.8), irrespective of dormancy breaking treatments. Some of the unemerged crown buds located at either the proximal or distal end of the cluster, may emerge in spring following clipping (Section 4.4.1 & Appendix IV) or application of GA_3. These crown buds are however, predominantly under paradormancy imposed by developing floral shoots, as chilling does not result in their emergence (Figure 8.6 & Figure 8.9). As a possible survival mechanism, the unemerged crown buds that remain dormant provide an opportunity for plants to survive if the growing shoots are lost in spring, perhaps to browsing or weather extremes. If not stimulated to emerge during that growth cycle they decay (Section 3.5.1), and the new crown buds that are initiated from the apex provide meristems for the subsequent growth cycle (Figure 10.2) and, therefore, survival.

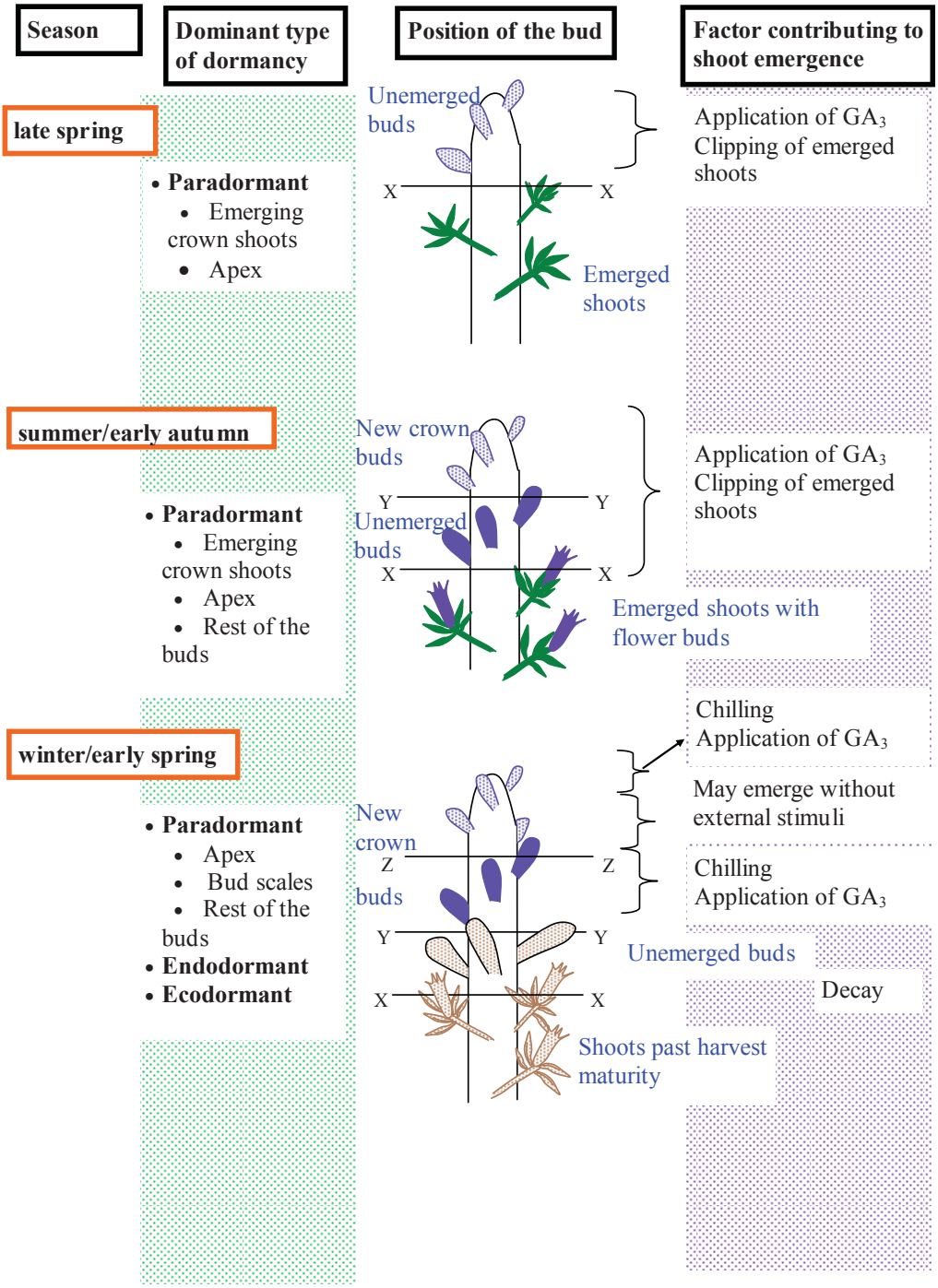

Figure 10.2 Potential external stimuli breaking dormancy in crown buds, depending upon the dominant type of dormancy exerted over a crown bud, its hierarchical position within the crown bud cluster, and time within a single growth cycle/season.

Due to the simultaneous existence of both paradormancy and endodormancy (Section 9.5.2), it was difficult to differentiate the specificity of response to chilling or GA_3 for a given type of dormancy, and/or determine the potential influence of bud maturity. With the new understanding of types of dormancy controlling the emergence of gentian crown buds developed in this thesis, it will be possible to study the type and degree of dormancy in isolation within the crown bud cluster as an individual crown bud differing in their position, as proposed within Chapter 9 (Section 9.5.3). The differential response between buds may also be due to a difference in capacity to attract assimilates (Bonhomme et al., 2010; Le Bris et al., 1998; Naor et al., 2004), based on sink strength. With the distance between source and sink important in some plants in determining their growth and development (Cieslak et al., 2011; Guo et al., 2011; Lai et al., 1989), carbon allocation between crown buds or vascular orthostichy within the cluster, with or without application of dormancy breaking treatments, will indicate how this translocation takes place relative to the hierarchy of buds within the cluster or level of their maturity. As noted within the preceding discussion, this research into assimilate translocation would be assisted by using a ^{14}C-labelling technique.

The response to GA_3 in promoting emergence of both crown buds (i.e. axillary buds within a cluster) at a wide range of developmental stages (Figure 8.3, Figure 8.6 & Figure 8.9), and axillary buds on floral shoots (Table 6.1 and Figure 6.4), suggests a role of GA_3 in overcoming both endo and paradormancy (Section 9.5.2). Although frequently reported as having a role in breaking endodormancy, the involvement of GA_3 in overcoming paradormancy is less well documented, e.g. breaking paradormancy imposed by leaves (Horvath, 1999), or indirectly via cell elongation following breaking of dormancy by cytokinins (Ali and Fletcher, 1970). As evident by the gradient in shoot emergence from proximal to distal ends of the cluster during the current study (Figure 9.5), GA_3 was not involved in apical dominance, however gibberellins appeared to alleviate paradormancy from the rest of the buds within the cluster, and the developing floral shoots. While this was hypothesized to be due to paradormancy mediated by abscisic acid (ABA), further studies are required to identify the exact mode of action (Section 9.5.2).

In addition to GA_3, the promotion of shoot emergence by ethephon/ethylene so soon after the formation of crown buds (Figure 7.6, Figure 7.7, Figure 7.9 & Figure 7.10), infers roles for ethylene in both the crown bud formation process and shoot emergence.

Although there are few published articles on the role of ethylene in initiation and development of adventitious buds (Van Aartrijk et al., 1985), ethylene is known to be involved in paradormancy (i.e. development of axillary buds (Yeang and Hillman, 1984)) and endodormancy (Sumitomo et al., 2008; Suttle, 2004). Reports documenting the involvement of ethylene in paradormancy were also limited. As discussed in Chapter 7 (Section 7.5.4), due to the involvement of ethylene in both induction and breaking of dormancy (Prange et al., 1998; Sumitomo et al., 2008; Suttle, 2004), and its involvement in other plant functions (Tonini et al., 2010; Van Aartrijk et al., 1985), it is questionable however whether early shoot emergence is a direct influence on breaking any particular type of dormancy or a separate phenomenon. With the understanding acquired during the current study about GA_3 and ethylene contributing to diverse roles in gentians, at different stages during the developmental process, the mechanisms of action of ethylene or GA_3 within these roles, and how their efficacy varies (Firn, 1986), can be further investigated as discussed in Sections 7.5.4, 8.5.1.2 & 9.5.2.

