Strigolactones and hormonal interaction in control of branching in *Zantedeschia* and other horticultural species

A thesis presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy in Plant Science

at Massey University, Palmerston North,
New Zealand

Sarina Manandhar

2016

Abstract

Shoot branching that involves development of lateral buds into shoots is one of the important factors influencing crop productivity. Strigolactones have recently been found to be involved in the control of branching, but the actual bioactive compound/s that inhibits bud outgrowth is still unknown. A germination assay utilizing the seeds of a parasitic weed (*Orobanche minor*), detected strigolactones within the xylem exudates of different horticultural crop species; the strigolactone concentration negatively correlated with branching of cultivars or mutants. In *Zantedeschia* grown *in vivo*, the concentration of strigolactones was independent on the volume of guttation fluid (xylem exudates) suggesting the difference in concentration of strigolactones in high and low branched cultivars was due to the difference in potential of producing strigolactones between these cultivars and not due to differences in volume of guttation fluid. While identifying a bioactive compound using germination and branching assays in combination with liquid chromatography and mass spectrometry, compounds containing 'N' were detected in the low branched wild-type *Petunia*, but not in the highly branched mutant, suggesting the possibility of such compounds being SL-conjugates which may be associated with bud outgrowth inhibition.

In *Zantedeshia* (*in vitro*) and pea stems, strigolactone reduced the axillary shoot number stimulated by the cytokinin suggesting an antagonistic interaction between these two hormones on bud release. However, as cytokinin may stimulate subsequent growth of released buds by increasing the auxin transport out of the bud, strigolactone may have reduced subsequent growth by reducing auxin transport. Since GA_3 enhanced subsequent growth of buds in pea stems, but not the release, an antagonistic interaction between strigolactone and gibberellins on subsequent growth is possible. Interestingly, strigolactone successfully reduced adventitious bud formation in *Zantedeschia* grown *in vitro,* adding a new role for strigolactones in plant development.

Despite correlation between strigolactone and branching inhibition in different horticultural crops such as apple, kiwifruit, *Zantedeschia* and *Acer*, further studies relating to strigolactone and its interaction with other hormones on branching of these crops could be performed using *in vitro* techniques for a clear understanding of strigolactones' role on branching inhibition. More importantly, quantification of strigolactones using the germination assay may have significant implications in horticultural crop breeding for obtaining desired shoot branching.

Extended abstract

Shoot branching, one of the important factors influencing crop productivity, involves development of lateral buds into shoots on an actively growing primary shoot. Recently, a new hormone, which may be a strigolactone, has been found that inhibits bud outgrowth, however, the precise chemical identity of the bioactive compound(s) is unknown. A bioassay based on the germination of a parasitic weed (*Orobanche minor*) was optimized to detect strigolactones. Although there has been controversy in the literature related to whether or not strigolactones are present in xylem exudates, in this thesis strigolactones were found in xylem exudates of a range of horticultural species. The strigolactone concentration correlated with branching of cultivars or mutants mainly at the stage of the growth cycle before the branches were visually evident.

As the germination assay detects all/most strigolactones, not necessarily specifically those associated with branching, a more specific bioassay based on branching was developed. This bioassay was combined with liquid chromatography and mass spectrometry in an attempt to identify a specific branching hormone, whether or not this was a strigolactone. In *Petunia,* four compounds containing 'N' were detected in the xylem sap of the wild-type, low branched, V26, but not in highly branched dad3 mutant, suggesting the possibility of such compounds being SL-conjugates which may be associated with branching inhibition. In *Zantedeschia* grown *in vivo*, since the concentration of strigolactones was independent of the volume of guttation fluid, it was suggested that difference in concentration of strigolactones in high and low branched cultivar was due to the difference in potential of producing strigolactones between these cultivars and not due to differences in volume of guttation fluid.

The synthetic strigolactone GR24 (0.1 or 1 mg L^{-1}) was able to reduce axillary shoot number stimulated by cytokinin in un-decapitated pea stems, and *Zantedeschia* grown *in vitro,* suggesting an antagonistic interaction between these two hormones on bud release, as opposed to subsequent growth. Likewise, strigolactone (1 mg L^{-1}) reduced decapitation-induced bud release, supporting the hypothesis that strigolactone may have interacted with endogenous cytokinin and/or sucrose. Strigolactone was also able to

reduce subsequent growth of the shoot, but the effect was stronger in buds of pea stems orientated horizontally, compared to those orientated vertically. Such disparity was possibly due to the fact that, in addition to vascular stream, the buds of horizontally orientated stems received strigolactones directly. Although exogenously applied cytokinin appeared to enhance subsequent growth of the released bud, the effect of cytokinin on subsequent growth may be via increasing the auxin transport out of the bud. Hence, rather than interacting with cytokinin, strigolactone may have reduced subsequent growth of the buds by reducing auxin transport. As GA_3 enhanced the subsequent growth of buds in pea stems, but not the release, an antagonistic interaction between strigolactone and gibberellins on subsequent growth is considered highly likely. Interestingly, strigolactone successfully reduced cytokinin-stimulated adventitious bud formation in *Zantedeschia* grown *in vitro*. Interaction studies of strigolactone with cytokinin, and probably ethylene, is recommended within highly branched cultivars of *Zantedeschia* spp. or other species, such as gentians and kiwifruit, to further explore the role of strigolactone in adventitious bud formation and development in order to obtain desirable shoots for commercial purposes.

Although strigolactone correlated with branching inhibition in different horticultural crops such as apple, kiwifruit, *Zantedeschia* and *Acer*, further studies for answering the direct role of strigolactone on bud outgrowth in these crops as well as its interaction with other hormones can be performed using *in vitro* techniques. More importantly, quantification of strigolactones using the germination assay may have significant implications in horticultural crop breeding for obtaining desired shoot branching. Since guttation fluid from *Zantedeschia* was found to give a true estimate of the concentration of strigolactones present in the xylem of the shoot system, future experiments may benefit through the use of guttation fluid for hormonal analysis and/or interaction studies *in vivo*. Consideration of the stages of shoot branching during such studies would be valuable for a clear understanding of the shoot branching mechanism and help modify the branching of commercially important crops.

Acknowledgments

Foremost, I would like to express my sincere gratitude to my chief supervisor **Dr David Woolley** for the continuous support of my PhD study and research; for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me during the research and writing of this thesis. His unending encouragement, constructive criticism throughout the course of my PhD as well as his kindness to understand me is highly appreciated.

Thanks to my co-supervisor Dr Keith Funnell for helping me learn technical things right from the beginning of my research until end of my thesis write-up. Your remarkable quality of planning, organizing and implementing things is really appreciated. Your constructive feedback and suggestions helped me drive towards achieving perfection.

I would like to extend my thanks to staff of the IAE, as well as at the Plant Growth Unit; especially Chris, Kay, James and Lesley for their technical help and moral support. Kay, also thank you for your advice when dealing with the health of my son.

I wish to acknowledge the staff of Plant & Food Research, especially Andrew Mullan for helping me prepare media during tissue culture experiments, Maree Debenham for teaching me skills associated with tissue culture, and Duncan Hedderley for statistical help while analyzing data. I am also grateful to Revel Drummond for the supply of petunia seed and helping me learn sample extraction in petunia plants. My sincere thanks go to Dr Tony McGhie and Dr Daryl Rowan for assisting me in learning LC/MS and also thanks to Dr. Janney Cooney and her team for analyzing cytokinins and strigolactones through LC/MS.

I would particularly like to acknowledge the financial support from a Massey University Doctoral Scholarship, without which I my PhD would not have run as smoothly. I am also grateful for the financial support covering the research costs provided by The New Zealand Institute for Plant & Food Research Limited Core funding: 1198 – “Fashionable Plants for the Ornamentals Industry”, 1972 – “Breeding Technology Development”, as well as both the Helen E. Akers PhD scholarship and Johannes Anderson PhD scholarship.

Special thanks to Marzieh, Fadhil and my senior colleagues for their help, sharing ideas and creating a friendly environment during the period of study. This thesis would be incomplete if I do not mention the help and support of dear friends Rachael, Mahesh, Bhawana, Rajiv, Srijan and my entire circle of friends in Palmerston North. Thank you so much my dear sisters Jamuna and Saraswati for looking after my son during such a busy time.

Most importantly, I would like to express my deep gratitude to my husband Bishad for your many sacrifices, constant support and encouragement. My son Aaron, his innocent words and funny activities were a tonic to recover from having a frustrated mind. My heart-felt thanks to both of you boys. At last, my mother, brothers, sisters, and in-laws, who are far from me; thank you so much for your constant support.

Table of contents

4 Transport of strigolactones in guttation fluid of *Zantedeschia* 96

5 Interaction between strigolactone and cytokinin in shoot branching of *Zantedeschia* grown *in vitro* ... 121

List of Figures

List of Tables

List of Abbreviations

ABA	Absisic acid
ANOVA	Analysis of variance
BAP	Benzylaminopurine
BPC	Base peak chromatogram
BRC1	Branched1
CCD	Carotenoid cleavage dioxygenases
CK	Cytokinin
CRD	Completely randomised design
D27	Dwarf27
DAD	Decreased apical dominance
DMRT	Duncan's multiple range test
DW	Dry weight
FW	Fresh weight
GA	Gibberellin
GA_3	Gibberellic acid
GLM	General linear model
GR24	a synthetic strigolactone (3aR*,8bS*,E)-3-(((R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IAA	Indole-3-acetic acid
IPT	Isopentenyl transferase
LC/MS	Liquid chromatography/ Mass spectrometry
LSD	Least significant difference

M.9	Malling 9
MAX	More axillary shoot
MRM	Multiple reaction monitoring
NAA	Naphthalene 1-acetic acid
NMR	Nuclear magnetic resonance
NPA	1-N-Naphthylphthalamic acid
NZ	New Zealand
PAT	Polar auxin transport
PIN	Pin-Formed
PPFD	Photosynthetic photon flux density
RO	Reverse osmosis
RMS	Ramosus
RWC	Relative water content
SAM	Shoot apical meristem
SE	Standard error
SL	Strigolactone
TW	Turgid weight
V26	Velm 26
X-CK	Xylem cytokinin

1 General Introduction

1.1 Importance of research on architecture of horticultural crops

Horticulture is an important sector of New Zealand’s national economy with exported merchanidise accounting for 8% of total export (FreshFacts, 2013). Fresh fruit is New Zealand's largest horticultural export sector, with revenues of $1.52 b, mostly from kiwifruit ($934 m) and apples ($475 m). Kiwifruit (*Actinidia* Lindl.) is New Zealand’s top horticultural export and New Zealand is the third largest kiwifruit exporter in the world. In contrast, cut flower production, flower and foliage exports from New Zealand was only around $32.4 m (FreshFacts, 2013), but the international trade in floriculture is estimated to be about $150 b with a high global demand for this sector (Bhattacharya et al., 2010), indicating a large potential growth of this sector for New Zealand.

Crop and/or plant architecture is an important factor determining productivity of plants. Plant architecture is defined as the three dimensional plant structure that includes branching pattern, size, shape and position of plant components such as leaves and flowers (Reinhardt & Kuhlemeier, 2002). Crop architecture denotes the spatial arrangement of the the plant architectural characteristics such as the number, shape and orientation of plant components in a canopy. In kiwifruit, vigorous shoot growth of vines is a major constraint for the industry as it leads to heavy and closed canopies that result in reduced fruit quality (e.g. fruit size) (Miller et al., 2001). Proper crop architecture allows adequate light penetration through the canopy during flowering, and ultimately results in good quality fruit production in terms of larger fruit size (Sale & Lyford, 1990). Thus a major cost of production in kiwifruit goes into manual pruning for managing the vigorous shoots. Utilization of dwarfing rootstocks could reduce shoot growth and improve fruit yield but, unlike other fruit crops e.g. apple (*Malus domestica* Borkh), there are no good dwarfing rootstock cultivars available for kiwifruit

(Clearwater et al., 2006; Miller et al., 2001). Likewise, excessive vegetative growth is antagonistic to flower bud initiation in fruit crops like apple and some other temperate fruit trees (Forshey & Elfving, 1989). Manipulation of branching is often practiced commercially in crops like kiwifruit and apple in order to obtain an optimum level of branching to maximize light interception for yield and within canopy light distribution for fruit bud longevity and fruit quality.

Plant architecture is important in ornamental crops because consumer choice is greatly influenced by plant shape and number of branches (Bhattacharya et al., 2010). In relation to the importance of branching pattern on production, highly apically dominant horticultural plants produce few shoots, including flowering shoots, resulting in reduced yield. In calla lily (*Zantedeschia* spp.), depending on the cultivar, only a few secondary shoots may develop on the tuber, while the rest of the buds remain inhibited (Naor, Kigel, Ziv, & Flaishman, 2005). Hence, flower yield of calla lily is affected by the limited number of secondary shoots. In contrast to the calla lily, in tomatoes (*Solanum lycopersicum*) where apical dominance is weak, removal of side shoots are required for generating quality fruit yield (Heuvelink & Buiskool, 1995). Hence, irrespective of whether the crop is regarded as a fruit crop or ornamental, research on plant/crop architecture is important for generating desirable branch number and canopy density in order to obtain high yield in terms of quality and quantity.

Branching of shoots, one of the major components of plant architecture, is mainly defined by genetics (Chen et al., 2009) but highly regulated by endogenous and environmental cues (Umehara et al., 2008). Plant hormones are regarded as the major endogenous signal that affect branching of shoots, with the hormones known to have a major influence being auxin, cytokinin (CK), gibberellins (GAs) (van Hooijdonk et al., 2010) and a novel hormonal group, the strigolactones (SLs) (Gomez-Roldan et al., 2008). However, the underlying mechanism of how hormones operate in the branching process is still not fully understood. Better understanding of the role of hormones in the branching mechanism should help to modify shoot architecture of commercially important horticultural crops.

This thesis is basically focused on the physiological aspects of branching in shoots, in a number of horticultural crops, taking into account interactions between different plant hormones (refer Section 1.5).

1.2 Shoot branching

Shoot branching is the process by which the axillary buds develop into shoots on an actively growing primary shoot (Ongaro & Leyser, 2008). The branching of shoots involves a sequential process of initiation of axillary meristems, formation of axillary buds, bud release and subsequent growth of the axillary shoot (Rameau et al., 2015; Sae Shimizu-Sato & Mori, 2001) (also refer Figure 1.1). However, after formation of buds, the buds may become dormant/correlatively inhibited or grow into shoot without entering a period of dormancy i.e no specific release phase. Generally, in many horticultural species such as *Zantedeschia*, *Petunia hybrida*, and kiwifruit (*Actinida* spp.*)*, buds undergo a period of dormancy. Bud dormancy can be divided into paradormancy (caused due to the effect of other parts of the plants, e.g. apical dominance), ecodormancy (caused due to environmental effect e.g., lower temperature) and endodormacy (caused due to internal factor(s) of the bud e.g., abscissic acid) (Lang, 1987). In this thesis, shoot branching affected by paradormancy and the mechanism regulating the release and subsequent growth, either combined or separately, is discussed. When discussed as a single entity the term ‘bud outgrowth’ (or ‘outgrowth of bud’) is used.

Suspension of bud outgrowth may be due to environmental effects or inhibition by another plant organ. Inhibition by a shoot apex or any vigorously growing lateral shoot over the release and/or growth of other bud/branches is known as correlative inhibition (Cline, 1997). For example, in the two-shoot system of pea (*Pisum sativum*) or bean (*Vicia faba*) plants in which one shoot grows vigorously while the other shoot remains inhibited, once the vigorous or dominant shoot was decapitated, the inhibited shoot started to grow (Li & Bangerth, 1999; Snow, 1937), thereby showing correlative inhibition. The more specific term ‘apical dominance’ denotes correlative inhibition of lateral buds caused by the apex of the primary shoot (Cline, 1997), as removal of the primary shoot apex promotes lateral bud outgrowth (Snow, 1937; Thimann & Skoog, 1934). Considering outgrowth of lateral buds via removal of the shoot apex, Cline (1997) divided the shoot branching process into four stages: (I) lateral bud formation, (II) imposition of inhibition on lateral bud growth, (III) release of apical dominance following decapitation or release of lateral buds, and (IV) branch shoot development (Figure 1.1). In some cases, before entering the fourth stage, the buds can re-enter inhibition suggesting the release of a bud can be temporary (Shimizu-Sato and Mori 2001). Several studies make mention that axillary buds are released in the presence of CK, but do not grow further unless GAs (Catalano & Hill, 1969; Subbaraj et al., 2010) or sucrose (Wickson & Thimann, 1958) are applied. Since CK activates the shoot apical meristem (SAM) via cell division (Shani et al., 2006), the release of buds due to CK is quite likely. GAs however, enhance the subsequent growth of the released bud or the shoot extension as the hormone promotes the sub-apical meristem via cell division and/or cell elongation (Talon et al., 1991). The third stage is also known as the "transition stage" because, at this stage, buds may either undergo subsequent growth of the branch or revert back to the inhibition stage (Shimizu-Sato, 2001). Hence, in plant systems, the degree of branching does not depend on one stage but all the four stages including the subsequent growth.

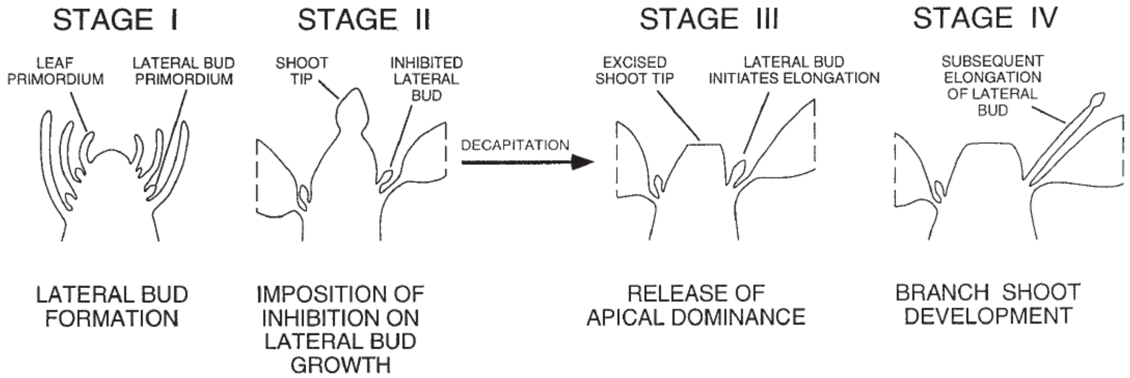


Figure 1.1 Developmental stages of apical dominance before and after axillary bud release caused by decapitation of the shoot apex (Cline, 1997).

Unlike apical dominance, the term apical control as proposed by Cline (1997) best describes the inhibition on growth of the released buds where the inhibited shoots do not need to pass through the stage of bud release inhibition. In woody perennials, apical control occurs in many species, especially in plants following the first growing season (refer Section 1.5.3). Within this thesis the general term "shoot branching" has been used, which may involve, apical dominance, correlative inhibition and factors influencing subsequent growth.

The mechanism of branching may be affected by plant hormones at any of the four stages (refer Figure 1.1). However, many papers have not indicated the particular stage of shoot branching that was being investigated. Generally in such literature, the third (release) and fourth (subsequent shoot growth) stages are combined as a single length measurement, even though each stage may be contolled by different mechanisms. For instance, in many papers, shoot length was measured a few days after decapitation or hormonal application rather than bud release *per se* (Cline, 1997). The actual interpretation for the release or subsequent growth of the shoot in decapitated plants appeared missing. A further short coming in the existing literature is that the rate of growth should not be used as a proxy for determining the release from apical dominance. For example, reduced growth rate due to SLs (also refer Sections 1.3 and 1.4.3.1, Theory C) may not indicate that SLs are involved in bud release *per se*. Within the current thesis therefore, the opportunity to increase understanding of the branching mechanism by recognizing each of these different stages was considered worthy for inclusion.