10.3.2 Shoot development post emergence

The factors that influence the growth and development of plants subsequent to emergence (Figure 10.1), may also influence the spread in time to flower harvest maturity. This was found to be cultivar dependant. As explored within the current research, cultivars varied in response to temperature during shoot emergence (Table 2.2, Table 2.3 & Figure 2.3C), as well as during subsequent shoot development through to harvest maturity (Table 2.2, Table 2.3, Figure 2.3A & Figure 2.3B). The cultivar 'Starlet' was more responsive to temperature during shoot development, with delayed harvest maturity due to cold temperature during autumn, while the phenotypic plasticity evident in 'Diva' meant that the duration to harvest maturity was more compact, irrespective of the date of emergence (Table 2.1 & Figure 2.3). In contrast, shoot development of the early-season flowering cultivar 'Spotlight' was not influenced by temperature during shoot development (Figure 2.4).

Cultivating 'Spotlight' in a warm growing environment following emergence enabled production of floral shoots which were almost twice as long, without affecting duration to harvest (Table 3.1). 'Spotlight' flowers relatively early in the season, but the existence of a possible promotional influence of high temperatures on shoot development was not investigated in late flowering cultivars, i.e. those with the potential for exposure to decreasing temperatures in autumn. With differences in

temperature response noted between cultivars, in order to develop grower-orientated strategies to manipulate harvest maturity, it will be beneficial to know the optimum temperatures for shoot emergence and development for each cultivar.

The response to chilling and/or GA_3 on shoot development, varied based on the developmental stage of the plant at the time of treatment application (Sections 8.4.2, 8.4.3 & 8.4.4), and the hierarchical position of crown buds within the cluster from which the shoots arose (Section 9.4.2). If following the start of shoot emergence (i.e. when crown buds are paradormant and/or partially endodormant), exposure to chilling had no influence on either duration to harvest maturity or shoot length (Sections 8.4.3 & 8.4.4). If chilling was encountered prior to emergence (fully endodormant) however, the duration to harvest maturity was reduced by 28 days (Figure 8.3B), shoot length increased by 20 cm (Figure 8.3), and production of floral shoots increased up to eight-fold (Figure 8.3A). GA_3 could substitute for chilling, except for any influence on shoot length (Figure 8.3). Unlike chilling however, GA_3 improved production of floral shoots (Figure 8.6A & Figure 8.9A) and shoot length (Figure 8.9B), even if applied when shoot emergence had started. Due to this differential response to treatments based on status of shoot emergence of a plant, the number of shoots produced, their quality, as well as their duration to harvest, was dependent upon the stage of development at which treatments (chilling and/or GA_3) were applied. As evident by decreasing the shoot length (Figure 9.6A) and number of nodes (Figure 9.6A), as well as an increase in duration to harvest (Figure 9.5B), i.e. from buds positioned from the distal to the proximal end of the cluster, shoot development was also influenced by the hierarchical position of a bud within the cluster. This hierarchical response was evident despite chilling or GA_3 being applied. The potential commercial implications of shoot development in response to treatments with chilling and GA_3 are discussed further in Sections 10.4.2 and 10.4.3.

While flower initiation was not the primary focus of this thesis, the different responses between cultivars to cold raises the possibility that future research might utilise such differences to investigate what if any role vernalization may have in members of this genus. While some cultivars displayed a facultative requirement for chilling, a requirement for vernalization *per se* was not found (Section 8.5.1 & Appendix IV), despite some cultivars with an obligate requirement remaining as a rosette if not exposed to chilling (Section 3.4.7 & Figure 4.4). If the role of chilling or GA_3 was

investigated relative to shoot emergence and development in a cultivar with an obligate requirement for chilling, e.g. 'Spotlight' (Section 3.5.4 & 4.5), any requirement for vernalization would be more readily identified by applying these cold treatments when not endodormant (e.g. as proposed in Section 4.5). The presence of a greater number of floral nodes following exposure to chilling (Table 9.2) however, infers there could be an influence of temperature on flower initiation, even in cultivars with a facultative requirement for chilling. Although the environmental signals, if any, associated with flower initiation, or the relationship between floral initiation with duration to harvest maturity, have not yet been identified, the genes responsible for floral initiation and differences in gene expression based on timing of floral initiation between gentian cultivars, have been reported (Imamura et al., 2011). If in the future gene expression was analysed, within the context of environmental changes (chilling and/or photoperiod) and diverse cultivar responses (such as 'Spotlight' and 'Diva'), it might be possible to identify what causes flower induction, and whether there are any differences evident between cultivars.

10.4 Potential commercial implications

10.4.1 An inadequate number of crown buds

One of the broad horticultural goals of the current thesis was to identify factors influencing the crown bud formation process, with a view to overcoming the present failures in crop establishment when using vegetative propagules. In the current thesis the inability to survive and regenerate was hypothesised to be dependent upon failure of the development of a transition zone on which bud initials are initiated (Section 5.5). If so, any failure or delay in development of the transition zone, and in initiation of bud initials, leads to an inadequate number of crown bud clusters. As noted above (Section 10.2), the present study provides the means of manipulating the number of crown buds arising from multiple locations (shoots, transition zone/rhizome, roots) and their growth and development, via photoperiodic or exogenous growth regulators (Figure 7.14). As photoperiodic changes were the main environmental stimulant, as compared with temperature, future research should aim to determine the optimum photoperiodic regime and, therefore, the optimum time of year/conditions to establish plants for propagation during the growing season.

Even if a short photoperiodic regime was not available, application of exogenous growth regulators provides a means of early initiation of crown buds of greater quality. This was illustrated most clearly by treatment with ethephon, since a single application led to development of a greater number of individual crown buds and clusters under the inhibitive long photoperiodic regime (Figure 6.5, Figure 7.3 & Figure 7.14). Persistent qualitative effects of shoots in the next growth cycle were achieved by application of ethephon in combination with PBZ or TDZ (Figure 7.4, Figure 7.14 & Table 7.4). The use of a combination of exogenous growth regulators, such as PBZ followed by ethephon or TDZ followed by ethephon, has not previously been reported to effect initiation of crown buds in gentians or other species. These combinations however were found to be very effective as evident by up to 6 more crown buds and shoots than within the Control treatment (Figure 7.3 & Figure 7.4). Further studies are required, so as to optimise dosage and timing of application of these exogenous growth regulators (TDZ, PBZ and ethephon) or their combinations.

Other than *in vivo* techniques utilised in the current thesis, it may also be possible to overcome the poor plant establishment following deflasking by addressing this issue *in vitro*, by promoting formation of a storage organ with bud initials prior to deflasking, as achieved with *Watsonia* spp (Ascough et al., 2008). If so, it is likely to be possible to achieve this using photoperiodic control and exogenous growth regulators that were identified during the current study (Figure 7.14 & Figure 10.1).

10.4.2 The variation in spread of flower harvest maturity

The current study enabled identification of sources of variability associated with the duration to harvest maturity, at the levels of; cultivars, individual plants and, individual crown buds. As the sources of variation, and the extent of influence, varied between cultivars, e.g. ‘Starlet’ (41 days), ‘Diva’ (35 days) and ‘Spotlight’ (29 days), the commercial strategies to alter the variation need to be determined based on the most causative factor(s) for each cultivar. While temperature during shoot development was not a primary cause that affects the spread in ‘Diva’ (Figure 2.4; Section 2.5), due to a wider spread in shoot emergence (Figure 2.3C) identifying strategies to reduce the spread in shoot emergence was considered important for this cultivar. With ‘Starlet’ however, it will be important to implement strategies during both shoot emergence and development (Section 2.5, Figure 2.3C & Figure 2.4), as discussed in Section 10.3.2. The spread in time to harvest maturity in ‘Spotlight’ however, was primarily due to

factors intrinsic to the plant itself (Section 2.4.2.; Figure 2.5). In the following paragraphs, potential strategies to narrow down spread in these cultivars will be discussed, based on shoot emergence and development, and factors intrinsic to a plant such as hierarchical development of buds within a cluster.

Shoot emergence

With the cultivars presenting a facultative or obligatory requirement of chilling for shoot emergence (Section 3.5.4, 8.5.1.1 & 9.5.2), having adequate chilling prior to emergence is a key determinant in reducing spread in time to harvest maturity. As discussed previously within Section 10.3.2, cultivars with a facultative requirement displayed wider spread in flowering (e.g. 'Diva') compared to the cultivar with an obligate requirement, e.g. 'Spotlight' (Figure 2.3). The warm temperate climate in New Zealand (NZ) however, could cause problems in cultivars with both obligate (low or delayed shoot emergence) and facultative (wide spread in flowering) requirements for chilling, due to the potential exposure to an inadequate number of chill units. Although shoot emergence could be early in a cultivar with a facultative requirement (e.g. 'Diva'), greater shoot emergence (Figure 8.3A & Table 9.1), reduced duration to harvest maturity (Figure 8.3B), and a narrower spread, was achieved when exposed to the longest duration of chilling prior to emergence (Figure 8.5 & Figure 9.3). Inadequate chill units could, therefore, be one of the reasons for the wider spread in the duration to harvest maturity reported by growers within the commercial industry. In a situation where chill units are inadequate however, GA_3 could substitute the requirement to achieve a similar effect on spread in time to harvest maturity as normally achieved with full chilling (Figure 8.5 & Figure 9.3). While, during the current study, the chilling requirement was primarily determined based on one cultivar, i.e. 'Diva', an assessment for other cultivars in the future, could be used to identify management strategies specific for each.

Shoot development

Cultivars displayed different responses to temperature during shoot development (Table 2.1, Table 2.2, Figure 2.3 & Figure 2.4). As evident by positive correlations between duration to shoot emergence and harvest maturity in late flowering cultivars, i.e. 'Starlet' (Figure 2.4), the declining temperature during shoot development in autumn also contributed to the spread in time to harvest maturity. Promoting early shoot

emergence through chilling and/or GA_3 (Section 10.4.1), or cultivating in a warm growing environment (Section 10.4.2.), are potential strategies to address this.

Hierarchical arrangement

While the above factors influence the spread, variation in duration to harvest maturity was also observed between individual crown buds within a bud cluster. As identified in the current thesis, crown buds located at the distal end took a longer duration to reach harvest maturity (Figure 9.5B). This was evident by 15% of the shoots harvested originating from buds at the distal end, leading to an extension of the spread in harvest by one third in 'Diva'. This positional effect however was observed only when treatments to break endodormancy (Chilling or GA_3) were applied. While for 'Diva' there is a contribution to the spread (10 days) from these buds located at the distal end (Section 9.5.1), since chilling or GA_3 was capable of narrowing the spread in time to harvest maturity (Figure 8.5A) by 83 or 63 days, the contribution to spread from the hierarchical position of a bud can be considered to be less significant than that resulting from inadequate chilling. As compared with 'Diva', 'Spotlight' however had 6 times more late maturing shoots contributing to the spread (Section 2.4.1.4. and 2.4.2.). Based on the conclusions presented within Chapter 9, it could be hypothesized that in 'Spotlight' these shoots developed from crown buds located at the distal end. In future research therefore, it will be important to investigate the strategies to control the spread within a cluster in cultivars like 'Spotlight', as the within plant contribution to variation to the spread in harvest maturity, was greater in this cultivar (Section 2.5).

Plant to plant variability

Despite the differences in variation in time to harvest maturity between cultivars, the lack of plant to plant uniformity cannot be overlooked in clonally propagated plants of gentians. This was evident in 'Diva' with an average spread in duration to harvest maturity of 25 days, with 16 days spread between plants (Figure 2.5; Section 2.4.2). Although not quantified during the current study, this could potentially be due to plants with a variable number of clusters at variable stages of development (i.e. presence of clusters initiated in different growth cycles), as well as a variable number of crown buds within clusters (Chapter 6 and 7). The successful establishment of an adequate number of high quality crown buds earlier in the season, while maintaining uniformity among plants, would be the first step in narrowing the spread in duration to harvest maturity.

The current study provides the means of addressing this issue in the future, via manipulation of the crown bud formation process. Further studies are required to understand how uniformity and early initiation of crown buds in their first growth cycle, may contribute to the spread in time to flower harvest maturity in subsequent growth cycles.

10.4.3 Overall floral productivity

As explored within the current thesis, numerous factors influencing the plant at initiation, development, and emergence of shoots, could influence the timing, quality and quantity of floral productivity (Figure 10.1). Within a commercial situation, if adequate environmental stimuli are not present, the identified roles of exogenous hormones on the crown bud formation process, shoot emergence, and development, will enable an improved level of manipulation of crown buds and floral productivity. As explored within the current thesis, the potential for timely formation of high quality crown buds through exogenous application of ethephon, or PBZ/TDZ followed by ethephon (Section 10.2), and the stimulation of emergence of these crown buds as shoots by application of GA_3 (Section 10.3.1), provide a means of achieving this.

As discussed in Section 10.3, the application of GA_3 increased the number of floral shoots irrespective of the developmental stage of the plant, while chilling resulted in a greater number of shoots as well as increased shoot length, but only if applied prior to emergence (Figure 8.3A, Figure 8.6A & Figure 8.9A). Greater shoot length however could not be achieved by GA_3 when applied prior to emergence and, therefore, application of both chilling and GA_3 appear to be beneficial in achieving both quality and quantity of floral shoots. With further improvements of the treatments identified during the current research, therefore, growers will have some ability to manipulate quantity, quality and/or timing of floral shoots required by the market.

Within a commercial situation, another potential problem is that low temperatures may occur after shoot emergence. As evident by 25% survival during the current study, plants were extremely susceptible to low temperatures experienced immediately after shoot emergence, i.e. after completing the chilling requirement for endodormancy (Section 8.5.3). Within a commercial situation in NZ, this could occur in spring. Additional to the plant death during establishment in the first growth cycle (Section 1.3.2) therefore, the plant death observed in mature plants in commercial fields by the

growers could be due to plants getting exposed to low temperature in spring after shoot emergence has started. In fact, having a mild facultative requirement in a cultivar like 'Diva', could be a disadvantage under such circumstances, because crown buds will emerge earlier in the spring (Table 2.1 & Figure 2.3C), when the likelihood of periods of damaging cold temperature still remains high.

Plant functional and architectural models enable understanding of plant-environmental interactions (Guo et al., 2011). Multiple factors, both plant and environmental related, influence growth and development of gentians, based on different developmental stages (Figure 10.1). These factors could be used in future research to identify the parameters to develop a growth model for gentians, as in other species (Graefe et al., 2010; Vos et al., 2010). With extension of such studies, it will be possible to integrate causative factors of yield with components such as quality, as well as duration to harvest maturity, and its spread in gentians. This could be undertaken as a whole plant or on the basis of individual buds within the cluster. Due to the wide genotypic differences however, it will be crucial to implement this research strategy on multiple cultivars.

10.5 Conclusion

The current study enabled establishment of a detailed base of statistically validated data relative to the developmental process of gentians, which was lacking previously (Figure 10.1). As per the objectives of the current thesis, an understanding has now been achieved of both plant and environment related factors influencing different developmental stages within this process. This information will enable resolution of the problematic situations examined, i.e. establishment of adequate number of crown buds, and control of the spread in time to harvest maturity. Such strategies could be based on utilising the natural environmental changes, or any lack of availability of environmental stimuli could be substituted with the exogenous growth regulators identified. The limitations of available time prevented identifying the optimum degree of the required environmental stimuli, dosage of exogenous growth regulators, or the internal mechanisms of some physiological phenomena. The current study therefore, presents an array of opportunities for future research. These future avenues do not necessarily need to be aimed at gentians, as these research approaches could be applicable to many other perennial plant species. Hence, while providing a knowledge base which will be valuable for gentians scientifically and commercially, the present study pinpoints gaps

in existing research pertaining to ecophysiology and hormonal physiology of perennial plant species.

Appendices

Appendix I; Stages of flower bud development and macroscopic evidence for flower initiation in 'Spotlight'

Background, objectives and methodology

Under growing conditions in New Zealand (NZ), and with the recent new varieties, how the timing of the various developmental phases relate to the annual growth cycle, was identified as being unknown for gentians (refer Section 1.2). So that it could be utilized in experiments targeting the timing of flower initiation, identification of when flower initiation had occurred, via indirect morphological features (macroscopic), was considered to be a potentially useful tool. If successful, it would conserve valuable plant material, as well as being easier than using dissection or any other microscopic techniques during these subsequent experiments. In order to develop such an experimental tool, the morphological features of flower bud development at different stages needed to be described, so as that they could be related to the corresponding macroscopic features.