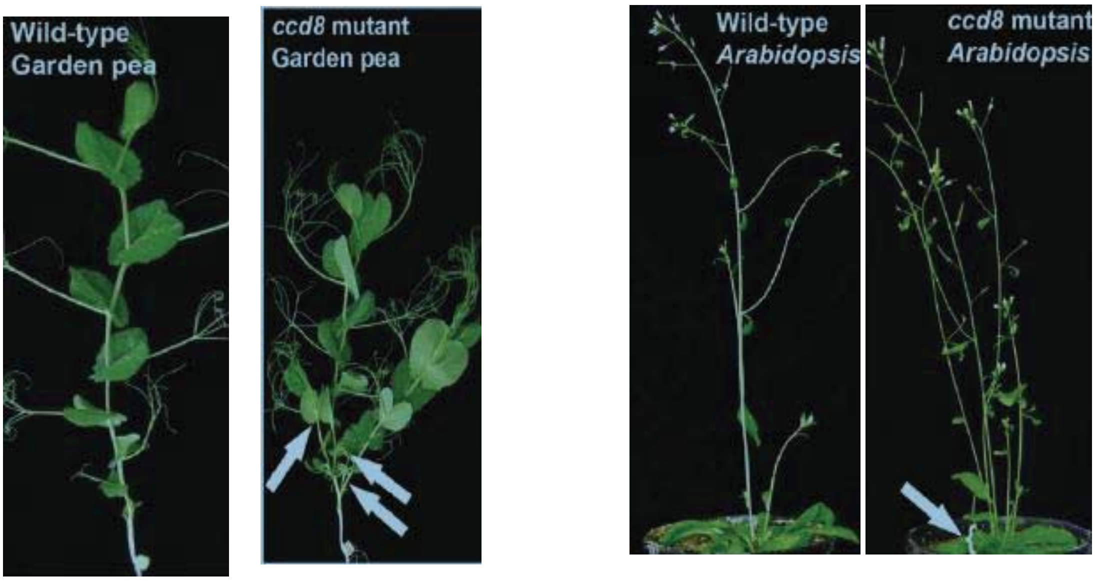
1.3 Strigolactones

Historically, auxin and cytokinin were thought to be the most important hormones known to influence shoot branching, particularly bud release, however many experiments such as those of Snow (1937) indicated that another unknown hormone may be involved. Recently, SLs, a new class of hormones, have been considered important in influencing shoot branching mechanisms (Gomez-Roldan et al., 2008).

Strigolactones (SLs) are carotenoid-derived compounds produced by the combined action of carotenoid cleavage dioxygenases, CCD7 and CCD8 (Matusova et al., 2005). Initially, SLs were isolated as germination stimulants of parasitic plant seeds of *Striga* spp and *Orobanche* spp. (Bouwmeester et al., 2003; Cook et al., 1972; Muller et al., 1992), and were later recognized for inducing spore germination and hyphal proliferation of arbuscular mycorrhizal fungi (Besserer et al., 2006). In 2008, SLs were first reported as a putative hormone involved in controlling shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). Hence, SLs act as a signaling molecule both within and outside a plant. Recently, SLs were also known to influence different physiological processes such as root growth, lateral root formation, root hair elongation, adventitious rooting, leaf senescence, drought and salinity responses and stem elongation (reviewed in Waldie et al., 2014), hence demonstrating their multiple roles within plant systems, like many other plant hormone groups. However, the review within this chapter will mainly focus on presenting an understanding of the role of SLs in shoot branching.

1.3.1 Strigolactones and branching

Before the discovery of the involvement of SLs in shoot branching, isolation/characterization of branching mutants in different plant species, as well as several grafting studies, revealed the likely presence of a branching inhibitor, possibly synthesized in roots and moving to shoots to repress axillary bud outgrowth (Gomez-Roldan et al., 2008). The mutants that lacked either CCD7/CCD8 genes or genes for branching response, exhibited enhanced shoot branching as compared to the wild-type (Figure 1.2; Dun, Brewer, & Beveridge, 2009). Those mutants were “ramosus” (rms) in pea (Beveridge 2000; 1997; Beveridge et al., 1996), “decreased apical dominance” (dad) in *Petunia ×hybrida* L. (*Petunia*) (Napoli, 1996; Napoli et al., 1998) and “more axillary growth” (max) in *Arabidopsis* (Sorefan et al., 2003; Stirnberg et al., 2002; Turnbull et al., 2002).



A B

Figure 1.2 Wild-type and branching mutant phenotypes of 'Garden pea' (A) and *Arabidopsis* (B). Mutant-induced branches on these plants are indicated by arrows (Dun, Brewer et al., 2009).

Through a series of grafting experiments using both wild-type and branching mutants, the inhibitor was considered to be SLs, despite the active form of SL not having been identified. In CCD8 mutants of pea, SLs were not detected, but were present in root exudates of wild-type plants (Gomez-Roldan et al., 2008) (Figure 1.3). In addition, application of GR24 (synthetic SLs analog) onto the buds of these mutant plants was also able to repress the bud outgrowth (release and subsequent growth, refer Section 1.2) (Gomez-Roldan et al., 2008; Umehara et al., 2008) (Figure 1.4) suggesting a role for SLs in repressing shoot branching. The presence of SLs in root exudates suggests that this hormone moves from root to shoot to inhibit axillary bud outgrowth, hence also fits well with the inhibitor proposed by Snow more than 70 years ago. Snow (1937), based on his two-shoot system of *Vicia faba* experiment (refer Section 1.4.2), proposed the hypothesis that an inhibitor moves acropetally to inhibit the growth of axillary buds.

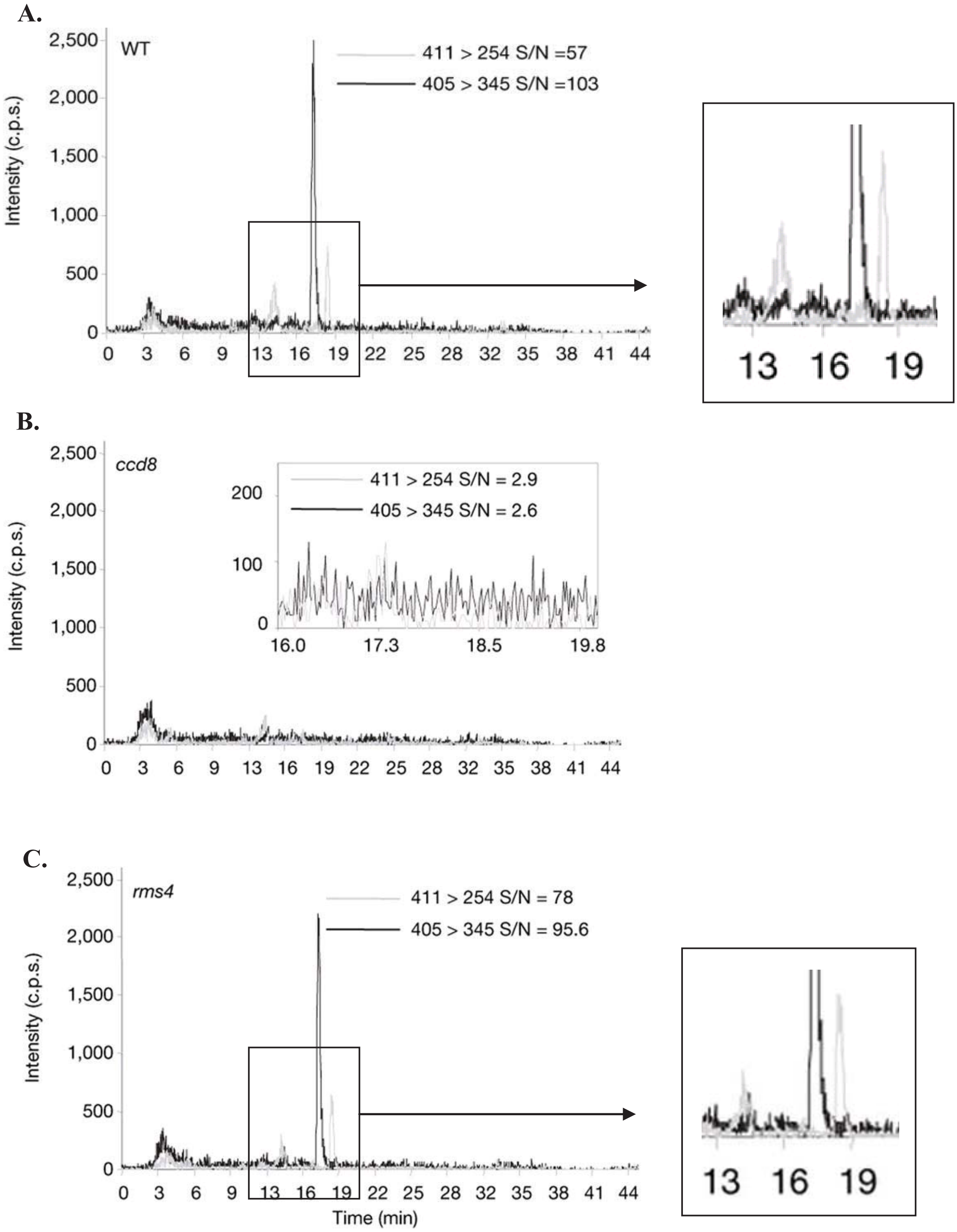


Figure 1.3 Root exudates of ccd8 mutant of pea is deficient in SLs (B) compared to wild-type (A) and rms4 plants (B). Characteristic transitions for orobanchyl acetate (grey line) and the second strigolactone (black line) were monitored in the MRM mode (Gomez-Roldan et al., 2008). Grey lines in Figures A and B are magnified.

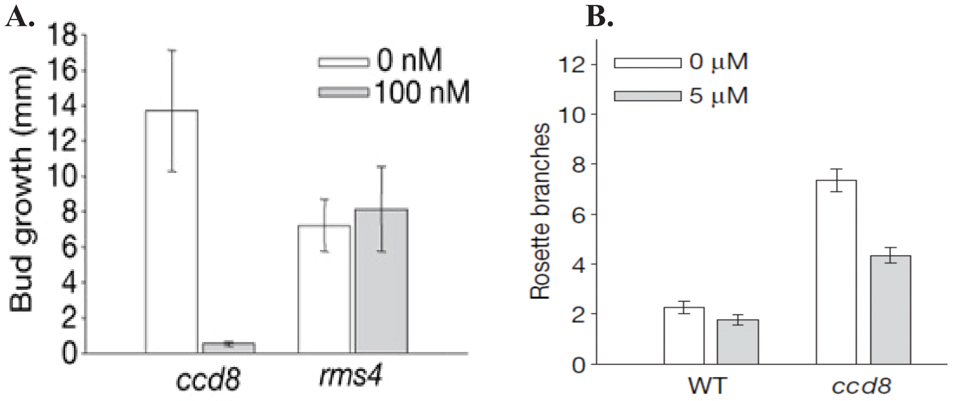


Figure 1.4 Effect of GR24 on growth of axillary bud/shoot of; (A) strigolactone deficient mutant rms1-10 and response mutant rms4-3 of pea seedlings, (B) strigolactone deficient mutant max4-1 (ccd8) and wild-type (WT) of *Arabidopsis.* GR24 reduced the shoot length/number of branches of ccd8 plants but not of rms4-3 of pea plants and wild-type of *Arabidopsis*. GR24 was applied directly to the bud at node 4 of pea plants or to the rosette axillary buds and leaf axils of *Arabidopsis*. Observations were taken 10 days and 25 days after GR24 application in pea seedlings and *Arabidopsis*, respectively (Gomez-Roldan et al., 2008).

Mutants 'rms5/dad3/max3' and 'rms1/dad1/max4' of pea/*Petunia*/*Arabidopsis* are deficient in SL synthesizing genes CCD7 and CCD8 (Beveridge et al., 1996; Napoli, 1996; Sorefan et al., 2003) (Figure 1.2, 1.3). Grafting of these mutants onto their wild-type rootstocks restores the wild-type phenotype (Figure 1.5). Inhibition of axillary bud outgrowth in mutants by grafting onto wild-type rootstock, suggests that SLs from wild-type rootstock moves into the shoot of these mutants. However, axillary bud outgrowth of these mutants was not affected when these mutants were used either as a scion or rootstock (Figure 1.5) (Beveridge, Murfet et al., 1997; Simons et al., 2007; Sorefan et al., 2003) suggesting that the mutants are deficient in SLs both in the root and shoot. Interestingly, the wild-type scion did not change its branching phenotype when grafted onto the mutant scions (Beveridge et al., 1994), suggesting that the branching inhibitor was present in the shoot. Supporting this hypothesis, when a small segment of the wild-type was used as an interstock within the mutant scion, axillary bud outgrowth was completely inhibited (Napoli, 1996). Hence, it can be concluded that SLs can be synthesized in both the root and the shoot, as well as transported from root to shoot.

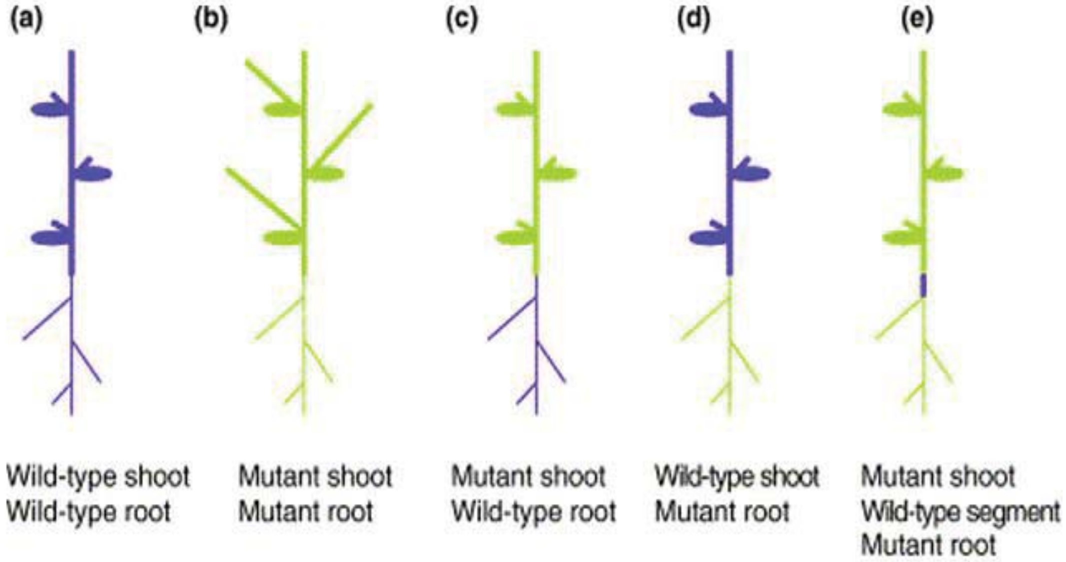


Figure 1.5 Schematic representation of results of reciprocal grafting between mutant and wild-type pea plants. (a) branching in self grafts of the wild-type, (b) branching phenotype in self grafts of the mutant, (c) mutant grafted over wild-type stock, (d) wild-type grafted over mutant stock and, (e) mutant scion with wild-type stem segment (i.e. interstock) and mutant stock (Leyser, 2003).

Although the synthetic strigolactone 'GR24' inhibited bud outgrowth of the ccd8/ccd7 mutants of rice and *Arabidopsis* (Umehara et al., 2008), further confirmation is required that the SLs within plants are actually the inhibitors and, if so, which particular SL. It is worth noting that detection of SLs within the wild-type, but not in rms mutants, does not by itself directly indicate that SLs are actually the inhibitors. It is possible that SLs might only correlate with branching inhibition, and that downstream compounds possibly related to SLs are actually more important for inhibiting bud outgrowth. In support of this hypothesis, a recent study by Boyer et al. (2012) has shown that the natural strigolactones tested were able to reduce the axillary shoot length of pea seedlings, but it is yet to be determined whether or not SLs also inhibit bud release.

There are also mutants, such as rms4 of pea (Beveridge et al., 1996), max2 of *Arabidopsis* (Stirnberg et al., 2002) and dad2 of *Petunia* (Hamiaux et al., 2012) that lack response to SLs. Despite the presence of SLs (Gomez-Roldan et al., 2008), these mutants are highly branching. Grafting mutants like rms4 of pea, max2 of *Arabidopsis* and dad2 of *Petunia* onto their wild-type rootstocks/interstocks caused no difference in their branching phenotype (Gomez-Roldan et al., 2008; Simons et al., 2007; Stirnberg et al., 2002). Interestingly, grafting SL-deficient mutants like rms1 of pea, max3 of *Arabidopsis* and dad3 of petunia onto rms4 max2 and dad2, respectively, restored the inhibition of axillary bud outgrowth. Hence, SLs present in rms4 (Figure 1.4), max2 and dad2 roots, was able to move to the shoot of the SL-deficient mutant to inhibit the bud outgrowth. Due to the lack of responding genes however, the rms4, max2 and dad2 failed to exhibit the wild-type phenotype. In mutants which were deficient in the branching response, the downstream signaling F-Box protein encoded by RMS4 and MAX2 and α/β hydrolase encoded by DAD2, which are required for SLs to respond for inhibition of bud outgrowth, was not present (Hamiaux et al., 2012; Stirnberg et al., 2002).

1.3.2 Biosynthetic and transduction pathway of strigolactones

Based on the genes likely to be involved in SL biosynthesis and inhibition response (refer Section 1.3.1), a pathway for SLs biosynthesis and transduction has been proposed (Figure 1.6). In this pathway, carotenoid cleavage dioxygenase CCD7 and CCD8 encoded by RMS5/MAX3/DAD3 and RMS1/MAX4/DAD1 respectively (Johnson et al., 2006; Napoli, 1996; Sorefan et al., 2003), act on 9-cis-β-carotene to form a mobile compound called carlactone (Alder et al., 2012). However, a first step is likely to be the conversion of trans-β carotene (a carotenoid precursor) into 9-cis-β-carotene with a help of an iron-containing protein encoded by D27 (DWARF 27) of rice (Alder et al., 2012) and ATD27 of *Arabidopsis*. This gene, however, has yet to be found in species such as pea and petunia. After formation of carlactone, cytochrome P450 encoded by MAX1 converts it into SLs (Booker et al., 2005). A F-box protein encoded by RMS4/MAX2 and α/β hydrolase encoded by DAD2 are required for SL for branch inhibiting response (Hamiaux et al., 2012; Johnson et al., 2006; Stirnberg et al., 2002) of either bud release, the subsequent growth or both. Recently, a transcription factor BRANCHED1 (BRC1) of TCP family, CYCLOIDEA, PCF domain, known to inhibit bud release (Aguilar-Martínez et al., 2007) is proposed to act downstream of SL action (Braun et al., 2011) (also see Section 1.4.3.1, Theory C). Upon SL application, bud outgrowth is not inhibited in psbrc1 (pea mutants) whereas PsBRC1 is highly up-regulated in SL deficient mutants (eg. rms1), supporting that BRC1 is involved downstream of SL.

Despite having several genes associated with SL synthesis and signaling discovered, a complete pathway is yet to be found (Foo & Reid, 2013). The newly identified compound 'carlactone', which is yet to be detected in plant species, possesses SL-like activity in branching and germination assays (Alder et al., 2012). So, confirmation about SL being a bioactive inhibitor is still in question (also see Section 1.7). It may be possible that an unknown downstream product(s) of the SL pathway may be the active inhibitor; a perspective that is explored within the current thesis (Chapter 3).

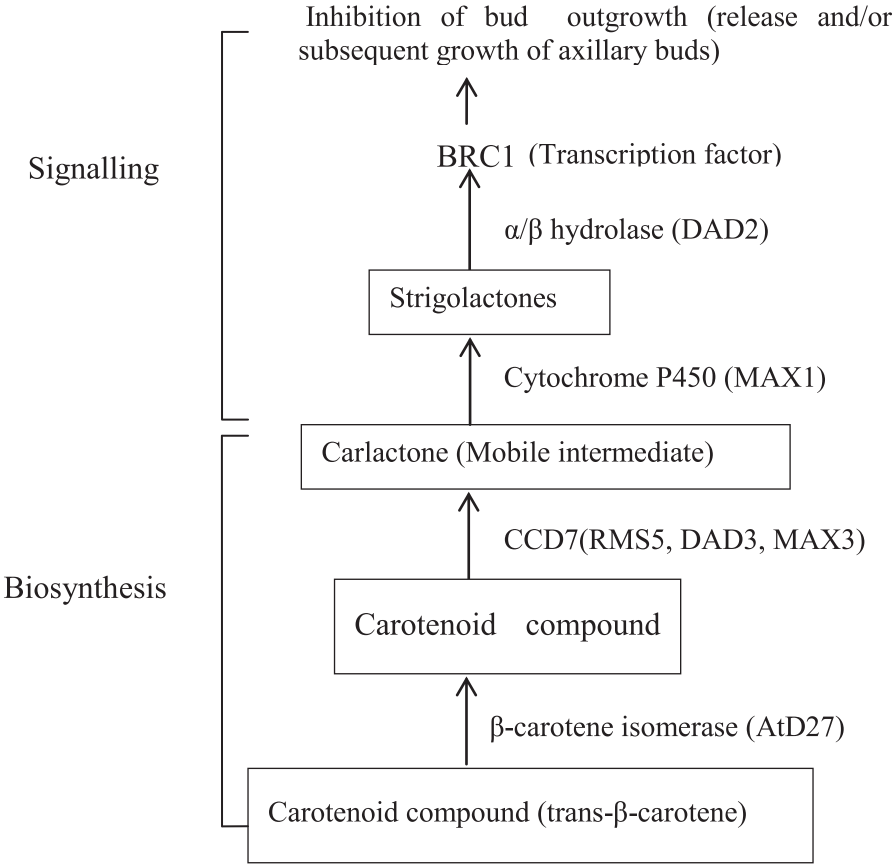


Figure 1.6 Proposed biosynthetic and transduction pathway of SLs to inhibit bud outgrowth (Foo & Reid, 2013). β-carotene isomerase and carotenoid cleavage dioxygenases (CCD7 and CCD8) are required for biosynthesis of SL, cytochrome 450 that encodes the apocarpetenous enzyme for SLs biosynthesis and downstream signaling, while the F-box protein and α/β hydrolase are required for responding to SLs. BRC1 that encodes for a transcriptional factor, is regulated by SLs.

1.3.3 Relationship between strigolactones and auxin

1.3.3.1 Auxin is required for biosynthesis of SLs

Auxin reduction either by decapitation of the shoot tip or application of a polar auxin transport inhibitor, caused a reduction in expression of the SLs synthesizing genes (Sorefan et al., 2003; Jonhson et al., 2006; Foo et al., 2005) suggesting that auxin is required for SLs biosynthesis. The expression of MAX3 (CCD7) in *Arabidopsis* was reduced by between 200 and 400-fold within 24 hours after decapitation or application of the auxin transport inhibitor NPA (1-N-Naphthalphthalamic acid) (Hayward et al., 2009). Likewise, expression of RMS1 (CCD8) was reduced by 10,000-fold below a stem phloem girdle in pea (Ferguson & Beveridge, 2009). When exogenous IAA was further applied at the site of decapitation, CCD8 expression was again restored (Sorefan et al., 2003). Hence, auxin is necessary for the expression of CCD8 and CCD7 genes responsible for the biosynthesis of SLs, suggesting the requirement of auxin for SLs production and that SLs act downstream of auxin. However, to confirm auxin's regulation of SL biosynthesis, direct quantification of SLs is still needed (Rameau et al., 2015).

1.3.3.2 Auxin may not be involved in action of SLs

In decapitated plants, which are therefore devoid of apically derived auxin, bud outgrowth was completely inhibited after SLs were directly applied to the buds (Brewer et al., 2009), suggesting auxin was not required directly for SLs to inhibit bud outgrowth. Supporting this hypothesis, GR24 also reduced branching in mutants of *Arabidopsis thaliana* that lack response to auxin (Brewer et al., 2009); hence auxin might enhance SLs synthesis, but SLs act independent of auxin.

1.4 Theories related to the mechanism of apical dominance and/or shoot branching

As explored further within the following sections, several theories have been proposed to explain the mechanism involved in apical dominance.

1.4.1 Nutritive theory

The earliest theory was the nutritive theory (Loeb, 1918) according to which, the apical meristems being the strongest sink for water and nutrients within the plant (i.e. for mineral ions and sugars), limit availability to axillary buds below some critical level. Thus when the apical meristem is present the axillary buds remain inhibited. Subsequent research threw considerable doubt on the theory, for example, in *Phaseolus*, axillary bud outgrowth was enhanced when soil nitrogen content was high (McIntyre, 1973), but this was unlikely to be due to lack of nitrogen in the bud as nitrogen applied directly to the inhibited buds (Cutter, 1972) did not influence bud outgrowth. Likewise, the concentration of mineral ions (Milborrow, 1974) and sugars (Wardlaw & Mortimer, 1970) within inhibited buds was not lower compared to those which were growing. In early studies, as reviewed by Phillips (1975), sugar was also thought to be important for bud outgrowth, but the role of sugar was considered to be secondary to that of hormones. During that time, no conclusive evidence was found to support sucrose being important for initial bud outgrowth. However, some recent papers (e.g. Mason et al., 2014) have again proposed a central role for sugars in rapid release of axillary buds. Having noted the existence of the nutritive theory with regard to apical dominance, futher details for and against this theory are described in Section 1.4.4.

1.4.2 Direct auxin action theory

The first classical model of apical dominance involving a plant hormone was proposed by Thimann & Skoog (1934), who suggested direct influence of auxin in inhibition of axillary bud outgrowth. It was based on the findings that decapitation of apical buds in pea plants released axillary buds but the bud outgrowth could be inhibited by auxin application to the cut surface. Evidence against this "direct theory" included that provided by Hall & Hillman (1975) who found radio-labelled auxin applied to the decapitated stump did not accumulate in axillary buds. Hence it can be concluded that auxin from the shoot apex does not enter the axillary buds to directly inhibit them.

A strong criticism against direct auxin theory is best explained in Snow's work published during the early 1930s. In his experiment using the two unequal shoot system of *Vicia faba*, the axillary buds on the inhibited shoot exhibited no axillary bud outgrowth as long as the dominant shoot was growing vigorously (Snow, 1937). When the dominant shoot was decapitated, then the axillary buds on the inhibited shoot grew out. Snow, himself at that time, criticized the direct role of auxin in inhibiting axillary buds by saying that if auxin moving down the stem inhibits axillary buds, then how could it inhibit the buds on the other shoot, as the major flow of auxin is basipetal rather than acropetal. Hence, as Snow himself concluded, an inhibitor other than auxin must be present that moves upward to cause inhibition of buds. At that time the identity of the inhibitor was unknown. Recently, SLs have been proposed as being the inhibitor (refer Section 1.3.1). Some people proposed that the inhibitor may be abscisic acid (ABA), but little good evidence for ABA being the inhibitor has been obtained (refer Section 1.4.3.1 Theory B).

1.4.3 Indirect auxin action theory

Despite auxin not directly inhibiting axillary buds, this hormone was still considered important for regulating the outgrowth of such buds. As explored further within the following sections, there are several theories based on an indirect role for auxin in inhibiting axillary bud outgrowth.

1.4.3.1 Hormonal interaction theory

A. Auxin - cytokinin interaction

The role of CK and auxin in the branching of shoots is well known to be antagonistic, with CK being promotive and auxin being inhibitive for bud outgrowth. CK directly releases lateral buds when applied directly to the buds (Wickson & Thimann, 1958). In contrast, auxin is thought to indirectly inhibit outgrowth of axillary buds, at least partly, by reducing CK availability in the buds. In support of this hypothesis, reducing apically derived auxin through decapitation leads to increased levels of endogenous CKs in the stem and/or xylem sap (Bangerth, 1994; Li et al., 1995). In *Phaseolus vulgaris,* CK transport from the root to shoot increased 40 times after decapitation (Bangerth, 1994). Likewise, the level of endogenous CK was raised 7 times within 6 hours, and 25 times within 24 hour, after decapitation of chickpea (*Cicer arietinum L*) seedlings (Turnbull et al., 1997). Levels of CK in the axillary buds increased about 6 hours after decapitation in pea, but whether or not such CK was delivery of root-produced CK to the axillary buds, or CKs within the stem, is still not known. However many ‘rms’ mutants of pea plants have reduced xylem sap CK but exhibit enhanced shoot branching (Beveridge, Murfet et al., 1997; Dun et al., 2006), suggesting that the CK derived from roots is not important for axillary bud outgrowth. The fact that the level of CKs in the shoot particularly at the stem near the node of pea plants started to increase three hours after decapitation, indicates *de novo* synthesis of CK in shoot tissue (Tanaka et al., 2006). However, the hypothesis of shoot-synthesis of CK was previously criticized as the CK present within the shoot may actually be derived from root-synthesized CK. This hypothesis was supported by the fact that axillary buds of rootless plants were not released into growth after decapitation when such plants were initially forced into growth by pre-treatment with GA (Woolley & Wareing, 1972). It was suggested that when the GA was applied to de-rooted plants, it reduced shoot-CKs by stimulating growth of the main axis. Hence, before decapitation, due to lack of endogenous CK, axillary buds were inhibited. It is therefore possible that CKs that are transported from roots are present in the shoot, but remain in an inactive form, such as glycosides, until the level of apically derived auxin is reduced. After decapitation, such CKs might have been activated and delivered to the corresponding axillary buds for their release.

Since the synthetic auxin 1-NAA (1-Naphthalene acetic acid) suppressed CK level when applied to the cut surface of a decapitated stem (Li et al., 1995), it could be suggested that auxin from the apical meristem is responsible for reducing the CK level. Supporting this hypothesis, the expression of Isopentenyl transferase (IPT1 and IPT2) CK biosynthesis genes was detected after decapitation, but was again repressed after applying indole-3 acetic acid (IAA) (Li et al., 1995; Tananka et al., 2006). Also, auxin could reduce CK level, either by stimulating the activity of CK oxidase that causes oxidative breakdown of CK (Palni et al., 1988; Zhang et al., 1995) or by glucosylation through inhibition of CK catalyzing glucosidases (Brzobohaty et al., 1994) (Figure 1.7). Thus, in intact plants, due to the presence of apically derived auxin, the level of CK may be reduced in the stem, resulting in less availability of CK to the axillary buds.

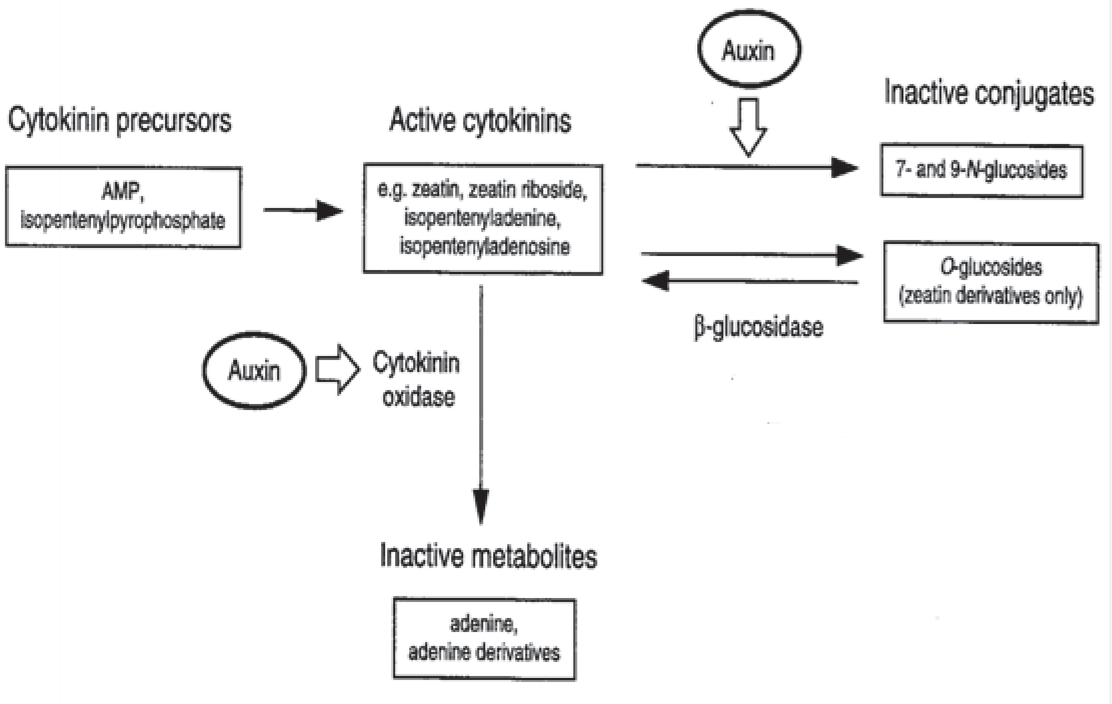


Figure 1.7 Potential points of control of CK biosynthesis by auxin (Coenen & Lomax, 1997). Open-shaped arrows showing the points where auxin could regulate enzyme activity to inhibit the formation of active CKs.

Despite much evidence to support the ability of CK to release axillary buds via reduced apically-derived auxin, there is still controversy about this mechanism in decapitation-induced axillary bud outgrowth; partly because release of the bud appears to be too rapid to involve hormone transport and metabolism (Turnbull et al., 1997). In chickpea, bud release was initiated 3 hours before the increase in level of endogenous CK after decapitation, suggesting that the level of CK in buds may not be responsible for the initial trigger of bud release. Likewise, in pea plants, the level of auxin was not reduced by decapitation within 4 hours, whereas bud release occured before any changes in auxin content in the stem near to the buds (Morris et al., 2005); supporting the existence of a non-auxin, decapitation–induced, rapid signal that stimulates bud release. The signal could be electrical or turgor pressure, as suggested by Urao (1999), as they could move faster than any hormonal signal. More recently, the the rapid signal is proposed to be sugars (Mason et al., 2014), which has been described in more detail within Section 1.4.4.

Despite the inhibitory effect of auxin on CK (refer Figure 1.7), there was also a significant increase in auxin level in buds after decapitation. In two-week old seedlings of *Phaseolus vulgaris*, removal of the apical bud lead to a five-fold increase in the auxin level in axillary buds within 4 hours (Gocal et al., 1991). Thus the increased level of both auxin and CK in buds and/or stem tissues about 4-6 hours after decapitation may have been important for growth of the released buds, rather than bud release *per se*. In fact, there is much evidence to support that CK alone is not enough to increase bud growth. Auxin or other hormones like GAs along with CK will be required for further shoot extension (Cody et al., 1985; Popenoe & Barritt, 1988).

It is true that there is a lack of strong evidence for the role of CK in bud release in decapitated plants. However, for intact plants, since CK released axillary buds when applied directly to the buds (Panigrahi & Audus, 1966; Wickson & Thimann, 1958), this hormone appears to be important in releasing axillary buds. Thus, one may need to draw a distinction between the mechanism operating in decapitated plants compared to intact plants. In the initial stage of the bud outgrowth process (Figure 1.1), CK could trigger bud release by influencing mitosis (Ferguson & Beveridge 2009; Zhang et al., 1996). It would be worth testing the influence of CK on the cell divison of buds at a very early stage of bud release. However, the experiments within this thesis have not explored this phenomenon as the focus of the thesis was on SL or branching inhibitor and its interaction with other hormones on shoot branching. However an acknowledgement of differences between decapitated and intact plants has been addressed within this thesis.

The hypothesis that axillary bud inhibition in intact plants was caused by less CK availability to the buds and higher levels of apically derived auxin (Li et al., 1995), was questioned (Beveridge et al., 1996). Highly branched mutants of pea and *Arabidopsis* possessed a high level of free auxin and reduced levels of CKs within the xylem sap, suggesting root-derived CKs might not be responsible for the increased number of shoots in such mutants. Highly branched SL mutants rms1, rms5 and rms4 (but not rms2) had highly reduced ‘xylem CK’ (X-CK) as compared to their wild-types (Beveridge, Murfet et al., 1997; Dun, Hanan et al., 2009) suggesting an unknown feedback signal regulated by RMS2 that reduces X-CK. Since SL branching mutants have elevated auxin, the feedback signal may be auxin that may have reduced the X-CK. However, decapitation of such mutants did not effect the level of X-CK (Foo et al., 2007). Also the rms2 mutant had elevated auxin (4.4 times more than wild-type) compared to other branching mutants (Beveridge et al., 1994) suggesting a feedback signal other than auxin (Dun, Hanan, et al., 2009). Like auxin, the feeback signal moves from shoot to root as grafting rms4 rootstock which have reduced X-CK did not reduce the X-CK in wildt-ype (scion), whereas the X-CK in rms4 scion was reduced when grafted onto wild-type rootstock (Beveridge, Murfet et al., 1997). Despite reduced CK, rms4 grafted over wild-type rootstock was highly branched, further confirming that root derived CK is less important for bud outgrowth. However, it does seem possible that the proposed feedback signal may have converted the active CK into inactive CK. As mentioned earlier in this Section, root CK may play an important role in bud release and the enhanced CK in the shoot may have been due to conversion of inactive CK into the active forms. In papers where reduced X-CK in rms mutant was reported, the measurements was undertaken for active CK such as zeatin ribosides (Beveridge, 2000; Beveridge, Murfet et al., 1997). In contrast to older pea plants (e.g. plants at floral initation stage) (Beveridge et al., 2000), X-CK in the rms mutant was not reduced in young pea seedlings (Foo et al., 2007) suggesting that root CK is important for bud outgrowth at the initial stage of growth when branching determines the phenotype of plants. Interestingly, shoot CK in rms mutants are not depleted compared to wild-type (Foo et al., 2007) and so it could be suggested that in the rms mutant, bud release may have been contributed by shoot-derived CK, but the origin of shoot CK may be the roots. The role of the proposed feedback signal in the rms mutant might be to maintain the CK status for homeostatic balance in branching. This also explains why highly branched rms mutants lack complete branching i.e., bud outgrowth at each node (Beveridge et al., 1996). Overall, the role of CK in bud release is highly supported, but the balance in CK status within a plant system may have been affected not only by auxin, but also other signals such as the proposed unknown feedback signal.