In a floral shoot, flowers are produced in apical, as well as in some axillary buds below the apex. Thus the apical buds and axillary buds of floral shoots of 'Spotlight' were sampled, and dissections were conducted to identify;

- the steps and corresponding morphological changes in the process of flower bud development, and
- the earliest macroscopic evidence corresponding to flower initiation.

Results & Discussion

Stages of flower bud development

Figure 1. Stage 1 of flower bud development within the apical bud of a floral shoot of 'Spotlight'; vegetative apex of a shoot with approximately 10 leaf pairs visible, but with no signs of flower initiation. Two leaf primordia can be seen on the opposite sides of the apical dome (40×).

Figure 2. Stage 2 of flower bud development within the apical bud of a floral shoot of 'Spotlight'; (A) Shoot tip with apical bud covered with leaves and showing axillary buds at different stages of development (30×), (B) Outer leaves covering the apical bud (40×), (C) Two inner leaf primordia with developing sepal primordia (40×) and, (D) Five sepal primordia (40×).

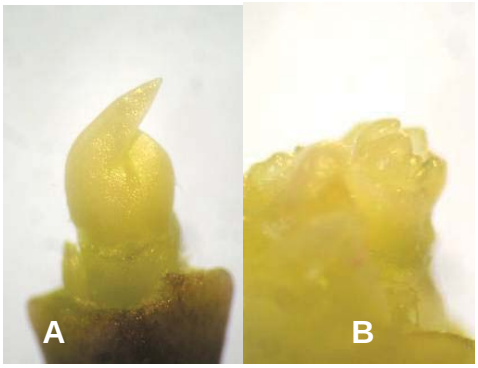

Figure 3. Stage 3 of flower bud development within the apical bud of a floral shoot of 'Spotlight'; (A) Uneven development of sepals (40 ×), (B) Development of five petal primordia (40×).

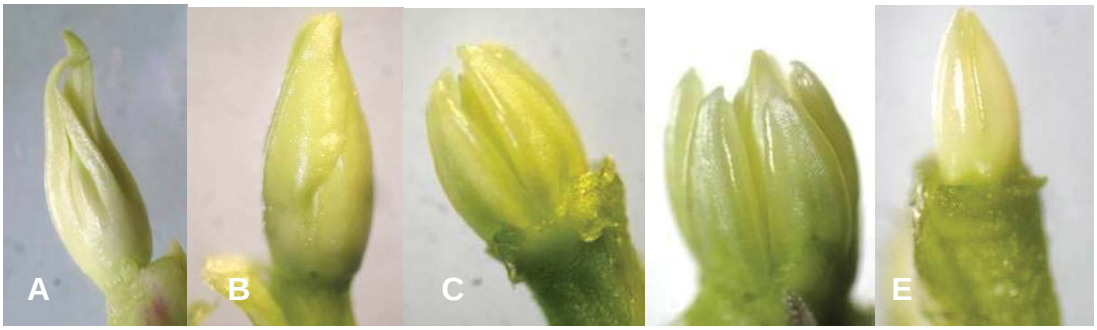

Figure 4. Stage 4 of flower bud development within apical bud of a floral shoot of 'Spotlight'; (A) Elongated sepals (30×), (B) Elongated petals (40×), (C) Development of stamens (40×), (D) Five Stamens with carpel in the middle (40×), (E) Two elongated carpals (40×). (All parts of flower bud were visible within the apical bud)

Figure 6. Stage 5 of flower bud development of a floral shoot of 'Spotlight' with flower buds visible at both the apex and upper leaf axils.

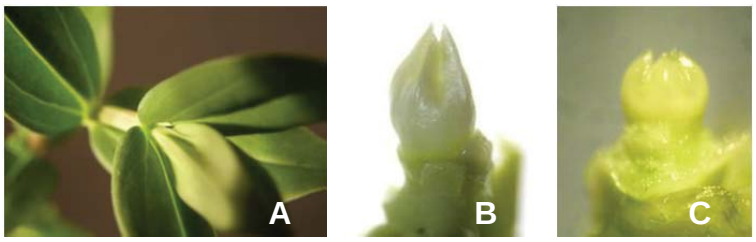

Figure 7. Tip of a floral shoot of 'Spotlight', (A) with first macroscopic evidence of appearance of an axillary bud, corresponding to the presence of, (B) sepal primordia and, (C) petal primordia, within the apical bud.

Dissection of buds at the time of first visible appearance of axillaries

Axillary buds started to become macroscopically visible at the distal end of the floral shoots of 'Spotlight' during November to December (Figure 6). The appearance of axillary buds were first observed at nodes, not necessarily those immediately closest to the apical bud. These axillary buds were at a stage of flower development from 1 through to 3 (Figures 1 through 3) at the time of their first macroscopic appearance.

At the time of first macroscopic evidence of axillary buds (Figure 7A), the apex of the floral shoot was found to be at either stage 2 or 3 (Figure 7B & C). As flower initiation had, therefore, already started in these shoots, it was concluded that the appearance of axillaries can be used as an indication that flower initiation has commenced in a shoot. As such, this macroscopically visible stage of development, which occurs well before any actual flower bud was visible, was used as a guide in experiments presented within the current thesis. The morphological descriptions of floral developmental stages described in this appendix, were similar to those reported for *Eustoma grandiflorum (*Raf.) Shinn., (Islam et al., 2005) which is also a member of the *Gentianaceae*.

Appendix II; Plant to plant variability in number of crown buds

Based on a sample of plants utilized for the experiments reported in Chapter 3, the greater the number of buds per plant observed prior to shoot emergence, the greater the number of shoots that emerged (Figure 1; $P < 0.001$, $R^2 = 0.94$). The number of crown bud clusters varied between plants, and those plants with multiple clusters typically comprised a greater number of buds that developed into thin, short shoots (Figure 2). In contrast, plants with fewer shoots, which emerged from a single crown bud cluster, had thicker and longer shoots.

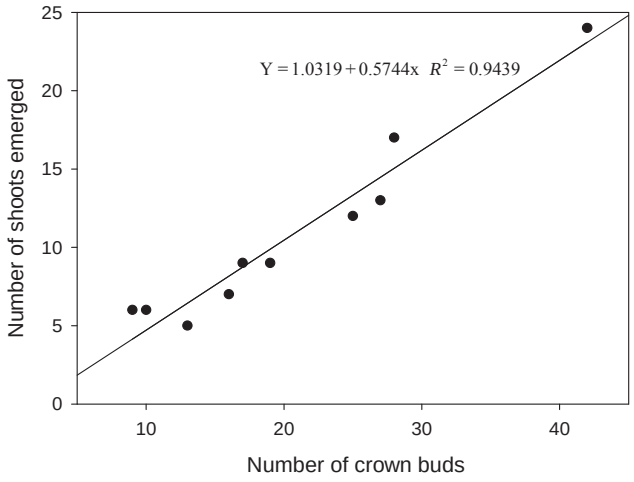

Figure 1. Correlation between the number of crown buds visible within a plant of 'Spotlight' prior to shoot emergence, and the number of shoots emerged. n = 10.

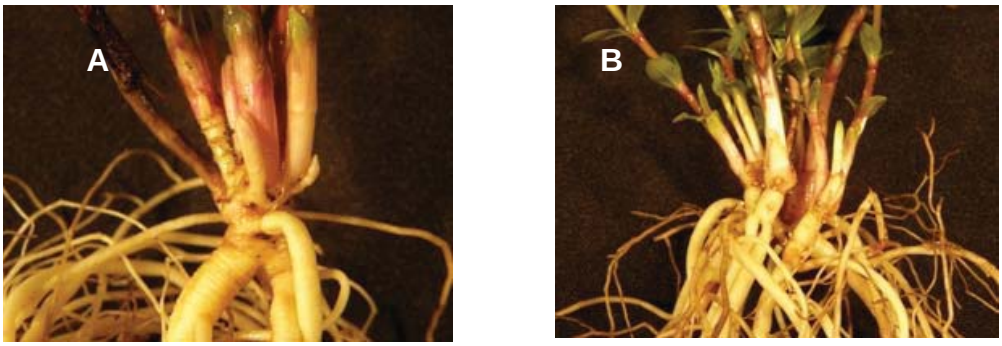

Figure 2. Crown region following shoot emergence of a plant of 'Spotlight' with; (A) one crown bud cluster and, (B) multiple crown bud clusters.