B. Interaction between auxin and other hormones

The role of abscisic acid (ABA) in apical dormance has been suggested, due to its role in inducing endogenous bud dormancy (Hocking & Hillman, 1975). Several early works have suggested the auxin-induced ABA inhibition of bud outgrowth (Eliasson, 1975; Tucker & Mansfield, 1971). Tomato plants grown under increased far red light contained high levels of IAA and ABA in young leaves and buds, respectively (Tucker, 1976). Such plants had suppressed branching, suggesting these two hormones may have been associated with each other for inhibition of branching. Later, much evidence failed to establish the correlation between ABA and the inhibition of branching. For example, studies have found no decrease in ABA content in lateral buds before and after decapitation (White & Mansfield, 1977), suggesting ABA was not important in bud inhibition imposed by apical dominance. Phillips (1975) suggested that ABA is mainly produced in mature foliage, so ABA is less likely to act as an inhibition signal from shoot apex. Thus, it seems reasonable to conclude that ABA may well be important in bud dormancy induced by mature leaves, but not in apical dominance imposed by the apex and young leaves.

Regarding the possible role of ethylene in apical dominance, there was no change in endogenous ethylene levels in buds or nodes after decapitation (Prasad & Cline, 1985), and direct application of ethylene promoters or inhibitors to inactive or to induce buds was without effect (Harrison & Kaufman, 1982). Hence ethylene may not be involved in axillary bud outgrowth.

With regard to GAs, auxin appears to increase synthesis of GAs (Ross et al., 2000), as both decapitation (Sherriff et al., 1994) and auxin transport inhibitors applied to elongating internodes (Ross, 1998) reduced GAs (GA_1) below the treatment site, while applying IAA to the decapitated stump further restored the GA_1 content. Increased GAs with IAA may indicate an influence of GAs in apical dominance. It could be argued that GAs may enhance growth of the primary/main shoot but without any effect on the inhibited buds. Since GA_1 enhances cell division or cell elongation in the subapical meristem (Talon et al., 1991), there is little doubt that primary shoot elongation is stimulated by GAs. On the other hand there is considerable evidence to support a role for GAs in subsequent growth of the released bud (Ali & Fletcher, 1970; Leakey & Longman, 1986; van Hooijdonk et al., 2011) but not in release of axillary buds.

C. Direct localized action between CK and SLs

According to this theory, auxin indirectly inhibits axillary bud outgrowth by enhancing the level of SLs (refer Section 1.3.2.1; Foo et al., 2005) and reducing CKs (Palni et al., 1988; Zhang et al., 1995). After removal of apically-derived auxin through decapitation in pea plants, there was increased expression of IPT1 and IPT2 below the treatment site, whereas expression of RMS1 and RMS5 reduced by 10,000 and 10–fold, respectively (Ferguson & Beveridge, 2009). Hence, it is possible that SLs and CK interact with each other in an antagonistic way. However, it is unknown whether or not SL and CK reduce the level of each other. Work by Beveridge & Ferguson (2009) strongly suggested that CK is always associated with bud release, although there may or may not be reduction in RMS1 and RMS5 gene expression. In their experiment, where axillary buds were released at the nodes, there was increased expression of IPT1 and IPT2 irrespective of RMS1 or RMS5 expression. It was suggested that for release and/or outgrowth of buds, SLs and CKs interact via regulating ‘BRC1’ (Braun et al., 2011), a gene that encodes transcription factor containing a TCP domain (Angular-Martinez et al., 2007). SLs up-regulate BRC1 expression whereas CK down-regulates BRC1 expression (Braun et al., 2011). Mutants lacking this gene are highly branched (Aguilar-Martizez et al., 2007). The direct localized action of SLs and CK at the bud (Dun et al., 2012) is strongly supported because ‘BRC1’ is mainly expressed in axillary buds. In pea, the transcript level of PsBRC1 (the gene analogue to BRC1) was high in axillary buds which were about 100-1000 times more than in floral buds, shoot apex and nodal tissue (Braun et al., 2011). However, this gene was not present in root tissues. Hence, in my view, anything that affects the delivery of CK or SLs to the vicinity of axillary buds via the vascular stream, may determine whether or not axillary buds are released.

This theory of “direct localized action between CK and SLs” can explain why axillary buds are released when CK is directly applied to the buds. For releasing buds, if xylem sap CKs in SLs mutants in pea plants are not involved (Beveridge, Murfet et al., 1997), then the endogenous CKs in nodes and/or buds (refer Section 1.4.3.1, Theory A) of those mutants are more important. Due to a reduced level of SLs in SL-mutant plants, the expression of BRC1 is also reduced. Hence, buds might have increased sensitivity to respond to the CK present in buds and/or nodes.

In contrast however, the "direct localized interaction between CK and SLs" theory is not able to explain initial bud release after decapitation, where the reduction of SLs and increase of CK is too slow to cause reduced expression of BRC1 for rapid initial bud release. Another criticism of direct localized action of SLs on bud inhibition was that SLs cannot directly inhibit axillary bud outgrowth if apically-derived auxin is absent (Crawford et al., 2010). GR24 applied basally to a one node segment of *Arabidopsis* (Crawford et al., 2010) and *Chrysanthemum* (Liang et al., 2010) was unable to prevent axillary bud outgrowth. However, after auxin was supplied apically to the node segment, GR24 significantly reduced the axillary bud outgrowth compared to that of auxin alone or without auxin (Crawford et al., 2010). Likewise, in two node segments, GR24 reduced the shoot length of the lower node, but not the upper node, suggesting that auxin transport from the axillary shoot of the upper node stimulated GR24 to reduce the axillary bud outgrowth of the lower node. Hence, if there was direct localized action of SLs on bud inhibition, SLs would have repressed buds of both upper and lower nodes. The authors mentioned that the auxin-strigolactone canalization theory could well address this problem (refer Section 1.4.3.2 Theory B). However, the experiments by Crawford et al. (2010) and Liang et al. (2010) were based on the resulting shoot length rather than bud release *per se*; so it is more appropriate to say that the direct localized action of SLs was criticized for subsequent growth of the released buds rather than bud release/outgrowth. Failure to indicate the stage of shoot branching in these papers is one of the examples where the mechanisms of shoot branching described is actually misleading. Further explanation to address this issue is mentioned in Section 1.4.3.2 Theory B.

1.4.3.2 Auxin transport theory

This theory is further divided into Theory A and Theory B, as described below.

A. Auxin canalization theory

The auxin canalization theory was first proposed by Sachs (1981); according to this theory, auxin transport from the apex in the main stem limits the flow of auxin transport from the axillary buds and, as a result, the axillary buds are unable to establish their own auxin transport stream into the main stem, resulting in inhibition. After decapitation, depletion of apically derived auxin in the main stem allows vascular connection between lateral buds and the main stem (Sachs, 1968). As a result, auxin travels from lateral buds to the main stem, hence buds are released. However, in soybean (*Glycine max*) the large axillary buds have well developed vascular connections with the main stem, but were still inhibited (Ali & Fletcher, 1970); and hence the hypothesis that auxin transport was associated with vascular connection for control of bud outgrowth was not fully accepted.

This canalization theory proposed by Sachs was later modified (Bangerth, 1989) to a model that emphasized the establishment of an auxin flow out of the bud, as compared with the establishment of vascular strands as originally proposed. Based on the requirement of auxin transport for the development of a sink such as fruits (Bangerth, 1989), it was hypothesized auxin flow from the first dominating shoot restricts the transport of auxin from the later-formed buds, and thus the buds remained inhibited. Hence, auxin export from a bud to the stem is necessary for bud outgrowth (Li & Bangerth, 1999). However, this theory emphasized only the importance of auxin in repressing axillary buds, without considering the role of an inhibitor as proposed by Snow in the 1930s (refer Section 1.4.2). Later both Prusinkiewicz et al. (2009) and Crawford et al. (2010) integrated SLs into the auxin canalization theory thus incorporating the hypothesis proposed by Snow.

B. Strigolactones and the auxin transport canalization theory

According to this canalization theory, in the presence of basipetal flow of auxin from the primary shoot apex, SLs moving acropetally in the main stem restricts the auxin flow out of the axillary buds into the main stem, and hence buds are inhibited (Prusinkiewicz et al., 2009, Crawford et al., 2010). As described in Section 1.4.3.1, using *Arabidopsis* (Crawford et al., 2010) and *Chrysanthamum* (Liang et al., 2010), basally applied GR24 failed to inhibit the axillary shoot length of a one-node stem segment, unless apically applied auxin was present. Likewise, on two-node segments of the same species, GR24 reduced the shoot length of the lower node but not the upper node. It was suggested that in the presence of a strong competing source of auxin, eg. the shoot apex (Prusinkiewicz et al., 2009), GR24 was able to reduce the auxin transport out of the axillary buds, but not when an apical supply of auxin was absent.

Prusinkiewicz et al. (2009) hypothesized that increased branching of SL mutants is associated with increased auxin transport. In *Arabidopsis*, SL deficient branching mutants (max4) had an increased accumulation of polar PIN-FORMED (PIN) proteins, an auxin efflux facilitator family for the polarity of auxin transport in the Polar auxin transport (PAT) (Bennett et al., 2006; Crawford et al., 2010) (Figure 1.8B). Supporting this hypothesis, a synthetic strigolactone 'GR24' reduced auxin transport out of the bud in wild-type as well as max mutants of *Arabidopsis* (Figure 1.8A) via reducing accumulation of PIN1 in xylem parenchyma (Crawford et al., 2010; Shinohara et al., 2013). It was suggested that GR24 reduced PIN1 accumulation via a clathrin-dependent mechanism (Shinohara et al., 2013). In presence of the clathrin inhibitor, A23, GR24 failed to reduce the PIN accumulation in the plasma membrane. However, unlike pharmalogical inhibition of PATS (for example application of N-1-naphthylphthalamic acid, NPA) (Brewer et al., 2009), GR24 requires a competing source of auxin (e.g. shoot apex) and acts systematically by dampening the canalization of auxin to the sink i.e., the main stem. Hence, as mentioned earlier in this section, the SL auxin canalization model is able to explain why basally-applied GR24 inhibits the axillary bud outgrowth of the lower node (e.g. in two node segments of *Arabidopsis* or *Chrysanthemum*) in the presence of auxin supplied from the shoot apex or lateral shoot of the upper node but not in the absence of apically derived auxin (Crawford et al., 2010; Liang et al., 2010).

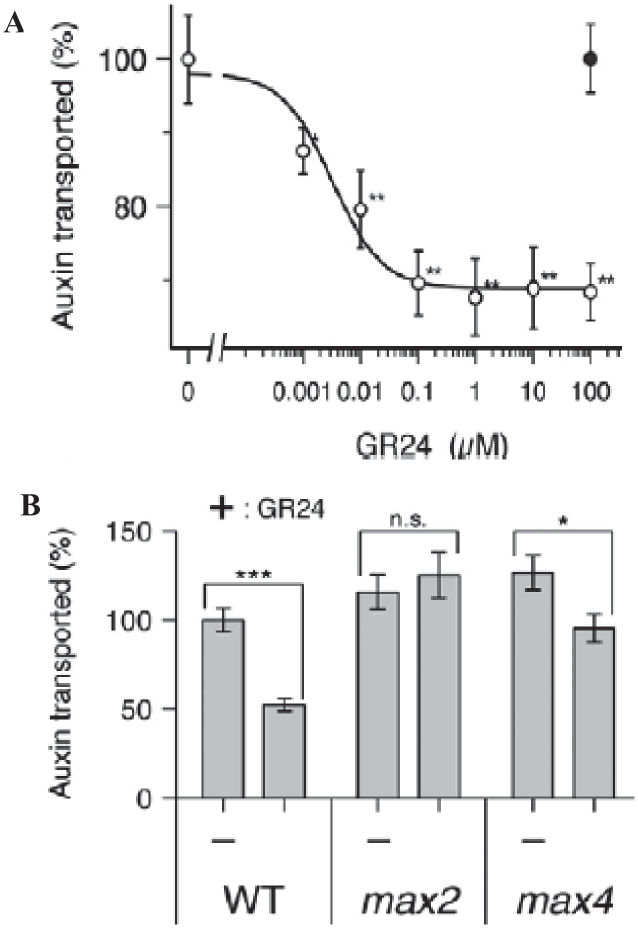


Figure 1.8 Effect of GR24 on polar auxin transport. (A) Dose-response of basipetal indole-3-acetic acid (IAA) transport to GR24. (B) The effect of 10 μM GR24 on basipetal IAA transport in wild-type, max4 and max2 stems (Crawford et al., 2010).

Hence, this theory explains why SLs mutants contain a higher level of free auxin than than that present in wild-type pea plants (Beveridge, Simons et al., 1997). In addition, the reason as to why that ccd7/ccd8 SL mutants, despite the presence of a shoot apex delivering apically derived auxin to the main stem, are highly branched (Beveridge, Simons et al., 1997) is made more clear through this theory. The integration of SLs with auxin canalization thus suggests that auxin alone could not inhibit bud outgrowth, and that an inhibitor that moves from the root to the shoot is required in order to explain the phenomena reported in the literature.

Failure to establish auxin transport from an individual bud, due to a high level of PAT from the growing apical bud (Sachs, 1981) or less auxin transport capacity of wild-type plants compared to the SLs mutants (Bennett et al., 2006), was criticized. Brewer et al. (2009), who found similar transport rates of radiolabelled IAA in both wild-type and mutant pea stems, suggests that the main transport stream of pea stems was not fully saturated.

The above two canalization theories strongly support the importance of auxin transport out of the buds for the development of shoot growth, but not the initial release. It is possible that CK stimulates bud release and helps released buds to transport auxin to the main stem for subsequent growth (Li & Bangerth, 2003). Since the auxin transport studies in branching mutants were carried out when plants had already developed their branches, the cause of the initial release of axillary buds in such branching mutants remains unknown. One could therefore conclude that the level of free IAA (Beveridge, Symons et al., 1997) or higher auxin transport from buds (Crawford et al., 2010) in branching mutants, might be due to SL deficiency or lack of SL responses resulting in increased auxin transport capacity from released buds to the main stem rather than from the buds that were not released. Several studies related with the auxin transport hypothesis were based on auxin transport capacity of released buds, rather than the buds at release or before initiation of release (Figure 1.1). Indeed, measurement of auxin transport out of the buds prior to release is required to prove whether or not auxin transport has occurred at the initial stage of release. As reported by Balla et al. (2011), the export of radiolabelled auxin ([^{14}C]IAA) from the buds of pea plants was evident mainly four hours after decapitation although a slight (not significant compared to the bud of intact plant) rise in such transport was observed in between 2 to 4 hours. Hence, auxin transport from the buds before the release in decapitated plants is still unknown as the initial release occurs within 2 hours after decapitation (Morris et al., 2005, also refer Section 1.4.4). However, measuring auxin transport out of the buds during the initial stage of bud release is rather challenging and seldom reported. As an alternative approach, application of NPA (an auxin transport inhibitor) has been used to provide some insights to the potential effects of changes in auxin transport. When NPA was applied to the buds at the earliest possible stage of release after decapitation, or CK application, it did not prevent the release (Brewer et al., 2009). Since NPA can be presumed to have reduced auxin transport from the buds (Bennett et al., 2006), failure to prevent the bud release by NPA supports the idea that auxin transport may not necessarily be important for bud release. Nevertheless, fast growing shoots produce more auxin and are able to inhibit other shoots. Such a phenomenon is best described as the correlative inhibition of a dominant shoot over inhibited shoot. In a two-shoot system of pea plants, the dominant shoot possess more auxin flow compared to that of dominated ones (Li & Bangerth, 1999). Hence the apical bud, which has strong PAT, grows out faster and becomes dominant, whereas the lateral buds remain inhibited.

For intact plants, the integrated theories implying importance of CK as well as auxin transport is quite convincing, but in decapitated plants where the initial release occurs before increase in CK level in or near buds, some quicker, non-hormonal initial release mechanism is required to explain the phenomena reported.

1.4.4 Non hormonal / sucrose theory

According to this theory, the rapid, initial release of axillary buds following decapitation is caused by sucrose. Since changes in cytokinin and auxin concentrations appear to be too slow to account for rapid bud release following decapitation, (Morris et al., 2005; Turnbull et al., 1997) a rapid non-hormonal signal was proposed as being a cause of decapitation induced bud release (Turnbull et al., 1997) (refer Section 1.4.3.1 *Theory A*). Recently it has been proposed that sucrose may be the rapid signal (Mason et al., 2014). The flux of sucrose is able to move 150 times faster than auxin in the stem and increases rapidly in axillary buds after decapitation, before observable growth of the bud. According to this non-hormonal theory, the apical bud is a strong sink for sucrose from the source (eg. leaves), but decapitation induces translocation of sucrose towards the axillary buds within an hour, and induces bud release. To further support sucrose being involved in bud release, externally applied sucrose released axillary buds of intact pea plants when applied through the cut petiole (Mason et al., 2014). Hence, deficiency of sucrose in axillary buds might have enforced inhibition of buds. Although sucrose may be important in the rapid release of axillary bud when the shoot is decapitated, in intact plants CK may well be the initial release stimulant. This idea is supported by many observations of plant in tissue culture. For example in plantlets of *Zantedeschia* with intact apices, grown *in vitro* with a high concentration of sucrose (30 mg dm^{-3}) in the media, axillary shoots were not released unless CK was supplied (Kozak & Stelmaszczuk, 2009).

For bud release associated with decapitation, it is possible that sucrose might have increased the sensitivity of axillary buds to respond to CK already present in the buds and/or nodes via reducing the effect of BRC1, the gene that may act downstream of SLs). Indeed, sucrose was found to inhibit the expression of BRC1 within two hours after decapitation (Mason et al., 2014). Thus CK present in stem tissues, even if at low levels (Chen et al., 1985), might influence the release of axillary buds once the expression of BRC1 is down-regulated. Hence, although sucrose appears to be the initial trigger for bud release following decapitation (Mason et al., 2014), the role of CK in bud release cannot be ignored. Future research on the link between sucrose and CK in stimulating bud release would therefore be useful, both in decapitated and intact plants.

1.5 General morphology and growth habit of different horticultural plants in relation to branching

In Chapter 2, a range of horticultural species were selected for quantifying the relationship between branching and the novel hormonal group, the SLs. However, much of the published research reported for SL in relation to shoot branching are based on annual (e.g., pea, rice) or biennial plants (e.g., *Arabidopsis*), but may not be relevant to perennial, woody plants. For example, woody perennials possess multiple phases of vegetative growth having proleptic shoot developing from lateral buds of the previous season and sylleptic shoots developing from lateral buds of the current season. So, branching control mechanisms for plants such as kiwifruit, apple and Japanese Maple, studied in Chapter 2, may not be exactly the same as that of annual or biennial species. The role of SLs in shoot branching is further elaborated for the herbaceous perennial *Zantedeschia* (Chapter 4 and Chapter 5) and the annual *Petunia* (Chapter 3). Hence, within the sections below, it was intended to describe the basic morphology of the species studied in relation to branching.

1.5.1 *Zantedeschia*

Zantedeschia are one of the most important cut flower and potted flowering plant in the world (Kuehny, 2000). The coloured *Zantedeschia* are tuberous day-neutral plants, having determinate shoot growth, with a sympodial shoot habit (Funnell & Go, 1993). The tuber carries dominant buds of both the previous and the current season accompanied with axillary buds (Figure 1.9). Within an intact plant (i.e. dominant buds remain intact), the dominant buds typically grow into primary shoots, with growth of secondary shoots arising from the leaf axils of the primary shoot (Figure 1.9). As in other perennials such as kiwifruit (see Section 1.5.3.2), *in Zantedeschia* the bud developed in previous season produce a primary shoot (or the proleptic shoot) and axillary buds arising during the current season's growth produce sylleptic shoots (Halligan et al., 2004).

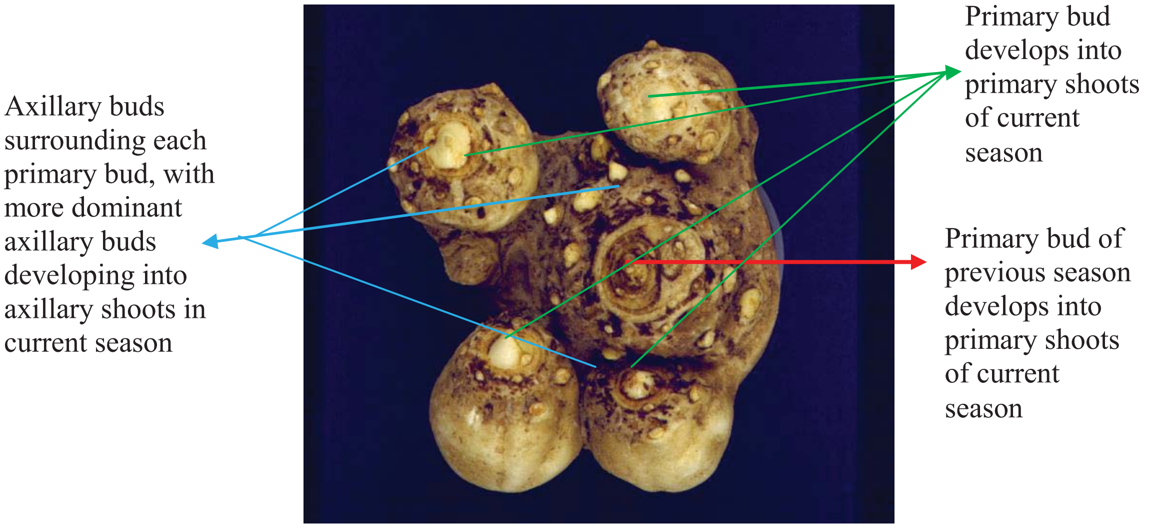


Figure 1.9 Flowering sized tuber of *Zantedeschia* with dominant and axillary buds. Dominant buds are contributed by the previous season (at centre of a tuber) as well as the current season (at periphery within a tuber). Figure contributed by Keith A. Funnell.

Since the number of buds which outgrow to become shoots is related to the flower yield (Funnell et al., 1992; Subbaraj, 2011)), it is of primary horticultural importance that a high number of buds are released. One of the approaches to achieve an increased number of shoots is application of plant hormones to the tubers or the plants.

Application of GA onto the tubers has been found to enhance the flowering shoots (Funnell et al., 1992) whereas the combination of GA and CKs stimulated more shoots when applied to the plants (Subbaraj et al., 2010). However, depending upon the growth stage of *Zantedeschia*, the response of the plants to the hormones varied. During leaf emergence externally applied CK was effective in producing more sylleptic branching, whereas simultaneous application of GA was required for increasing axillary shoots during later stages of the annual growth cycle. The difference in efficacy of CK in different stages was due to the type of bud dormancy; paradormancy (bud inhibition imposed by apical dominance) in early stages and endodormancy (bud inactivity due to interal bud status) at later stages. Due to endodormancy at later stages of the annual growth cycle GA was required along with CK for inducing more sylleptic branches from within the primary shoot (Subbaraj et al., 2010). Since GA helps in breaking dormancy (Cody et al., 1985), this hormone was essential for inducing branching in later stages. Hence, given the evolving understanding of hormonal interactions (refer Sections 1.3 and 1.4), as well as CKs and GA, other hormones such as strigolactones should be considered for understanding shoot branching of *Zantedeschia*. Hence, within this thesis, *Zantedeschia* was taken as a model plant for studying the role of SLs, CKs, gibberellins and their interactions in shoot branching.

1.5.2 *Petunia*

Petunia ×hybrida (*Petunia*) is often used in studying genetic control of plant form due to its distinct shoot architecuture (Napoli, 1996) as well as the availability of mutants having branching phenotypes (Napoli & Ruehle, 1996). The vegetative growth of *Petunia* is monopodial followed by sympodial branching of the reproductive shoot axis, comprising other shoot modules known as anthoclades (Child, 1979; Napoli & Ruehle, 1996). From these anthoclades, axillary branches arise that terminate in a flower (Snowden & Napoli, 2003). The extensive growth of the apical shoot illustrates the apical dominance of the wild-type *Petunia*, with the underlying genetic control illustrated by mutation of the branching gene ‘DAD’ (decrease apical dominance) evident by induced growth of axillary buds in various mutants. The dad mutants possess highly branched phenotypes with few pleiotropic effects (Figure 1.10) (Napoli & Ruehle, 1996), as evident within the ramosus (rms) mutants of pea (Morris et al., 2001), and the more axillary growth (max) of *Arabidopsis* (Stirnberg et al., 2002; Turnbull et al., 2002).

Three independent DAD loci controlling branching in petunia have been identified so far, and mutations in any of these loci result in plants with an increase in number of basal axillary shoots accompanied with decreased height (Figure 1.10) (Napoli, 1996; Simons et al., 2007; Snowden & Napoli, 2003). The pattern of branching in the dad1 mutant involves increased branching in basipetal and acropetal directions, and also second-order branching, which is absent in wild-type *Petunia* plants (Napoli et al., 1998). In comparative analysis of branching between dad mutants and wild-type, it was noted that the wild-type lacks branching in all four zones of the vegetative shoot axis, except zone II (Snowden & Napoli, 2003), whereas other mutants exhibit branching in other zones, viz I, III and IV (refer Figure 1.10). Furthermore, the branching potential of wild-type ‘V26’ has low (less frequency) branching potential in zone II as compared to other mutants, supporting the fact that wild-type ‘V26’ exhibits a higher degree of apical dominance as compared to branching mutants (Snowden & Napoli, 2003). Thus, given the depth of detailed understanding of the underlying genes and gene expression involved, this variation in branching among different varieties of *Petunia* makes this species useful as a model plant for studying shoot architecture within this thesis. Several reciprocal grafts between ‘dad’ mutants and wild-type *Petunia* (as described in Section 1.3.1.) has revealed the presence of an inhibitor that moves from the root to shoot. The putative inhibitor is thought to be SLs as the gene ‘DAD’ encodes CCD7/CCD8 required for SL synthesis. In addition, a synthetic strigolactone, GR24, reduced the bud outgrowth in SLs-deficient dad mutants (Hamiaux et al., 2012); further supporting SLs as being important in inhibition of branching. Despite showing inhibition of bud outgrowth by application of SLs, the active compound is not yet known, but may be a specific SL, or an SL precursors (Boyer et al., 2012).

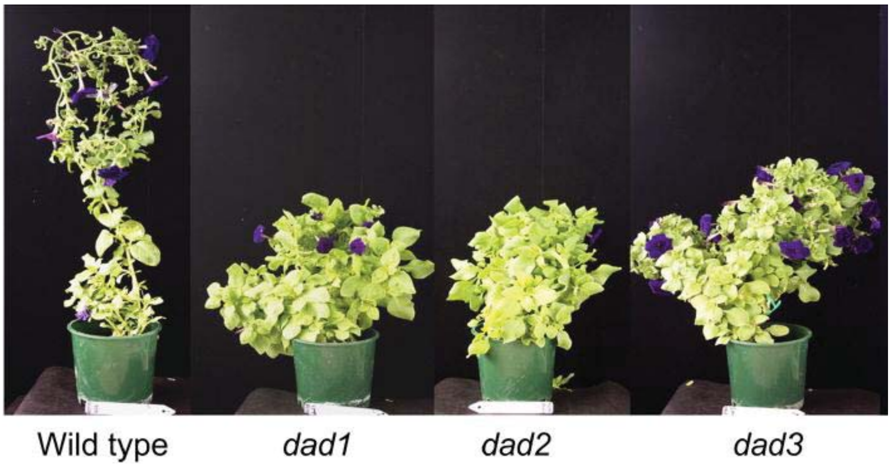


Figure 1.10 Growth habit of different mutants of *Petunia* including wild-type. Wild-type *Petunia* showing high apical dominance while the rest; dad1, dad2 and dad3, are showing a profuse branching phenotype (Simons et al., 2007).

1.5.3 Woody perennials

Branching of woody perennials is affected by both apical dominance and apical control; where apical dominance represents dominance of apical buds over release of lateral buds and apical control is dominance of the terminal/apical shoot over growth of released lateral buds (Cline, 2000; Wilson, 2000) (also refer Section 1.2). In woody perennials, it is often found that plants with weak apical dominance in the current season's growth may give rise to plants with strong apical control in the following season; a phenomenon known as excurrent branching (Brown et al., 1967). On the other hand, plants with strong apical dominance in the current season, or complete inhibition of axillary buds may become become highly branched in the following growing season (Brown et al., 1967). Hence, applying similar approaches to study the model of shoot branching in herbacious plants may not be always relevant for studying the model of shoot branching in woody perennials. Likewise, interpretation of the shoot branching model of woody perennials is often compared with that of herbaceous plants without addressing the morphological and physiological complexity of woody perennials. However, within this thesis, while shoot branching of woody perennials was not studied in detail, an attempt has been made to determine what if any role may exist for the newly identified hormonal group of SLs. This may help further consideration of the phenomenon of apical dominance and apical control in woody perennials. As a representative group of woody perennials, three species that are commercially important were investigated (Chapter 2), and are considered below.

1.5.3.1 Apple

During the juvenile phase, branching of apple (*Malus domestica* Borkh.) is monopodial (Lauri, 2007), with increased vigour of the vegetative proleptic branches at distal positions**.** The differential growth of laterals along the parent shoot occurs as early as bud burst; however only the distal buds develop and grow vigourously, inhibiting other lateral shoots, suggesting correlative inhibition of basal shoots over upper shoots.

Control of shoot vigor in terms of reduced branch number and shoot length through the use of dwarfing rootstock, is a common horticultural practice with apple. Such rootstock-induced dwarfing is mainly related to modification of endogenous hormonal transport. It was suggested that dwarfing apple rootstocks reduced scion vigour by reducing IAA transport from shoot to root, and limiting the availability of root-produced CK in the shoot (Lockard & Schneider, 1981). Such a shoot-root-shoot signaling mechanism was tested by blocking the basipetal flow of auxin (van Hooijdonk et al., 2010, 2011). Following NPA application there was a decrease in the number of secondary i.e., axillary shoots, whereas exogenous application of CK increased shoot number, suggesting reduced auxin might have reduced the synthesis of CK in the roots. Hence, these studies convincingly emphasized the importance of CK, auxin and shoot-root-shoot signaling in branching of apple, but did not include the possible role of SLs. As a first step in exploring what if any role SLs may have in apple, within the current thesis the amounts of SLs were comparatively analysed in dwarfing and vigourous apple rootstocks (Chapter 2). In fact, this study was the first to identify the presence of SL within apple.

1.5.3.2 Kiwifruit

Kiwifruit (*Actinidia* Lindl.) is a dioecious, deciduous, perennial vine with vigorous vegetative growth (Ferguson, 1999). Shoot branching within the kiwifruit vine is composed of mixed axes, and has primarily a sympodial branching pattern (Halle et al., 1978). A commercially grown kiwifruit vine consists of a trunk in its proximal part, while the distal part consists of two main horizontal branches (proleptic shoots) oriented in opposite directions along the row. Such branches bear several buds which contain a number of preformed leaf primordial (Walton et al., 1997) and, upon bud break, they give rise to axillary shoots (sylleptic shoots) in the next growing season. When they subsequently develop, these axillary shoots may be terminated or non-terminated.

As in other fruit crops like apple, recent studies have focussed on understanding the rootstock-scion relationship, including the influence of plant hormones to control vegetative vigour (Clearwater et al., 2004). However, studies to understand hormonal influences in shoot growth and branching of kiwifruit vines are still limited, except some studies performed *in vitro* (Vattiprolu, 2012) where the response to exogenously applied CK and gibberellins are basically the most studied hormones. In another study, the hypothesis that shoot-root-shoot signalling (as described for apple (van Hooijdonk et al., 2010, 2011), (refer Section 1.5.2.1) being responsible for branching, was investigated (Vattiprolu, 2012). In contrast to apple (van Hooijdonk et al., 2010), restricting PAT from shoot to root of the kiwifruit primary shoot, increased the number of sylleptic axillary shoots, and suggested that it might be due to the possible role of other root-produced plant hormones like strigolactones (Vattiprolu, 2012). Within the context of the current thesis therefore, in this scenario a reduced flow of auxin from the shoot may have reduced the synthesis of SLs in roots, resulting in increased formation of secondary/axillary shoots. Hence, as explored further within this thesis, quantifying endogenous SLs in kiwifruit may help to confirm this hypothesis.

1.5.3.3 Japanese Maple

There are different cultivars of Japanese Maple (*Acer palmatum*) that exhibit different branching phenotypes. Some are highly branching whereas others are highly apically dominant with few or no axillary shoots (Funnell, 2014). In addition, there are delayed branching cultivars of Japanese Maple. This delayed branching habit is characterized by a complete inhibition of buds in the current season, followed by outgrowth of the buds in the following season. In cultivars with high apical dominance, possibly either bud release may have been inhibited or the axillary bud may not have developed. There has been some work on understanding the shoot architecture of *Acer* (Sakai, 1990; Verdú &

Climent, 2007), but research on plant hormones in relation to shoot architecture of Japanese Maple is limited. Within the current thesis therefore, studying shoot branching in relation to plant hormones including a novel hormone SLs, a new insight might be gained.

1.6 *Orobanche*: Life cycle and host interaction

In Chapters 2, 3 and 4, for studying the potential involvement of SLs in shoot branching, a bioassay based on the germination of seeds of *Orobanche minor* was developed and utilized. As illustrated previously with plant root exudates (Joel et al., 1995), once developed this type of assay would actually help in identifiying as well as estimating the relative concentrations of SLs present, because SLs are the potent germination stimulants of *Orobanche* spp (Matsuura et al., 2008; Matusova et al., 2005). In contrast to species like *Orobanche densiflora*, *O. gracilis* and *O. hederae,* seeds of *Orobanche minor* were previously noted as being able to germinate in root exudates of a wide range of plant species (Fernández-Aparicio et al., 2009). Hence, within this thesis the advantage of selecting this species for developing a bioassay is that the analysis of endogenous SLs can be undertaken for different crop species.

Broom rape belong to the family *Orobanchaceae*, which has more than 150 species represented within it (Barker et al., 1996). While collectively all known as broom rape, the major species *O. ramosa*, *O. aegyptiaca*, *O. minor, O. cernua and O. crenata* are widely distributed throughout the Middle East, India and large parts of Europe and North America (Visser, 1989). They are the notorious root holoparasitic plants, that lack chlorophyll and obtain all the assimilates they need from the host plant species (dePamphilis & Palmer, 1990; Kusumoto et al., 2007). They have a broad host range, affecting the major crops like legumes (chickpea, and pea), Solanaceous (eggplant, tomato, tobacco, potato), Umbelliferous (carrot, parsley, celery), Brassicaceae (cabbage, lettuce and cauliflower) and *Helianthus* (sunflower) species. More specifically, *Orobanche minor*, being one of the most devastating species, is noted as being able to infect hundreds of a wide range of host species from Ranunculaceae to the Poaceae (Rumsey & Jury, 1991).

1.6.1 Life cycle of *Orobanche* spp.

The life cycle of *Orobanche* passes through different phases (Figure 1.10) and begins with seed germination, which is stimulated by host-root-exuded chemical signals (Kebreab & Murdoch, 2001) and, in the case of *O. minor*, this chemical signal specifically being SLs. However, the seed responds to stimuli after being exposed to moist and suitable temperatures for several days, called ‘preconditioning’ (Joel et al., 1995). Following germination, seed radicles attach to the host plant’s root with the help of haustorium (Joel et al., 2000; Thorogood et al., 2009a; Westwood et al., 1998). The haustorium penetrates the host tissue to connect to the vascular system, followed by tubercle development that helps to absorbs nutrients from the host. Once established, the parasitic plant develops a shoot which emerges above the soil, flowers and then produces seed. These seeds may either disperse or remain in the soil for many years, and again, each seed begins its new growth cycle after it perceives the presence of a host (Matusova & Bouwmeester, 2006).

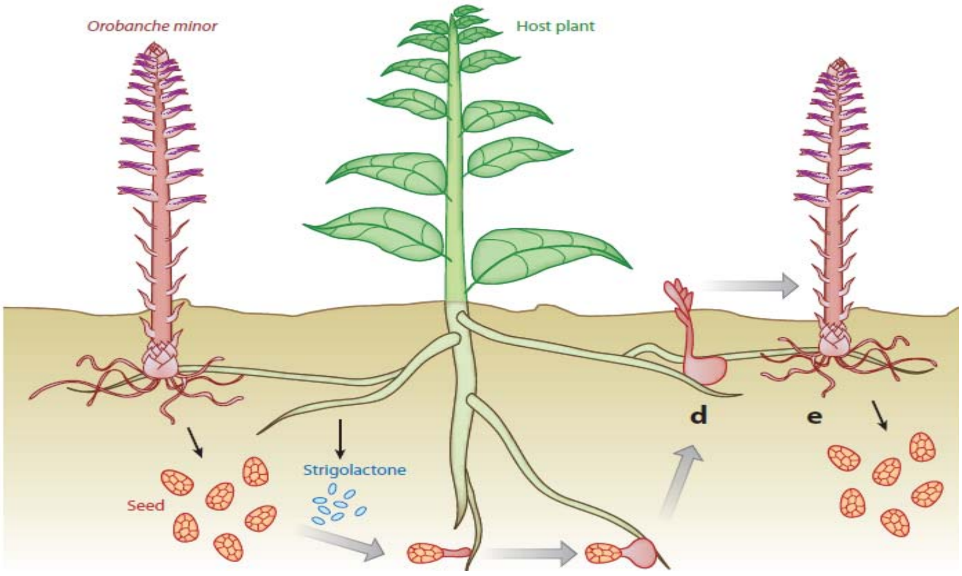


Figure 1.11 Life cycle of *Orobanche* spp. *and Striga* spp. (a) the seeds germination is stimulated by host-derived strigolactones, (b) seed radicle attached to host root via haustoria, (c-d) the seedling develops into a flowering shoot, (e) the weed produces a large number of seeds which get dispersed (Xie et al., 2010).

1.6.2 Germination stimulants

Host plants produce SLs, which act as a germination stimulant during interaction between the host plant and the parasite (Bouwmeester et al., 2003). The first germination stimulant identified was the SL known as 'Strigol', which was identified in the false host cotton, and induced germination of S*triga* spp (Cook et al., 1972). At present, more than 20 different forms of SLs have been isolated from root exudates of different plant species (Xie et al., 2013).

All of the natural SLs have a tricyclic lactone (ABC part) but the subsituents on A- and B- rings may vary (Figure 1.12). For example, the A- and B- rings carry methyl, hydroxyl, acetyloxly etc. The C- ring binds to a butenolide group (D ring) through an enol ether bridge to (Xie et al. 2010) which is the common structural feature preserved in all natural SLs (Zwanenburg et al., 2009). Likewise, all SLs contain the same D ring moiety (Figure 1.12), thus potentially enabling detection of unknown SLs using liquid chromatography/mass spectrometry (LC/MS) (Xie et al., 2010). In Chapter 3, detection of SLs within plant samples was attempted based on the presence of ions corresponding to D ring moiety.