Appendix III; Morphological features of seedlings of parental lines

Seedlings of the parental species of the varieties of gentian used in the current thesis were managed as per Section 5.3.1.1, and were assessed in their first and second growth cycles.

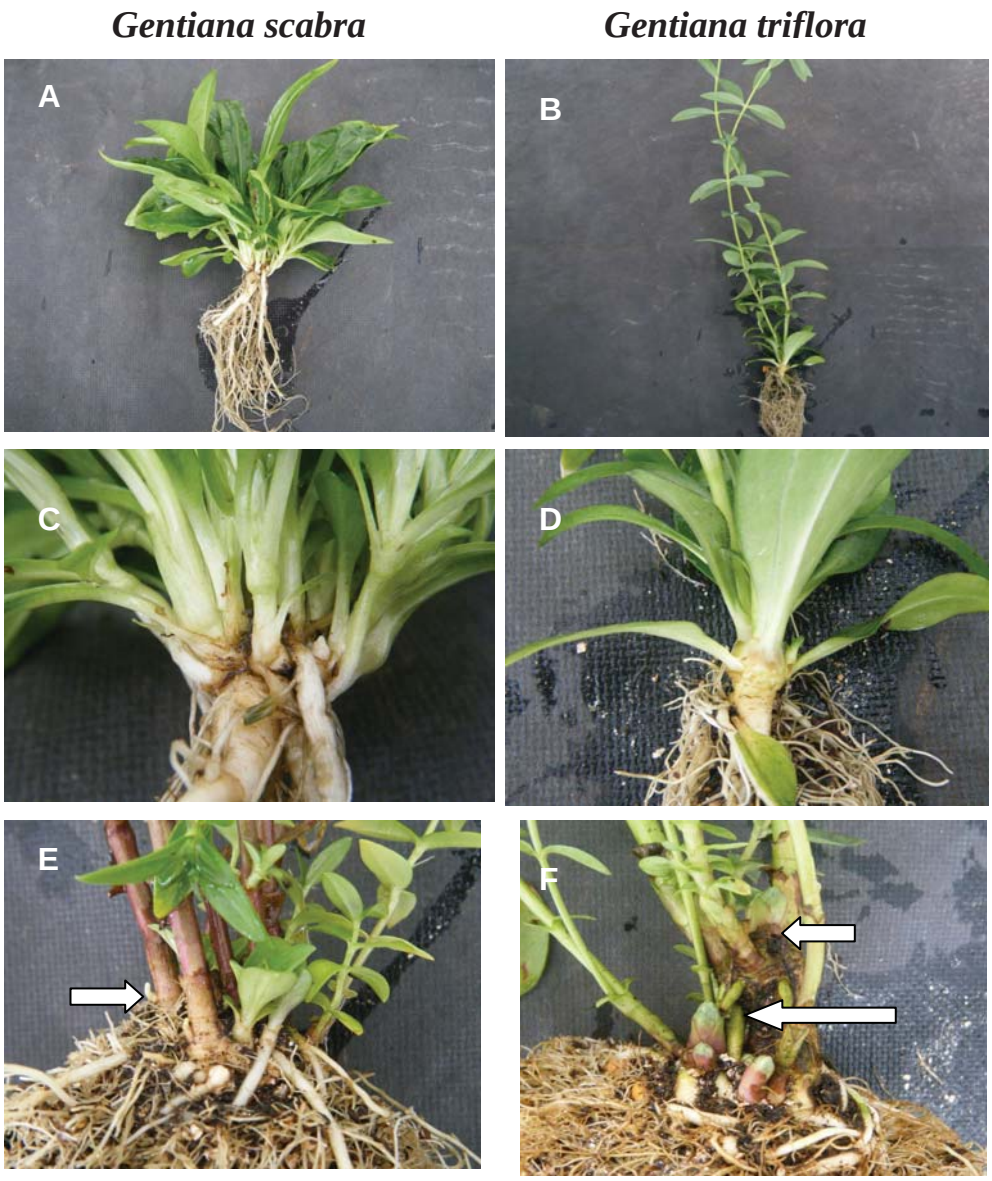

Figure 1. Morphological features of seedlings of *Gentiana scabra* and *Gentiana triflora*, (A – D) in the first growth cycle and (E & F) in the second growth cycle. (A & C) whole plant and crown of *Gentiana scabra* with multiple (10-15) shoots and, (B & D) of *Gentiana triflora* with 1-3 shoots. (E & F) crown buds (white arrows) evident during the second growth cycle.

Appendix IV; Shoot emergence and development as influenced by clipping, chilling and GA_3 in genotype '03/04-114'

Background, objectives and methodology

This experiment was originally designed to determine if there was a requirement for vernalization for gentian genotype '03/04-114'. In doing so it was designed to quantitatively describe the impact of both chilling and GA_3 on the crown shoots arising from clipped plants (i.e. paradormancy removed). The experiment was designed following the observation within Chapter 3 that plants that were clipped formed rosettes in the absence of chilling, i.e. an indication that vernalization might occur in gentians. Regrettably genotype '03/04-114' developed floral shoots without forming rosettes, even in the absence of chilling, therefore this experiment has been relegated to an Appendix.

Plants of '03/04-114' were propagated as per Section 6.3.1, prior to transferring outside for one growth cycle. Plants with unemerged crown buds were selected and repotted at the beginning of second growth cycle (13th October 2008), into black polythene bags (3.4 L) using the growing medium as described in Section 6.3.1. Throughout the experiment plants were grown in a greenhouse (heated at 15 ºC, ventilated at 20 ºC) and managed under an irrigation system as per in Section 6.3.1. When the original shoots were beginning to indicate the presence of floral axillaries (19th December 2008), all the shoots were either clipped, as described in Chapter 4 (refer Section 4.3.2.), or not clipped (i.e. non-clipped control). When new shoots subsequently emerged (20th January 2009), plants within both clipping treatments were allocated to chilling (5 ºC) treatments of 0, 7 or 35 days. Three days following the completion of each chilling treatment, plants were sprayed to run-off with either 100 mg l^{-1} GA_3 or water. So as to study any carry over effect of treatments into the next growth cycle, all shoots were clipped (i.e. a second clipping) at their base on 4th August 2009 i.e. at the beginning of third growth cycle. The experiment was conducted as a CRD utilising seven single-plant replicates.

Results

Following the first clipping treatment, shoots emerged and elongated in all plants within three weeks, i.e. without developing rosettes. In contrast, plants within the non-clipped control treatment did not show evidence of any new shoot emergence at that time. Of the shoots which emerged from plants which were clipped, 92 ± 5% flowered, and no difference was observed between treatments ($P > 0.05$). By the end of second growth cycle plants treated with 35 days of chilling had reduced survival rates with (42%), or without (i.e. 28%) application of GA_3 (while all other treatments had 100% survival following clipping. The number of crown buds at the beginning of third growth cycle was however 7-16 more within the remaining plants of the treatment with 35 days of chilling than all other clipped treatments (Table 1). Shoot emergence in the third growth cycle following second clipping, was also greater in the treatment with 35 days of chilling than rest of the treatments. Plants within the control treatment which had two clippings (Clipped control) displayed twice the number of shoots as compared with the treatment which had a single clipping (Non-clipped control). Between the plants that had the first clipping treatment, all plants that were not applied with GA_3 displayed 3-10 less shoots, as compared with plants treated with GA_3.

Table 1. Numbers of visible crown buds per plant before the second clipping, and the subsequent shoot emergence in the third growth cycle following treatment application prior to second growth cycle.

Treatment	Crown buds present prior to emergence	Buds emerged as shoots
non-clipped control	15 ± 1^{ab}	5 ± 1^{c}
clipped control	12 ± 2^{bc}	10 ± 1^{b}
7 days of chilling	8 ± 1^{c}	7 ± 1^{bc}
35 days of chilling	19 ± 3^{a}	14 ± 3^{a}
7 days of chilling + GA_3	10 ± 2^{bc}	1 ± 1^{d}
GA_3	6 ± 1^{c}	4 ± 1^{cd}

[Z] For each parameter means indicated by different letters was significantly different. Mean separation by DNMRT at $P < 0.05$

Appendix V; Fresh weight of crown buds in response to photoperiodic regime, growth regulators and their interactions

Figure 1 presents additional results from Experiment One within Chapter 7.

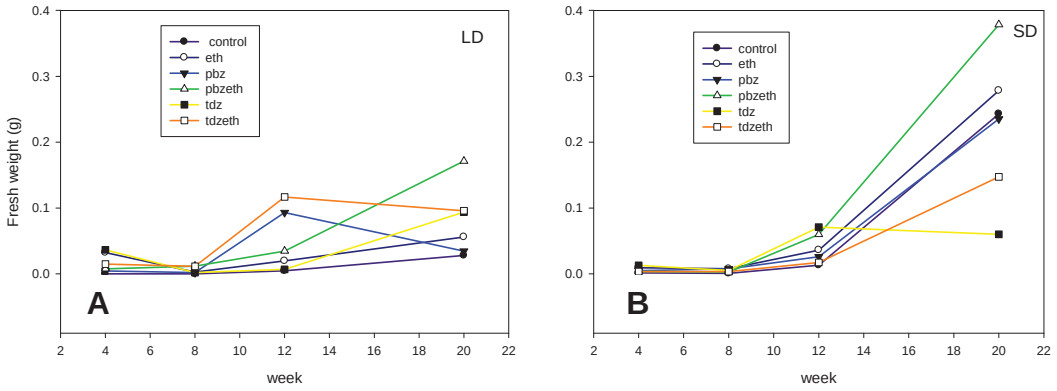

Figure 1. Fresh weight of crown buds on plants of 'Diva'; (A- B) during the 20 weeks following the last treatment application within two photoperiodic regimes. Abbreviations for treatments as described in Table 7.1

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Crown Buds in Gentians: Appearance, Shoot Emergence and Development

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Key words: cyclic periodicity, flowering, temperature, herbaceous perennial, preformed buds.

Abstract

Increased productivity and scheduled flower timing of hybrid gentians will help growers further expand New Zealand's exports of this new crop. The current lack of information on the physiology of growth and development limits the development of management strategies to achieve such expansion. Information on the ontogeny of buds may help in developing strategies to reduce the spread in timing of anthesis between flowering shoots, as well as increasing shoot production. In order to identify and quantify bud types, and the time of their appearance, emergence and development to flowering, these parameters were tracked over time, in two growing environments. Although different types of buds were identified, only preformed primary-crown buds produced flowering shoots. Irrespective of the growing environment 27% of these crown buds did not emerge. Appearance of new crown buds for the following season's flower production occurred over an eight month period, from summer through to early winter. Cultivation in a protected environment resulted in longer shoot length and increased appearance of buds, but did not alter the timing of flowering within the current growth season.

INTRODUCTION

New Zealand (NZ) gentian growers are working to increase the exports of their crops and, to this end, cultivars with an extended colour range are being grown (Eason

et al., 2007). Growers observe a wide spread in the timing of anthesis within varieties resulting in inefficient use of labour at harvest, and difficulty targeting specific markets. Information related to the growth and physiology of this crop is limited. One review article (Ohkawa, 1983) provided a brief description of the growth cycle, and cultural information related to environmental control of growth, but only provided summary data with no statistical validation. Therefore it was considered necessary to identify factors, both physiological and environmental, that might influence both productivity and timing of flowering.

Gentians are usually grown outdoors, but using protected environments has proven advantageous for ornamental plant production in other crops as it provides opportunities for scheduling production, and can result in improved yields of high quality product. There appears to be no information available on the growth response of gentians to protected environments in relation to productivity and timing of flowering. Therefore it was expected the comparison of plant performance grown in two growing environments that differed primarily in temperature, would provide information about probable changes in rates of growth and development of gentians.

Gentians are herbaceous perennials with the perennating organ, a crown, comprised of a rhizome, crown buds, storage roots and feeding roots (Figure 1A). Crown buds remain dormant in winter and grow in spring to form flowering shoots (Ohkawa, 1983). Overwintering buds in other herbaceous perennials (De Hertogh and Le Nard, 1993) may have flowers already initiated at the beginning of the growing season, though these preformed buds may comprise vegetative initials only. Alternatively flowering shoots may also be derived from crown buds produced entirely within the current growing season. In contrast to preformed buds, we hypothesized that any gentian buds formed in the current season may result in shoots that flower later in the season giving an extended flowering period for the plant. It is also hypothesised that differences in the time of shoot emergence following winter might lead to differences in the timing of flowering of the resulting shoots during the current season. In this paper we investigate and describe the influence of the time of bud appearance and shoot emergence on the subsequent development of flowering shoots in the gentian variety ‘Showtime Spotlight’.

MATERIALS AND METHODS

Plants of *Gentiana* ‘Showtime Spotlight’ used in this experiment were propagated by *in vitro* culture at the NZ Institute for Plant and Food Research Ltd, Palmerston North. Following acclimatisation plants were grown in an unheated greenhouse for 18 months until June 2007 until transferred to cold storage at 10 ± 1 °C in darkness. The plants were removed from cool storage for repotting in September 2007, i.e. they were held for 12 weeks at 10 °C. Thus at the beginning of the experiment, the plants had experienced two growing seasons, and were in cold storage.

Plants were potted into plastic pots (5 L), using a medium (C.A.N. Bark Fines A Grade 50%; Fibre 30%; Pacific Pumice 7 mm 20%; Serpentine Super 1 kg/m^3) supplemented with 150 g L^{-1} of dolomite, 200 g L^{-1} of 8-9 month Osmocote® 16N–3.5P–10K (Grace-Sierra International, The Netherlands), and 100 g L^{-1} 3-4 month Osmocote® 15N–4.8P–10.8K. Throughout the experiment, irrigation was delivered by microtubes to each pot on a drained capillary bench, with a duration of 10 min, between three and five times a day, depending upon plant demand.

The growing medium on top of each plant's crown was removed so that buds on the crown could be easily observed. The crowns were subsequently covered with non-absorbent cotton wool and plastic woven weed-mat in order to allow easy access to the crown while limiting light exposure and maintaining humidity levels. Data comprising observations of bud number and development were collected at 14 day intervals. Bud development was tracked over time by producing a map of the crown region at each recording date (Figure 1). A shoot was defined to have emerged from the bud when 2.5 cm above the point of attachment at its base. When flowering shoots had reached commercial maturity (the top-most flower bud was not open but was coloured (Eason et al., 2004) they were harvested with any remaining shoots removed at the beginning of winter (15th June 2008).

Buds were classified into several classes depending on their origin and appearance. "Primary-crown buds" were thick (> 1 mm diameter) and purple-coloured; "Secondary-crown buds" were thinner than primary buds (< 1 mm diameter) and pale purple in colour; and "Root buds" arose from the more distal ends of storage roots.

Commencing 19th September 2007, plants were grown in one of two environments, i.e., either outside under ambient conditions at the Plant Growth Unit, Massey University, Palmerston North (40°20'S 175°37'E), NZ, or in a greenhouse heated at 15°C, vented at 25°C, with 20% shading and natural photoperiod. Mean monthly temperature outside ranged from 8°C to 18°C, and in the greenhouse it ranged between 16°C and 24°C (Figure 2a). In order to assess the impact of regularly exposing the crown for tracking, in both environments, control treatments comprised plants where the growing medium was not disturbed. There were four treatments (tracked in the greenhouse, tracked outside, control in the greenhouse, and control outside). The experiment comprised a CRD design, utilising five single-plant replicates in each treatment. Three additional plants were grown in each environment for dissections at the end of the growth cycle.

For tracked plants, the variables recorded for buds were; bud type (crown or root), diameter, status (alive or dead), and date of appearance (for new buds). For shoots, variables recorded were position of the previous season's senesced shoot, number of visible nodes, shoot length, number of nodes below the lowest flower bud, and date of appearance of floral axillary buds. For plants in the control treatments, shoot number was recorded at two week intervals. In addition, at the end of the period of harvesting flowering shoots, the crown was exposed, and the number and types of buds were noted. In all treatments the numbers of nodes, floral nodes, shoot length, and date of anthesis was recorded for each shoot.

Primary-crown buds (> 1 mm in diameter) were removed at the end of the first growth cycle (11th September 2009) from three additional plants grown in each environment. Buds were dissected (using dissecting microscope) and the number of scale leaves and types of primordial structures recorded for each bud.

Data were analysed using the general linear models procedure of SAS systems software version 9.13. Mean comparisons were conducted using LSD and Tukey's test.