Table 1.1 Different forms of strigolactones present in some of the plant species noted as acting as germination stimulants of *Orobanche* spp.

Germination stimulants	Plant species	Reference
Strigol and Strigyl acetate (First isolated strigolactone)	Cotton (*Gossypium hirsutum L.*) *	(Cook et al., 1972)
	Sorghum (*Sorghum bicolor)* Maize (*Zea mays*) Proso millet (*Panicum miliaceum*)	(Siame et al., 1993)
Sorogolactone	Sorghum (*Sorghum bicolor)*	(Hauck et al., 1992)
Alectrol	Cowpea (*Vigna unguiculata*)	(S. Müller et al., 1992)
Orobanchyl acetate	Cowpea (*Vigna unguiculata*)	(Matsuura et al., 2008)
Orobanchol (First germination stimulant of *Orobanche* isolated)	Red clover (*Trifolium pretense*)	(Yokota et al., 1998)
	Tobacco (*Nicotiana tabacum* L.)	(Xie et al., 2007)
	Arabidopsis thaliana *	(Goldwasser et al., 2008)
Solanacol (a tetradehydro-strigol isomer)	Tobacco (*Nicotiana tabacum* L.)	(Xie et al., 2007)
-Deoxystrigol	Sorghum (*Sorghum bicolor)* Maize *(Zea mays)* Pearl millet (*Pennisetum typhoideum)*	(Akiyama & Hayasi, 2006; Awad et al., 2006)

* Non-host species of *Orobanche spp*

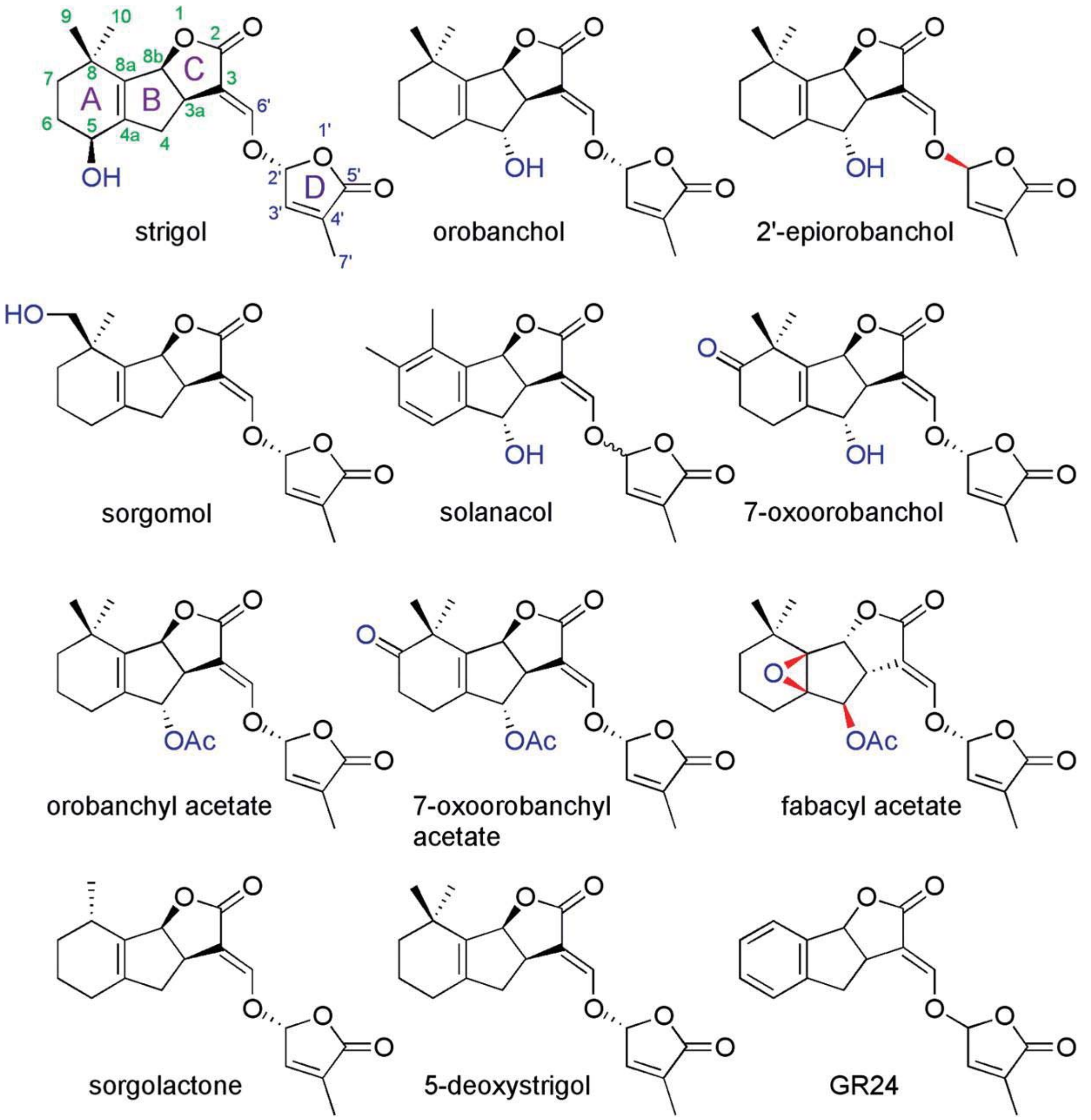


Figure 1.12 Chemical structure of different forms of Strigolactones (Yoneyama et al., 2010) including a synthetic strigolactone GR24.

1.7 Summary, rationale and thesis objectives

Since constraints to horticultural productivity is often associated with branching pattern (as discussed in Section 1.2), understanding plant hormone interactions that affect shoot branching potentially has importance for improved productivity of many commercially valuable crops. However, knowledge about the involvement of hormones in shoot branching and apical dominance is still limited. Recently, different grafting techniques and classical physiological approaches, combined with molecular genetics and transgenic approaches, have started to increase our understanding of the underlying mechanisms of apical dominance and/or shoot branching, as affected by plant hormones. From these studies, a novel signal postulated to exist many years ago (Snow, 1937), has been identified as probably being a strigolactone involved in controlling shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). This discovery may help to resolve a gap in our understanding of the mechanism of shoot branching, and could be of great significance in increasing production of different horticultural crops like kiwifruit, where canopy architecture is of considerable importance (Selenznyova et al., 2002).

In 2008, after the discovery of SLs being a putative hormone group probably involved in controlling shoot branching branching (Gomez-Roldan et al., 2008; Umehara et al., 2008), there were extensive studies on the involvement of SLs in shoot branching, particularly in the field of molecular biology. During this time rapid progress has been made in determining several genes associated with the biosynthetic pathway of SLs. Yet, knowledge of the complete biosynthetic and signaling pathway of SLs is still lacking. So, though widely accepted, it still requires confirmation as to whether SL is a bioactive compound that inhibits axillary buds (Foo and Reid, 2013), both in terms of bud release and subsequent growth. As more than one form of SLs are present in a single plant species (Xie et al., 2013), it is possible that different forms of SLs (Yonemaya et al., 2010) may not have the same physiological roles, e.g. controlling shoot branching. In order to confirm that SLs are bioactive branch-inhibiting compounds, as well as molecular genetics, other approaches should be used such as SL biosynthetic inhibitors, measurements of endogenous levels (Foo & Reid, 2013), and direct application. Rather than focusing on molecular approaches the investigations within this thesis therefore attempted to add to the existing and rapidly developing body of knowledge, by use of a physiological approach on horticularally important crops that included quantification of endogenous SLs (Chapters 2 & 4; Figure 1.13) and direct application (Chapters 3, 5 & 6). In this thesis, quantification of SLs was done using a biological assay based on germination of a parasitic weed seed *Orobanche minor*. In addition to the crops being horticulturally important, as a research strategy one further point of distinction in the current thesis was exploring the applicability of current models to perennial plant species, both herbaceous and woody (Chapters 2).

Recently, several studies reported the total level of SLs in root exudates but these levels may include SLs inactive in branching, inactive precursors, or metabolites of the putative active SLs (Foo & Reid, 2013). Since the SLs that may actually inhibit axillary bud outgrowth are still unknown, in the current thesis a series of experiments were undertaken that attempted to separate the bioactive compound(s) from inactive precursors, and chemically identify it utilizing a combination of biogical assay (included branching specific and SLs specific) as well as liquid chromatography/mass spectrometry (Chapter 3; Figure 1.13).

The broad aim of this study was to understand the hormonal mechanism of shoot branching for modifying shoot architecture, and the possible implications for crop yield and quality of relevant horticultural crop species.

The specific objectives were to:

- determine the physiological relationship between SLs and branching inhibition (Chapters 2 & 4),
- investigate further, the specific identity of the putative branch inhibiting hormone (Chapter 3),
- examine if externally applied strigolactone inhibits shoot branching, and further determining the stage of shoot branching at which strigolactone is important (Chapters 5 & 6).
- investigate the interaction of strigolactone with other hormones, particularly CK, in bud release and/or subsequent growth (Chapters 5 & 6).

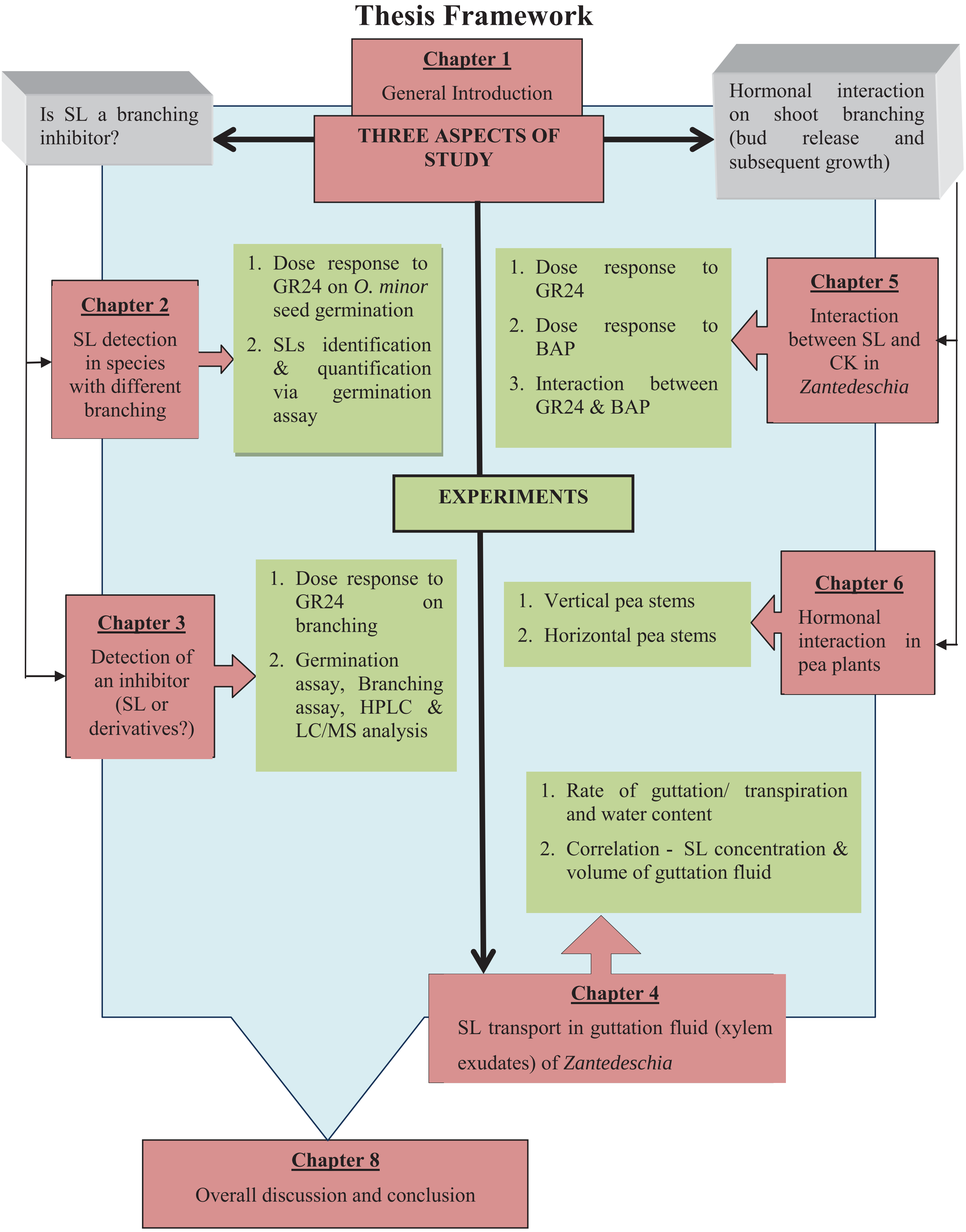


Figure 1.13 Thesis outline presenting chapters and experiments with associated research targets.

2 Germination assays using seeds of *Orobanche minor* for detection of strigolactones

2.1 Introduction

Strigolactones (SLs), recently proposed as a branch inhibiting hormone (Gomez-Roldan et al., 2008; Umehara et al., 2008), are the carotenoid-derived compounds produced by action of CAROTENOID CLEAVAGE DIOXYGENASES, CCD7 and CCD8 (Matusova et al., 2005). These compounds are also known to trigger the germination of parasitic plant seeds of *Striga* spp. and *Orobanche* spp. (Bouwmeester et al., 2003; Matusova et al., 2005; Muller et al., 1992) and induce hyphal branching of symbiotic fungi (Besserer et al., 2006). With regard to the role of SLs in branching, this novel hormone has the potential to become quite important in commercial horticulture where shoot branching is directly related with crop productivity. However, study of this hormone has been done only in few horticultural species and the identification of endogenous SLs is limited only to grass species and a few legumes as listed in Ćavar et al. (2014). Recent investigations have detected SL synthesizing genes that encode CCD7 and CCD8 in *Actinidia* Lindl. (kiwifruit) (Ledger et al., 2010), *Dendranthema grandiflorum* (chrysanthemum) (Liang et al., 2010) and *Solanum lycopersicum* (tomato) (Vogel et al., 2010), suggesting the existence of SLs in different horticultural plant genera and species. However, the precise physiological relationship between natural SLs and shoot branching is yet to be known.

It is likely that SLs are mainly synthesized in roots and move upward to inhibit axillary bud outgrowth, since all natural SLs have been detected from root exudates (Xie et al., 2010) and, one of the SLs, ‘Orobanchol’, has been detected in xylem sap (Kohlen, 2011). About 19 different forms of SLs have been detected so far (Koltai & Prandi, 2014); still detection and quantification of this hormone is difficult as SLs present *in planta* occur in extremely minute quantities (Boyer et al., 2012; Matusova et al., 2005; Rameau, 2010) and thus remains challenging in the context of plant developmental studies (Dun, Brewer, et al., 2009). In previous studies of SLs in shoot branching, a germination assay of seeds of the *Orobanche* family was utilized to determine whether SLs were present in different species such as *Pisum sativum* (pea) (Gomez-roldan et al., 2008) and *Oryzae sativa* (rice) (Umehera et al., 2008; Cardoso et al., 2014). During the identification process of SLs in such studies, the germination assay was useful for initially detecting SLs within the plant samples and once detected, techniques like LC/MS were useful to identify the SLs present.

SLs are the potent germination stimulants of parasitic weed seeds (Matusova et al., 2005). There are also two other non-SL compounds sesquiterpene lactones and dihydrosorgoleone that are known to stimulate germination of some parasitic weed seeds (reviewed in Boustweer et al., 2003). However, such compounds have been found to stimulate germination of *Striga* species which belong to Orobanche family. In fact, none of the sesquiterpene lactones were able to germinate different *Orobanche* species (de Luque et al., 2000) including *O. minor* (reviewed in Thorogood et al., 2009b). So far, only SLs and their synthetic analogues have been shown to induce *Orobanche* seed germination (Yoneyama et al., 2006). So, in contrast to *Striga*, *Orobanche* species appear to have more strict germination requirements. Hence, SLs for germination of *O. minor* is essential, suggesting that a biological assay based on germination of *O. minor,* for detecting SLs is highly specific.

Determination of a dose response curve is fundamental to understanding the hormonal response (Bradford & Trewavas, 1994), and represents an effective way of determining a tissue's sensitivity to respond to a hormonal stimulus (Weyers et al., 1995). With developing a bioassay being central to this chapter, a dose response curve was viewed as being potentially useful for both detection of SLs and determination of the comparative concentration in different horticultural species. A synthetic SL 'GR24' was used, as this chemical has commonly been used as a standard for germination of parasitic weed seeds (Wigchert et al., 1999).

For the germination assay of *Orobanche* spp., seeds require preconditioning via exposure to specific temperature and moist conditions for a certain duration, before they are able to uniformly respond to any germination stimulants (Joel et al., 1995). Without preconditioning, seeds failed to germinate, even in the presence of a germination stimulant (Lechat et al., 2012). Sensitivity of seeds to respond to a germination stimulant varies depending upon duration and temperatures during preconditioning (Matusova et al., 2004). In addition, the optimum preconditioning period varies in parasitic weed populations of different or the same species (Logan & Stewart, 1992). Hence, in the development of the assay used in this thesis, determination of the optimum preconditioning period for each population of *O. minor* seeds was considered in order to obtain the most sensitive seeds to the applied SL for detecting minute level of endogenous SLs.

Among a range of horticultural species examined in this thesis, *Zantedeschia* spp. K. Spreng was studied in most detail in relation to the existence of SLs at different stages of development. Previously in *Zantedeschia*, externally applied cytokinin (CK) alone increased axillary shoot development when at an early stage of the annual growth cycle (Subbaraj et al., 2010; also see Section 1.5.1). However, sequential application of CK and gibberellin (GA) were both required to increase the development of shoots during later stages. Since within this experimental system (i.e. *Zantedeschia*), the hormonal response appears to be dependent on the stage of the annual growth cycle and that SLs had not been studied previously, the opportunity existed to explain more clearly the mechanism involved in shoot branching of this herbaceous perennial. Also, knowing the endogenous concentration of SLs at different stages of the annual growth cycle may provide an additional clue to manipulate shoot branching of *Zantedeschia*, because this hormone is known to interact with CK (Dun et al., 2012), and maybe with GAs, in shoot branching.

In this chapter, the objectives were, to develop a seed germination bioassay for SLs and use it to:

- determine a dose response curve of germination of *O. minor* seeds to the synthetic SL ‘GR24’.
- detect SLs in different cultivars of different horticultural species having different branching phenotypes,
- quantify the approximate concentration of endogenous SLs in different cultivars of *Zantedeschia* at different stages of the annual growth cycle.

2.2 Materials and methods

Experiments throughout this chapter included germination assays based on seeds of *Orobanche minor* Sm. harvested from plants associated with *Trifolium pretense* L. (red clover) located at Castlepoint, New Zealand. At the time of collection, the inflorescence of the *Orobanche* plants had completed flowering and had started to dry. The species collected was identified as *Orobanche minor*, based on inflorescence structure and colour. As described by Webb et al. (1988), *“The inflorescence was 10-40 cm high, slender or stout with crisped, glandular hairs, corolla about 10-17 mm length, bluish mauve colour, whitish towards base having glandular hairs on the outside”* (Figure 2.1). Before use in germination assays, seeds were stored at room temperature (21°C ± 2) in air-tight glass tubes in the dark.