RESULTS

When the experiment began (September 2007) 94 ± 3% of the buds originated from stem/rhizome tissue and 6% from storage roots. Primary-crown buds accounted for 84 ± 1% of the crown buds arising from stem/rhizome tissue, and secondary-crown buds 16 ± 1%. Most shoots had emerged by the end of October (2007), i.e., within 28 days of removal from cold storage (Figure 1 and Figure 2b). With the exception of two shoots arising from root buds, all shoots observed emerged from primary-crown buds present at the start of the experiment.

The number of buds per plant ranged between 7 and 42 with a higher bud number at the beginning of the season resulting in higher shoot numbers (from 5 to 24; $r^2 = 0.94$). When the total number of shoots that emerged during the season was compared to the total number of crown buds (i.e. primary and secondary) observed on the first day of the experiment, only 73 ± 7% of crown buds had emerged as shoots. Thus 27% of primary and secondary-crown buds did not emerge. Following 8 months growth, during which the shoots that emerged had completed their development to anthesis, the majority of the non-emergent crown buds had senesced and degenerated (Figure 1F).

Shoot length on plants in the greenhouse was always longer than plants growing outside, both in tracked and control plants (Figure 3a). Differences were most evident ($P < 0.0001$) at harvest maturity where shoots were between 50% and 64% longer (Table 1). At 0.42 cm/day, the rate of increase in shoot length in the greenhouse was almost twice that achieved outside (Figure 3a). The final number of nodes on shoots (20 ± 1), was not different between growing environments ($P > 0.05$) and neither the number of floral nodes per shoot (6 ± 1) nor the shoot number per plant (10 ± 1) differed among treatments ($P > 0.05$). For both environments, 98 ± 2% of shoots which emerged were floral (Figure 2b). The few shoots that remained vegetative either developed from root buds or emerged from the crown later in the season (i.e., after 17th October 2007).

In tracked plants, time to anthesis was 11 days earlier in the greenhouse than outside ($P < 0.05$; Table 1), but this difference was not seen in control plants ($P > 0.05$). In the greenhouse tracked plants reached anthesis 12 days earlier than control plants ($P < 0.05$) but this difference was not observed between tracked and control plants outside ($P > 0.05$).

In both environments the rate of appearance of new crown buds was low in spring (e.g. November), but increased later in the growth cycle (December to June; Figure 1 and 2b). The increase in the rate of bud appearance coincided with the time when most of the shoots could be confirmed as floral (Figure 2b). From the date of observation of floral axillary buds in a shoot (98 days after start of experiment), the rate of appearance of new crown buds in the greenhouse was 0.0349 buds per plant per day compared with those outside achieving 0.0293 per day (Figure 3b). Appearance of new crown buds was initially greater ($P < 0.05$) in the greenhouse but, by the beginning of the following season, bud numbers were not significantly different with 20 ± 3 new buds in the greenhouse and 17 ± 2 outside for both tracked and control plants. This comprised 19 ± 3 and 14 ± 2 primary-crown buds in the greenhouse and outside environments, respectively. The diameter of primary-crown buds was greater ($P < 0.05$) on plants in the greenhouse (3.66 ± 0.144 mm) than outside (3.03 ± 0.09 mm). Secondary-crown buds were not measured individually as they were less than 1 mm in diameter. Buds recorded as having greater diameters on the first day of tracking

produced longer shoots, but the relationship was marginal in both the greenhouse (r^2 = 0.45) and outside (r^2 = 0.40). A relationship between bud diameter and final node number/shoot was not evident.

The first new crown buds appeared in December (summer) and they continued to appear until August (winter) (Figures 1 and 2). The 27% of crown buds, that hadn't emerged, gradually senesced during this period. The new crown buds sometimes appeared arranged as clusters on the rhizome (Figure 1). After initially appearing as a single crown bud, some differentiated further to form multiple buds (i.e. bud clusters). Bud clusters sometimes developed at the proximal end of storage roots, but most formed directly on the rhizome. When primary crown buds were dissected after the first winter, before emergence in the second growth cycle, primary-crown buds contained similar numbers of primordia, i.e., 10 ± 0.71 (including prophylls and primordial leaves), but no floral initials were found.

DISCUSSION

It was only the primary-crown buds that were formed the previous growing season that contributed to flowering shoots in the current season. No crown buds appeared and developed into flowering shoots in the same season; and no root buds from the previous season produced flowers. Therefore contrary to our hypothesis, the variation in time to flower was not due to either bud type or to buds initiated during the current season developing into floral shoots. In the current study the primary crown buds that develop into floral shoots had 10 ± 0.71 primordia (none floral) already developed prior to emergence. In woody perennials most shoot production, both after winter (dormancy) or following disturbance, is from preformed buds (Del Tredici, 2001). Hence factors that affect the spread of flowering time in gentian plants still require further investigation.

Only a proportion of the primary-crown buds emerged (Figure 1). The remaining primary and secondary-crown buds (27%) had all senesced and degenerated by the end of the growing period and did not contribute to next season's flowering shoot production. In other species, growth and development of one large bud may cause smaller buds in the cluster to cease growth and become dormant (Stafstrom, 1995). These dormant buds may only grow when the larger bud(s) is removed. Thus, it is hypothesized that in gentians some of the crown buds remain suppressed due to apical dominance of those buds emerging as shoots. If indeed apical dominance does suppress growth of the non-emergent buds, reducing apical dominance should be investigated as both a source of the variation in timing of flowering, and to increase the yield of flowering shoots per plant.

Crown buds were produced continuously over the 8-month growing season (Figure 1 and 2b) and each bud on a plant would have experienced different environmental conditions from appearance to emergence the following season. Hence, it seems reasonable to expect that crown buds could emerge and perform differently during their growth to anthesis. Further to this, cultural practises that disturb the crown during the growing season might influence bud initiation, appearance and shoot development. It is hypothesized that the time of actual bud initiation could lead to differences in shoot size and time of anthesis. Future investigations based on time of

bud initiation, appearance, and subsequent growth through to anthesis, is required to investigate this hypothesis further.

In contrast to plants growing outside in full sun, more buds were observed in the heated greenhouse with 20% shade (Figure 3b). We interpret this as an indication of the potential of the growing environment to influence bud initiation and/or appearance rates. Therefore, the influence of environment (temperature, daily light integral, and/or day length) and physiological factors (above-ground and below-ground changes in growth), which may be correlated with initiation and appearance of new crown buds in gentians, needs to be investigated further.

In the current experiment, following removal of endodormancy, shoot emergence was faster in greenhouse growing plants compared to those outside, but timing of anthesis was not consistently different among treatments (Table 1). Environmental conditions, primarily higher temperatures (Figure 2a) are likely to have stimulated rapid shoot emergence and development in the greenhouse environment. The difference in monthly average temperature between the protected environment and outside environments varied from 4°C in summer to 12°C in winter. Although the final shoot length differed, the number of nodes pre flowering shoot was not different between the two environments. Increased shoot length was due to the increase in internodal length in the protected environment. Temperature induced differences in shoot length, flower number and time to anthesis have been noted in several species (Davies et al., 2002; Funnell, 2008), so our failure to consistently observe similar effects with gentians may be due to the limited number of replicate plants used.

When compared with the outside environment in greenhouse grown plants, a greater number and larger new crown buds appeared. In other species larger buds with more primordia may be produced from plants with larger, more vigorous shoots (Remphrey and Davidson, 1994). In the current experiment plants with more crown buds produced more flowering shoots and, buds with a larger diameter gave longer shoots. It can be hypothesized that in gentians if a greater number of large primary crown buds are initiated in a given year, more, high quality floral shoots can be expected the following season. Although the growing environment did not consistently influence the time of anthesis, longer shoot length and increased appearance of crown buds, may make greenhouse cultivation of gentians attractive if other requirements for growth can be met.

CONCLUSIONS

Only primary-crown buds form flowering shoots in *Gentiana* ‘Spotlight’. These primary-crown buds are preformed, appearing in the growing season prior to their emergence as flowering shoots. Growing plants in a protected environment resulted in longer shoot length and initiation of new crown buds of a larger size, but did not influence the time to anthesis. These results indicate the future potential to control flower production of gentians by further exploring both environmental and/or plant-related factors.