Before performing the main experiments presented in this chapter, several pilot experiments were conducted. These pilot studies helped in determining the optimum conditions for conducting the main experiments, by identifying different factors that influenced the germination of *O. minor* seeds. Based on the results of pilot studies and the main experiments, different variable conditions that either did or did not affect germination of seeds were determined.

Figure 2.1 Inflorescence of *Orobanche minor* (inside the red oval) associated with the host (red clover) at Castlepoint, New Zealand. Seeds of this weed species were collected for the series of experiments presented in this thesis.

2.2.1 General methods - germination assay

The procedure for germination assays (from seed sterilization through to application of treatments to seed) followed the method as described in Matusova et al. (2004) with some modifications. Seeds of *O. minor* were first surfaced sterilized with 2% sodium hypochlorite and Tween 20 (0.02%) for 5 minutes, and then thoroughly washed with RO (Reverse Osmosis) water. Seeds were dried in a laminar flow hood (Oliphant Pty. Ltd., Adelaide, Australia) for 30 minutes just before preconditioning.

During preconditioning, seeds were transferred to sterile Petri dishes (9 cm Ø) lined with a double layer of Whatman No.1 filter paper, wetted with 3 ml of RO water. The Petri dishes with lids were sealed with parafilm, and covered with aluminium foil. Based on the results of preliminary trials (Appendix I), so as to optimise the germination response, seeds of different ages (length of storage after harvest) were preconditioned either for 14 days at 21°C or 7 days at 21°C in the experiments presented within this chapter. Generally, 'young' or newly harvested seeds were preconditioned for 14 days at 21°C and 'older' seeds (i.e. following storage for a few months) only required preconditioning for 7 days at 21°C to optimise their sensitivity.

After preconditioning, seeds were placed in a laminar flow hood for 20 minutes. Between 30 and 40 seeds were spread inside a glass Petri dish (5 cm Ø) lined with a double layer of Whatman No.1 filter paper, wetted with 800 µl of treatment solution (i.e. concentrations of GR24 or plant sample extracts). Another glass Petri dish of the same size was used as a lid and the treated seeds were then allowed to germinate at 25°C for 8 days in the dark.

2.2.2 Experiment One- Dose response of *O. minor* seeds to GR24

2.2.2.1 Preparation of GR24 solutions

Ten ml of GR24 (298.29 molecular weight, Chiralix, The Netherlands) stock solution (100 mgL^{-1}) was prepared, by dissolving one mg GR24 in 100% acetone and, once dissolved, making this up to 10 ml with distilled water so that the final stock solution contained 10% acetone. While making a stock solution, sonic vibration (Elmasonic, Germany) was used for effective stirring. After the stock solution was prepared, serial dilutions were immediately prepared so to obtain final concentrations ranging between 10 mgL^{-1} and 10^{-8} mgL^{-1}. All solutions were stored at 4 °C until used. The highly concentrated solutions, particularly those between 10 and 100 mgL^{-1} tended to precipitate after a few hours of storage. In order to ensure that GR24 was completely dissolved, as evident by the attainment of a clear solution, these treatment solutions were again treated with sonic vibration just before their application to preconditioned seeds.

2.2.2.2 GR24 treatments

The standardized dose response curve contained eleven different concentrations of GR24 from 10^{-8} mg L^{-1} (3.33×10^{-14} mol L^{-1}) to 100 mg L^{-1} (3.33×10^{-4} mol L^{-1}), and a control (water). Preconditioned seeds of *O. minor* were treated with GR24 solutions as described in Section 2.2.1. Each treatment was replicated three times. A single Petri dish (5 cm Ø) represented one replication.

2.2.2.3 Microscopic observations and data analysis

The number of germinated seeds of *O. minor* was determined after eight days using a binocular microscope. Seeds with the radicle protruded through the seed coat were considered germinated. In addition, the radicle length of the germinated seeds was categorized into three classes as either; short (equal or less than the length of the seed), long (more than twice as long as seed length) or, medium (radicle length in between short and long categories).

The percentage germination and standard errors were calculated using MS Excel (Microsoft Corp., USA). No statistical analysis was carried out for interpretation of radicle length as almost all seeds within a treatment had a similar treatment effect i.e., short, long or medium length.

2.2.3 Experiment Two- Existence of strigolactones in different horticultural species

2.2.3.1 Plant selection

For detecting SLs and quantitatively comparing the content of SLs between cultivars, different horticultural species and cultivars (Table 2.1) were selected. As reported previously (refer Table 2.1), with the exception of kiwifruit these cultivars within each species were selected because of their different branching phenotypes. In the case of kiwifruit, most of the cultivars used have a similar branching phenotype as described in Table 2.1.

Table 2.1 Descriptive branching and/or growth characteristics of cultivars/variety of different horticultural species used for detecting and quantitatively comparing endogenous strigolactones

Species (Common name)	Cultivars/variety	Branching and/or growth characteristics	References [z]
***Zantedeschia* spp. K. Spreng (Calla)**	Goldilocks	Highly branching	
	Best Gold	Low branching	Subbaraj, 2011
	GE45	Moderately low branching	
***Actinidia* Planch. *var. chinensis* (Kiwifruit)**	Hort 16A	both varieties of Kiwifruit have vigorous shoot growth with predominance of proleptic shoots	
***Actinidia* Lindl. *var. deliciosa* (Kiwifruit)**	Hayward		
***Malus domestica* Borkh (Apple)**	Royal Gala (RG)	Vigorous shoot growth	
	Malling Nine (M9)	Dwarfing growth habit	
***Acer palmatum* Thunb. (Japanese maple)**	Sango Kaku	High branching	(Funnell et al., 2014)
	Red Emperor	Low branching	
Petunia hybrida	Mutants (dad1, dad2 and dad3)	High branching	(Napoli & Ruehle, 1996)
	Wild-type (V26)	Low branching	(Napoli & Ruehle, 1996)

[z] branching and/or growth characteristics of each cultivar/mutant within the species is supported by the relevant 'References'

2.2.3.2 Plant growing conditions and sample collection

2.2.3.2.1 Zantedeschia

The environmental and cultural conditions under which plants were grown were as described by Subbaraj et al. (2010) (also see Appendix II). The flowering sized tubers of

‘Goldilocks’, ‘Best Gold’ and ‘GE45’ were grown in a glasshouse at the Plant Growth Unit, Massey University, Palmerston North (40°20°S) during spring (end of September) 2010 until the end of summer April 2010.

Xylem exudate was collected as guttation fluid from the leaves of *Zantedeschia* which were well watered (ten plants per cultivar), with samples per plant pooled together for subsequent analysis by bioassay. In the first year of experiments (Year 1), guttation fluid was initially collected early in the annual growth cycle, commencing 45 days after planting tubers, and again from the same plants on four further occasions; i.e. in total therefore;

- at the first date of collection, the plants were at leaf emergence, with one to two fully opened leaves per primary shoot (Figure 2.2).
- by the second date, branches (axillary shoots) were visible.
- at the third date plants were flowering and,
- during the last two collection periods the plants had progressed to either early natural senescence of foliage or,
- late foliage senescence, at the last period of collection.

In addition, the experiment was repeated the following year (Year 2), in order to test the consistency of results obtained from the first year. In this repeat experiment, guttation fluid was collected from all three cultivars at each of the different growth stages described above, except the late foliage senescence stage.

As the number and/or size of leaves varied with stage of development and cultivar, guttation fluid was collected from either one leaf or a group of two to eight leaves, of each plant sampled. Different sized plastic ‘zip locks’ bags, depending upon number and size of leaves, were used to envelop leaves to collect guttation fluid. Collection bags were put in place late afternoon (around 6-7 pm), with the accumulated guttation fluid collected the following morning. Samples of guttation fluid were stored at -20°C until analysis with the germination assay.

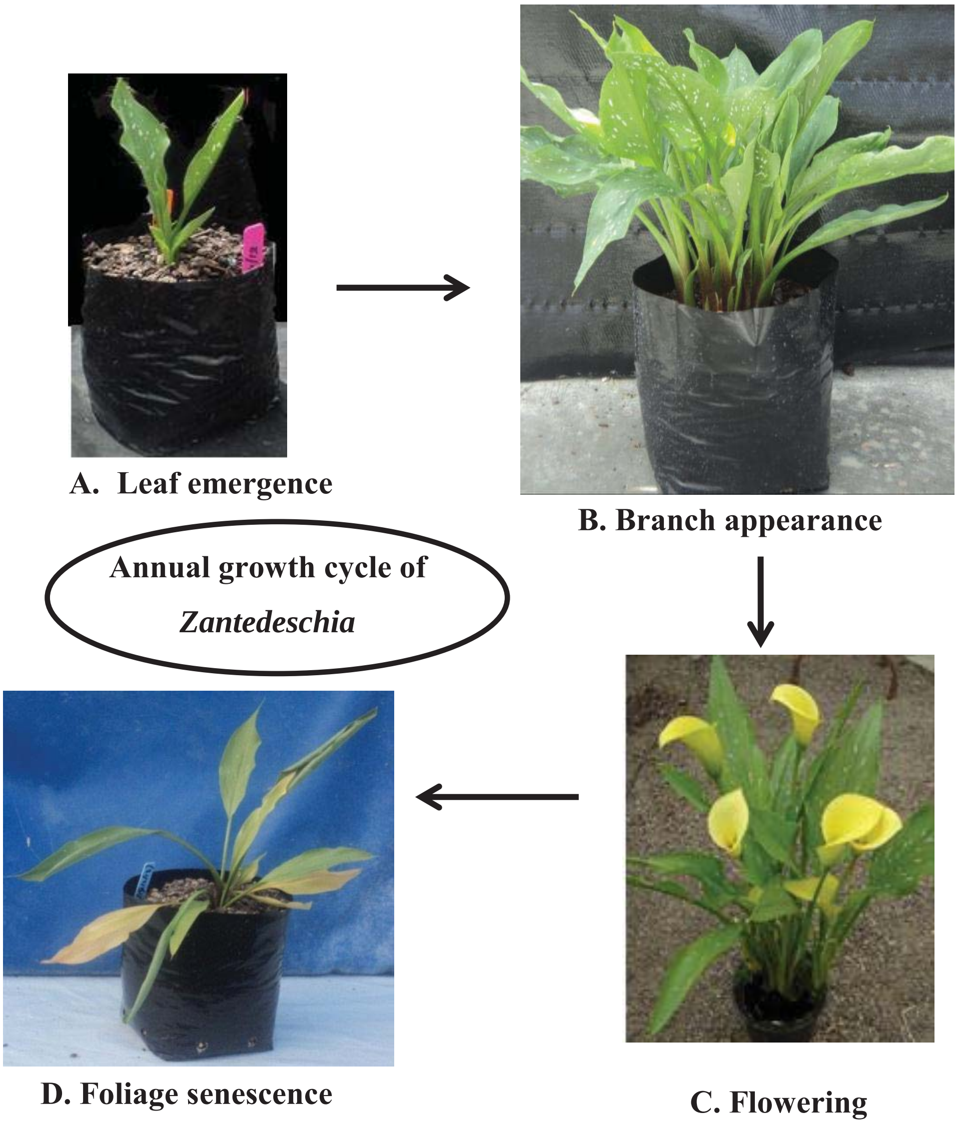


Figure 2.2 Different stages of annual growth cycle of *Zantedeschia* (A-D) at which guttation fluid was collected.

2.2.3.2.2 *Petunia*

The growing conditions for *Petunia* plants of wild-type ‘V26’ and three mutants (dad1, dad2 and dad3) was as described in (Snowden et al., 2005). As such, plants were grown under greenhouse conditions with a minimum/maximum temperature regime of 20 °C/30 °C and a minimum of 12 h photoperiod. Each plant received 80:80:60 mg N:P:K (Wuxal Super 8-8-6 plus micro liquid fertilizer; Aglukon, Düsseldorf, Germany) per litre, when irrigated. Extraction of root xylem sap was done about 50 days after the seeds had been sown.

As described below, the procedure for root xylem sap extraction from Petunia plants followed that described in Drummond et al. (2009). During collection of root xylem sap, the plants were first cut off just below the first leaf (Figure 2.3). A short piece of Masterflex® silicon tube (Cole-Parmer Instrument Co., Illinois) was fitted over the stump and tied in place. A syringe was then fitted to the other end of the tube, and also tied in place. The syringe was drawn open to its maximum extent and the plunger held in place with a pipette tip. The vacuum created, plus the root pressure, drew the sap from the roots. The vacuum was applied for about 2 hours. During this time the plant and syringe were protected from light by being covered with tinfoil. Finally the sap was transferred into 15 ml tubes and frozen in liquid N_2 before being stored at -80 °C.

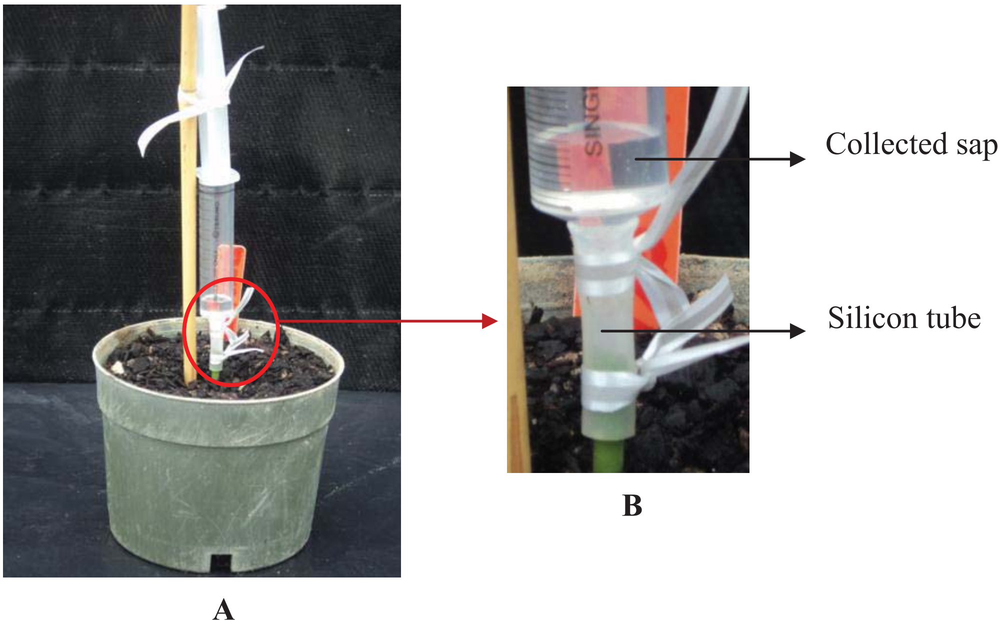


Figure 2.3 Equipment set-up for extraction of root xylem sap of container-grown plants of *Petunia* (A) and at right a magnified view of section within figure A highlighted within red circle (B). A syringe fitted over the mouth of the cut stump of the primary shoot at the base where a small silicon tube was used to connect the stem and the syringe ensuring an air tight condition was achieved.

2.2.3.2.3 *Acer, kiwifruit and apple*

Apple and kiwifruit plants were grown in an open field under standard commercial conditions at the Plant Growth Unit (PGU), Massey University, Palmerston North (latitude 40.2°S, longitude 175.4°E), New Zealand. The soil within the area was classified as Manawatu fine sandy loam (refer http://atlas.massey.ac.nz/soils/profiles/profile.htmlP54). Samples were collected from these plants during late spring (3rd November) 2010. For *Acer*, container-grown plants that had previously been established within a greenhouse (heated at 15°C, ventilated at 20°C) at The New Zealand Institute for Plant & Food Research, Palmerston North were used for sample collection. The growing media was composed of A-grade bark (50%), bark fiber (30%), 7 mm diameter pumice (20%), supplemented with 1 kg m^{-3} serpentine super, 150 g L^{-1} dolomite, 200 g L^{-1} 16N–3.5P–10K controlled-release fertilizer (Osmocote® 8-9 month; Everris International, Geldermalsen, The Netherlands), and 100 g L^{-1} 15N–4.8P–10.8K controlled-release fertilizer (Osmocote® 3-4month; Everris International). Each plant was supplied with 50 to 60 mL of water per day supplemented with one overhead watering 2-3 days before sap extraction. Samples from *Acer* were collected during winter (14th June) 2010 and again in summer (27th December) 2010. Additional sample analysis was carried out within the sap extracted from 22 months old *Acer* plants during early spring (12th August), 2013.

For apple, *Acer* and kiwifruit, and as described below, extraction of xylem sap was carried out as described by Bollard (1953). As such, one day before extraction the plants were fully watered, and sap extraction was done by inserting the proximal end of the harvested shoot into a vial placed in a sealed Buchner flask connected to a vacuum pump (Hivac, Auckland, New Zealand) (Figure 2.4). Starting from the distal end of the harvested shoot, 100 mm of shoot was removed with pruning shears every five seconds. The flow of xylem sap under vacuum was allowed to drip into the vial until the entire shoot was cut. As the xylem sap was contaminated with stem residuals, stem tissues, the sap was immediately filtered using Phenex filters (0.2 µm) (Phenomenex, North Shore City, New Zealand). Samples were stored at -20 °C until analysis.

Figure 2.4 Equipment set-up for xylem sap extraction from shoots, illustrating section of a kiwifruit shoot inserted into a vial, held within an airtight Buchner flask (front) connected to a vacuum pump (back) (red circle indicates vial placed in the Buchner flask for collection of fluid).

2.2.3.3 Treatments and experimental design

Between 30 and 40 preconditioned seeds of *O. minor* were treated with xylem exudate derived from each of the different species (Table 2.1) as described in Section 2.2.1. In pilot studies, undiluted xylem exudate of some species did not produce any germinated seeds, whereas upon dilution, the seeds germinated (data not presented). Hence, the bioassay was separately conducted using samples which were both undiluted and ten times diluted. Each rate of dilution was applied to a single Petri dish, i.e. single replicate sample.

In the second year of experiments with guttation fluid of *Zantedeschia* (refer Section 2.2.6.1), the experiment comprised a (3 × 4) factorial arrangement of treatments comprising the samples collected from each of the three cultivars (Goldilocks, Best Gold and GE45) at four stages (Leaf emergence, branching, flowering and leaf senescence, Figure 2.2) of the annual growth cycle. There were altogether 12 treatments, each replicated three times, with each replication comprising 30 to 40 seeds of *O. minor*.

2.2.3.4 Microscopic observations and data analysis

Microscopic observations of seeds after 8 days of exposure to treatment solutions were carried out as described previously (refer Section 2.2.2.2). The percentage germination was calculated using MS Excel (Microsoft Corp., USA).

Data obtained from the samples of guttation fluid of *Zantedeschia* plants in the second year (Year 2), were analysed by a two-way ANOVA, using the GLM procedure of Minitab 16 (Minitab Inc., State College Pennsylvania, USA). Comparison between the means was made at $P \leq 0.05$ using Tukey's method.

2.2.3.5 Quantification of endogenous SLs in *Zantedeschia* (Year 1 samples)

To estimate the amount of SLs present in the guttation fluid of *Zantedeschia* collected during Year 1, the resulting germination percentage of *O. minor* was converted to GR24 equivalent concentration, via interpolation of the dose-response curve (refer Appendix III).