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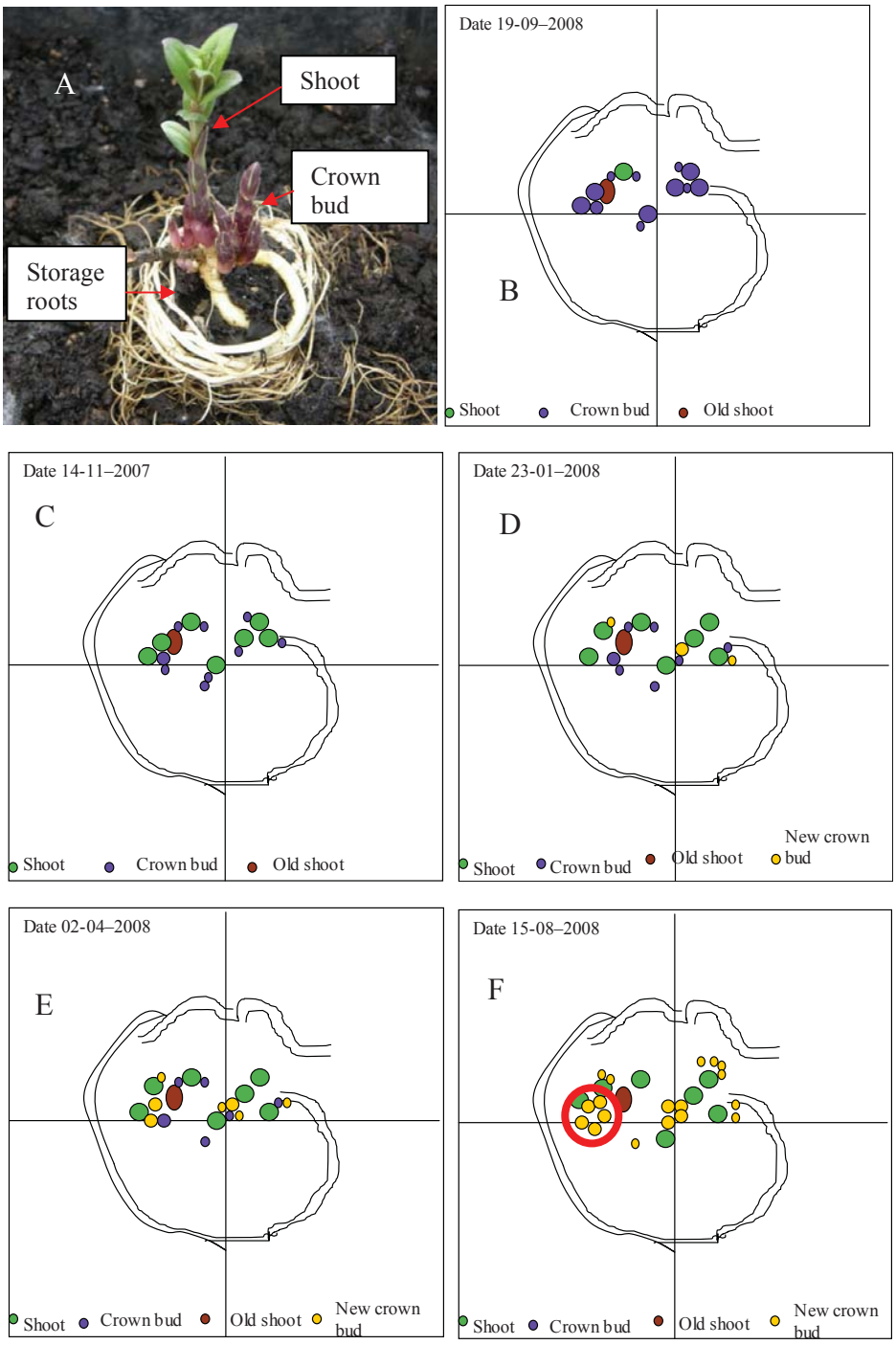

Fig. 1. A and **B,** respectively, photograph of a representative plant of *Gentiana* 'Spot light', and diagrammatic illustration of the same, illustrating key organs at the start of experiment, **B-F;** Developmental sequence and physical location of buds and shoots on the crown over a period of one year, on one representative plant (size of buds and shoots in the diagram are relative to the sizes observed during the experiment, where the smallest coloured dot is representative of less than 1 mm diameter in size); red open circle indicates a single bud cluster.

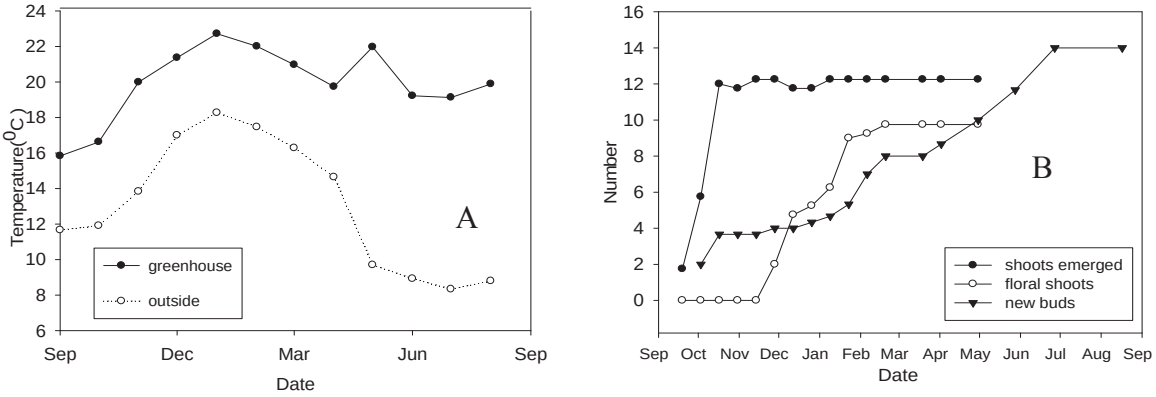

Fig. 2. Mean monthly temperature encountered in two environment treatments used **(A).** General pattern of changes in number of emerged shoots, designation of shoots as being floral and, appearance of new buds in the outside environment **(B)**. Vertical bars = standard error

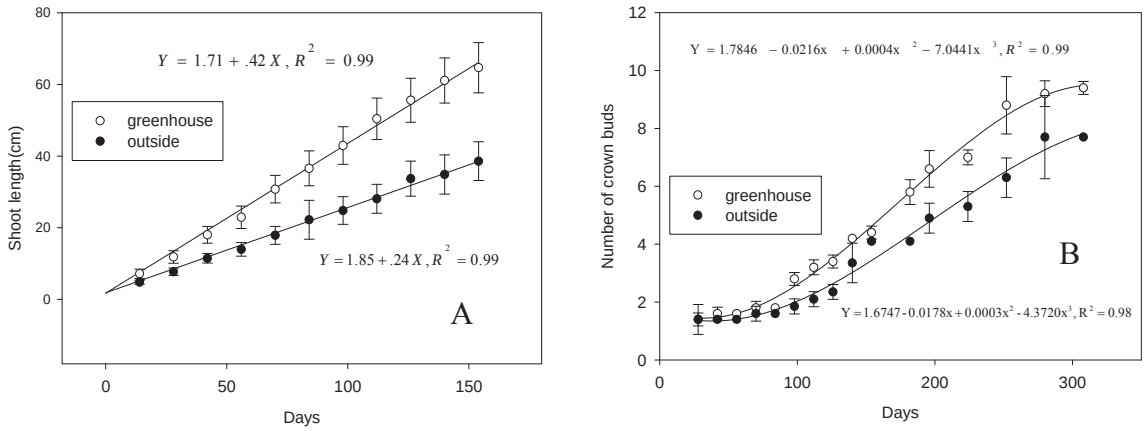

Fig. 3. Pattern of change in shoot height until harvesting commenced (**A**) and combined crown bud number (**B**), in two different environments. Vertical bars = standard error.

Table. 1. Shoot length at harvest, and average duration to harvest from the date of removal from the cold store, of flower stems of *Gentiana* 'Spotlight' in either the greenhouse or outside environments during the growing season.

	Shoot length (cm)		Duration to harvest (days)	
	Tracked	Control	Tracked	Control
Greenhouse	66.1 ± 2.5[a]	74.4 ± 2.5 [a]	178 ± 2 [b]	190 ± 2 [a]
Outside	44.2 ± 1.6 [b]	45.4 ± 1.7 [b]	190 ± 3 [a]	184 ± 2 [a]

[1] Tukey's groupings at $P \leq 0.05$

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