2.3 Results and discussion

2.3.1 Standardized dose response curve

The dose-response curve showed maximum germination in 1 mg L^{-1} (3.35×10^{-6} mol L^{-1}) GR24, and above this concentration the germination activity declined (Figure 2.5). The fact that higher concentrations can inhibit parasitic seed germination was supported by several other authors (Wigchert et al., 1999; Malik et al., 2010; Matusova et al., 2004; Joel et al., 1995). The dose-response also clearly indicated that the seed was sensitive to GR24 at concentrations as low as 0.0000001 mg L^{-1} (i.e. 3.35×10^{-13} mol L^{-1}), suggesting that GR24 is highly effective at very low concentrations. This is also supported by Malik et al. (2010) who reported germination was stimulated by concentrations of GR24 as low as 10^{-10} to 10^{-12} mol L^{-1}. In fact, the response of *Orobanche* spp. seeds to SLs were reported to be at least 100-fold more sensitive for the detection of SLs than liquid chromatography (LC) and mass spectrometry (MS) (Yoneyama et al., 2010). Hence the germination assay is a highly sensitive technique, and can be used for detecting SLs which might be present in a minute quantity within the extractable fluid of plant systems. In contrast, for isolating and identifying SLs, methods like LC/MS require a large amount of plant sample in order to obtain pure and detectable SLs (Joel, 2000). Generally, plant samples contain several other components apart from the target compounds hence, samples are used up during the purification and concentrating process (Weyers & Paterson, 2001). In contrast, the germination assay does not require a high volume of plant samples (e.g. 800 μl is sufficient; refer Section 2.2.1), and can be directly tested on crude plant samples (refer Section 2.2.3), avoiding any sample purification and concentration processes.

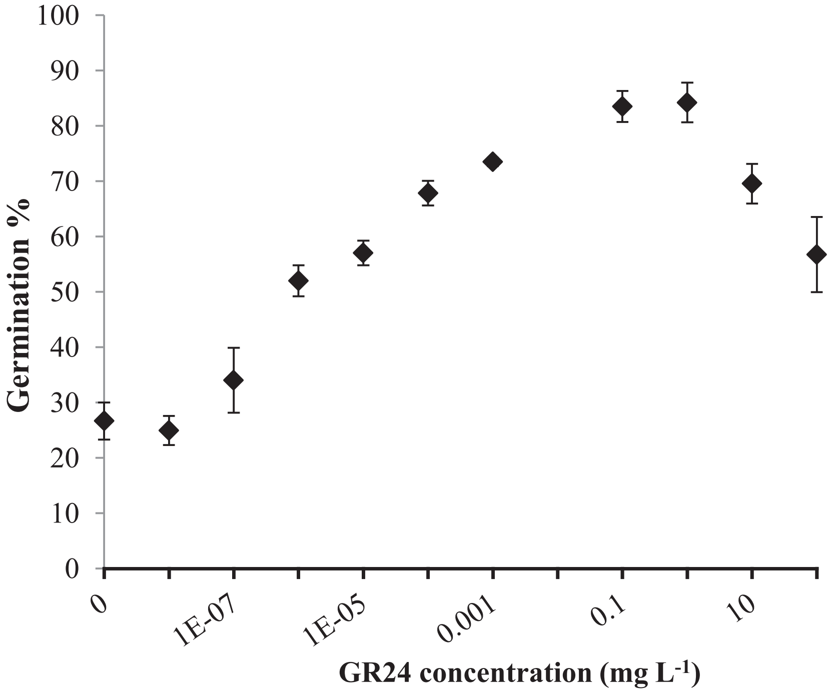


Figure 2.5 A standardized dose response curve of germination of *O. minor* seeds to different concentrations of GR24. Vertical bars represent standard errors (n=3).

Apart from measuring the sensitivity of the hormonal response (Weyers et al., 1995), the dose response curve can also be used in estimating the concentration of SLs (GR24 equivalent) present in plant samples. It is true that quantification of SLs via a dose response curve lacks accurate estimation as achieved by LC/MS and cannot be used for direct chemical identification. However, the dose response curve for the germination assay was successfully applied to quantify SLs present in *Zantedeschia* cultivars in Section 2.3.2.1. Further discussion related to the significance of this method has also been presented in Section 2.3.2.1.

2.3.2 Strigolactones in different horticultural species

When comparing cultivars within each of the horticultural species examined, germination percentage could readily be interpreted as indicating the relative concentration of SLs as being either high or low. Based on the assumption that the concentration present in these species was within that utilized in the dose response curve (a reasonable assumption if both diluted and undiluted samples are used), a higher germination percentage represents a higher concentration of SLs (Figure 2.5).

2.3.2.1 *Zantedeschia*

In both years, the highly branched cultivar Goldilocks produced less SLs as compared to the low branched cultivars, during the early stage of growth when leaves on the primary shoot had just started to emerge. In Year 1, during leaf emergence, guttation fluid of the moderately low branched cultivar GE45 contained about 70 times more SLs than the highly branched cultivar Goldilocks (Table 2.2). This difference in concentration of SLs between the high and low branched cultivars, before the branches had fully developed (Halligan et al, 2004), supports that SLs are involved in controlling shoot branching. In contrast however, guttation fluid of the low branched cultivar Best Gold, collected at this first stage of development in Year 1, appeared to have less SLs than the moderately branched ‘GE45’ (Table 2.2). Since the plants of ‘Best Gold’ developed more quickly as compared to the other two cultivars, it is possible that in ‘Best Gold’ the critical stage at which the concentration of SLs influences branching might have already passed at the time when samples were collected. Supporting this logic, it was evident that the concentration of SLs in ‘Best Gold’ tended to reduce as the branching stage commenced (Table 2.3). In Year 2, at the stage of development defined as “leaf emergence”, the guttation fluid of ‘Best Gold’ was collected seven days earlier than for the two other cultivars (when plants had one to two leaves), and produced a significantly ($P < 0.01$) greater number of germinated *O. minor* seeds than for ‘Goldilocks’. Hence in Year 2 at the leaf emergence stage, ‘Best Gold’ contained a higher concentration of SLs as compared to ‘Goldilocks’, supporting the hypothesis for a correlation between concentration of SLs and inhibition of shoot branching.

Table 2.2 Concentration of strigolactones (ng L^{-1} of GR24 equivalent; calculated using dose response curve; Appendix III) present in guttation fluid of three different cultivars of *Zantedeschia* at different stages of the annual growth cycle - Year One. (Number in parenthesis is germination percentage of *O. minor*; n = 30-40). About 20% seeds germinated in the Control (Water)

Cultivar	**Stage of annual growth cycle**				
	Leaf Emergence	**Branching**	**Flowering**	**Early Senescence**	**Late Senescence**
Goldilocks (highly branching)	0.18 (33.87)	20000 (93.33)	1.9 (50)	90 (69.44)	20 (63.16)
Best Gold (low branching)	0.28 (37.98)	1.9 (50)	100 (70)	0.4 (40)	0 (0)
GE45 (moderately low branching)	130 (71.05)	0.16 (33.33)	100 (70)	5 (56.67)	0 (0)

Table 2.3 Germination percentage (%) ±SE of seeds of *O. minor* as affected by guttation fluid of different *Zantedeschia* cultivars at different stages of annual growth cycle – Year Two. About 1% or near to zero seeds germinated with the Control (Water)

Cultivar	**Stage of annual growth cycle**			
	Leaf emergence	**Branching**	**Flowering**	**Senescence**
Goldilocks	17.7 ±5.78 b^{Z}	41.9 ±10.1 ab	52.0 ±7.75 a	20.0 ±4.75 b
Best Gold	61.2 ±4.26 a	40.5 ±1.47 ab	68.8 ±1.56 a	12.6 ±6.18 b
GE45	66.5 ±3.8 a	66.8 ±1.08 a	55.9 ±9.93 a	38.2 ±8.85 ab

^{Z} Within the same column different letters indicated significant differences at $P < 0.05$ using Tukey's test

In Year 1, the guttation fluid collected from 'Goldilocks', when branching was visibly evident, contained the highest concentration of SLs, being approximately 100,000-fold more than that produced when shoot branches had just started to develop (Table 2.2). Likewise, in Year 2, when branching was evident, guttation fluid of 'Goldilocks' produced more than twice the number of germinated seeds (indicating more SLs) compared to the guttation fluid collected at the leaf emergence stage (Table 2.3), and similar to the germination percentage produced by guttation fluid of 'Best Gold' collected at the branching stage (Table 2.3). Despite 'Goldilocks' being characterized as being naturally highly branched, the high concentration of SLs after branching had occurred may have been caused by high concentrations of auxin produced by the apex and young leaves of multiple shoots. Production of more SLs after the development of branches and leaves is supported by the fact that auxin stimulates biosynthesis of SLs (Dun et al., 2006).

During the late foliage senescence stage, for samples from 'GE45' and 'Best Gold' the germination was reduced to zero (Table 2.2). As bud endodormancy ensues rapidly after cessation of new leaf production (Subbaraj et al., 2010), it is possible that SLs may have been present, but the lack of seed germination may be due to high levels of abscisic acid (ABA) (also see Section 2.3.3), produced in plants entering dormancy (Ofir & Kigel, 1998; Yamazaki et al., 2002), including *Zantedeschia* (Naor, Kigel, & Ziv, 2005). This argument is supported by the fact that abscisic acid at concentrations of 10^{-5} mol L^{-1} and 10^{-6} mol L^{-1} has been found to reduce the germination percentage of *Striga* seeds (*Orobanche* family) when applied in the presence of germination stimulants (Yoneyama et al., 1998).

In general, the concentration of SLs (calculated using a dose response curve; Appendix II) varied between the different stages of the annual growth cycle as well as the cultivars of different branching phenotypes. Plants of highly branched 'Goldilocks' produced less SLs before the appearance of branches, but high SLs after the branches were fully developed. However, for the moderately low branched 'GE45' the concentration of SLs was opposite as compared to that of 'Goldilocks'. In general these results support those of Ledger *et al.* (2010) who reported varied expression of the SL synthesizing genes AcCCD7 and AcCCD8 in kiwifruit plants at different stages of growth. In early stages of continuous growth (4 and/or 8 months), there was higher expression of these AcCCDs, which had reduced by 13 months. However, except in the leaf emergence stage, the concentration of SLs was not significantly different among the three cultivars of *Zantedeschia*, at each of the other stages of annual growth cycle (Table 2.3). Thus, it could be predicted that, for at least this early stage of the annual growth cycle before branches were visibly evident, a higher concentration of SLs may be responsible for reduced branching. Since CK stimulated more axillary branches of the *Zantedeschia* cultivar Best Gold at leaf emergence stage (Subbaraj et al. 2010), it was suggested that reduced shoot branching in *Zantedeschia* was due to reduced CK. Hence, it is possible a higher concentration of SLs may also have decreased the CK level in *Zantedeschia* plants resulting in low branching.

Regarding the role of gibberellins, Subbaraj et al. (2010) suggested that the applied CK may have interacted with endogenous gibberellins (GAs) to produce more axillary branches at the leaf emergence stage. However, GA was needed to increase branching when applied at later stages of the annual growth cycle but not at the early stage during leaf emergence, possibly by preventing the onset of dormancy. In the early stage of growth GAs would naturally be high, and, as found by Naor et al., (2008), CK applied alone, was sufficient for enhanced branching. Despite GAs being present in leaf emergence stage, axillary branches did not develop at that stage unless CK was supplied (Subbaraj et al., 2010), suggesting CKs are required possibly to overcome the inhibitory effect of SLs, and that GAs do not stimulate release of axillary buds in *Zantedeschia*, but may help subsequent growth of shoots once released. Hence for release of axillary buds, SL may have interacted with CKs but not with GAs. Further studies relating to interactions between GAs and CK or SL and CK have been explored in Chapter 5 and 6. In Chapter 5, the interaction between SLs and CK is studied using *Zantedeschia* cultivars grown *in vitro*.

Accumulation of SLs during other stages of the annual growth cycle, e.g. when branches have fully developed, might have other roles (Foo & Reid, 2013) or possibly no physiological role at all (Garcia-Martinez et al., 1987). Another possible explanation for the presence of SLs in later stages of growth is that because there are different forms of SLs (Yoneyama et al., 2010) preferentially detected by germination assays, any particular form which influences shoot branching might not be present after the shoot branches have been fully developed. Supporting the existence of different forms of SLs at different stages of a growth cycle, Xie et al. (2013) reported that the SL, i.e., ‘7-oxoorobanchyl acetate’, was found in root exudates of rice during early growth, but not in later stages. It is possible that this form of SL may have a role in controlling the release of axillary bud. Hence, the concentration of SLs present in *Zantedeschia* was potentially correlated with shoot branching during leaf emergence, prior to when actual buds release and branches were visibly evident, but not when branching had already occurred.

This experiment, on detection and quantification of endogenous SLs in *Zantedeschia* via a germination assay now provides a useful base for using this method for measuring the comparative concentration of SLs in other species as well. Germination assays consume relatively less time and cost for detecting SLs as compared to LC/MS and therefore, makes this the potentially attractive. The limitation of the germination assay however, is the inability to characterize or chemically identify SLs. Hence, other methods like chromatography and mass spectrometry are required for chemical identification of SLs. With regard to *Zantedeschia* however, future research would benefit from chemical identification of the SLs present in the guttation fluid via LC/MS, at different stages of annual growth cycle. One could then speculate as to which form(s) of SLs may be related with branching. Attempts to follow this research strategy are reported elsewhere in this thesis (Chapter 3). In addition, identification of SLs based on a branching response assay would be beneficial.

2.3.2.2 *Petunia*

There was a marked difference in concentration of SLs between low branched wild-type 'V26' and highly branched 'dad' mutants (except dad2) (Figure 2.6). In highly branched mutants dad1 and dad3, the germination percentage was similar to that of the control (water) whether diluted or undiluted (Figure 2.6) and about 40 times less than 'V26'(when xylem sap was diluted 10 fold). Hence, SLs in these mutants is either very low or nil. Deficiency of SLs in these mutants supports the hypothesis that *Petunia* mutants defective in the SL synthesis genes DAD1 (CCD8) and DAD3 (CCD7) have an enhanced branching phenotype (Napoli, 1996; Simons et al., 2007). Interestingly, 'dad2' though, being a high branching phenotype, also contained high SLs as it produced the highest germination levels of up to 100% (Figure 2.6). The reason was that dad2 lacks the gene that encodes for the SL receptor (α/β hydrolase) required for branch inhibition response (Hamiaux et al., 2012). Similar to dad2, SL response mutants of pea and *Arabidopsis* rms4 and max2 respectively, that lack the SL receptor F-box proteins (Beveridge et al., 1996; Stirnberg et al., 2002). Hence while these mutants contain high levels of SLs they express highly branched phenotypes (Gomez-Roldan et al., 2008; Kohlen et al., 2011).

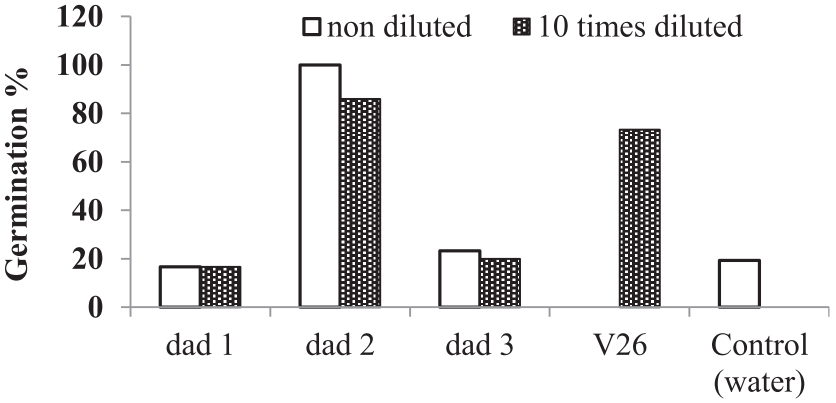


Figure 2.6 Germination of *O. minor* seeds as affected by root xylem sap (non diluted and 10 times diluted) of wild-type 'V26' and different dad-mutants of *Petunia*. Water was used as a control.

The presence of SLs in 'V26' and 'dad2' is supported by grafting experiments in which 'dad1', defective in CCD7, was restored to inhibition of branching when grafted onto either 'V26' or 'dad2' rootstock (Simons et al., 2007). The ability to inhibit shoot branching on branching scions is interpreted to suggest that root-produced SLs in 'V26' and 'dad2' rootstocks inhibited the release of axillary shoots after being transported to the 'dad1' scion. Thus, SLs detected in the root xylem sap of 'V26' and 'dad2' could have a role in controlling shoot branching in *Petunia*; it would be useful to identify and test the activity of these SLs.

None of the *O. minor* seeds germinated when treated with undiluted sap of the wild-type 'V26' (Figure 2.6), but did when the sap was diluted ten times. Since higher concentrations of the SL 'GR24' reduced the number of germinated seeds of *O. minor* (Figure 2.5), it was considered plausible that zero germination of undiluted sap of 'V26' may have been due to a supra-optimal concentration of SLs. An alternative or additional hypothesis is that the zero germination of 'V26' may also have been the result of other inhibitory substances present in the sap. As noted in the preceding discussion with *Zantedeschia*, one such possible inhibitor of germination present in the sap of 'V26' is ABA (refer Section 2.3.2.1). Production of higher ABA in blue-flowered *Petunia* before floral initiation as compared to the stage when flowers had fully developed (Ferrante et al., 2006), supports the hypothesis that ABA may be one of the causes of reduced seed germination. Hence, supra-optimal concentration of SLs as well as ABA in wild-type 'V26' *Petunia* could have strongly affected germination of the seeds. Clearly, in future experiments it would be useful to simultaneously analyse for both SLs and ABA.

2.3.2.3 *Acer*, apple and kiwifruit

All the three woody perennials *Acer*, apple and kiwifruit contained SLs (Figure, 2.7, 2.9 & 2.10) although the concentration of the hormone may differ with the cultivars or the stage of development. This is perhaps, the first time the presence of SLs been reported within these speces. In *Acer* xylem sap collected during winter (in Year 2010), the low branched 'Red Emperor' contained higher concentration of SLs as compared to highly branched 'Sango Kaku' (Figure 2.7A). Consistent with this result, there was a similar differential contrast in concentration of SLs between highly branched and low branched *Acer* during winter (in Year 2013) (Figure 2.7B) as seasonal regrowth was commencing. Thus, the concentration of SLs and branch inhibition in *Acer* appears to be correlated. However, an explanation for the fact that *O. minor* seeds did not germinate at all in 'Sango Kaku' saps collected during winter (both Year 2010 and 2013) (Figure 2.7A & B) is not known. Unlike in *Petunia,* the existence of an inhibitor (Section 2.3.2.2) is less likely as the sap samples collected in 2013 when diluted (10 times) did not increase the germination of *O. minor* seeds.

Interestingly, assays using sap collected in summer showed that sap from 'Sango Kaku' also achieved 30% seed germination (Figure 2.7C), suggesting that the production of SLs could vary at different stages of development, as noted for *Zantedeschia* (Table 2.2, 2.3). In kiwifruit, SL synthesizing genes are highly expressed in four months old compared to thirteen month-old plants (Ledger et al., 2010), further supporting that production of SL varies at different stages of growth cycle. Thus, as in the highly branched cultivar of *Zantedeschia*, the highly branched 'Sango Kaku' produced considerable SLs only at a stage of development when axillary shoots had already developed during summer (late December); the possible reasons for production of SLs at this stage of development has been discussed elsewhere (see Section 2.3.2.1). Hence, during late winter, when axillary buds of *Acer* plants were near to or at the stage of release, the highly branched *Acer* contained less SLs, supporting the role of SLs in inhibition of shoot branching particularly release of bud.

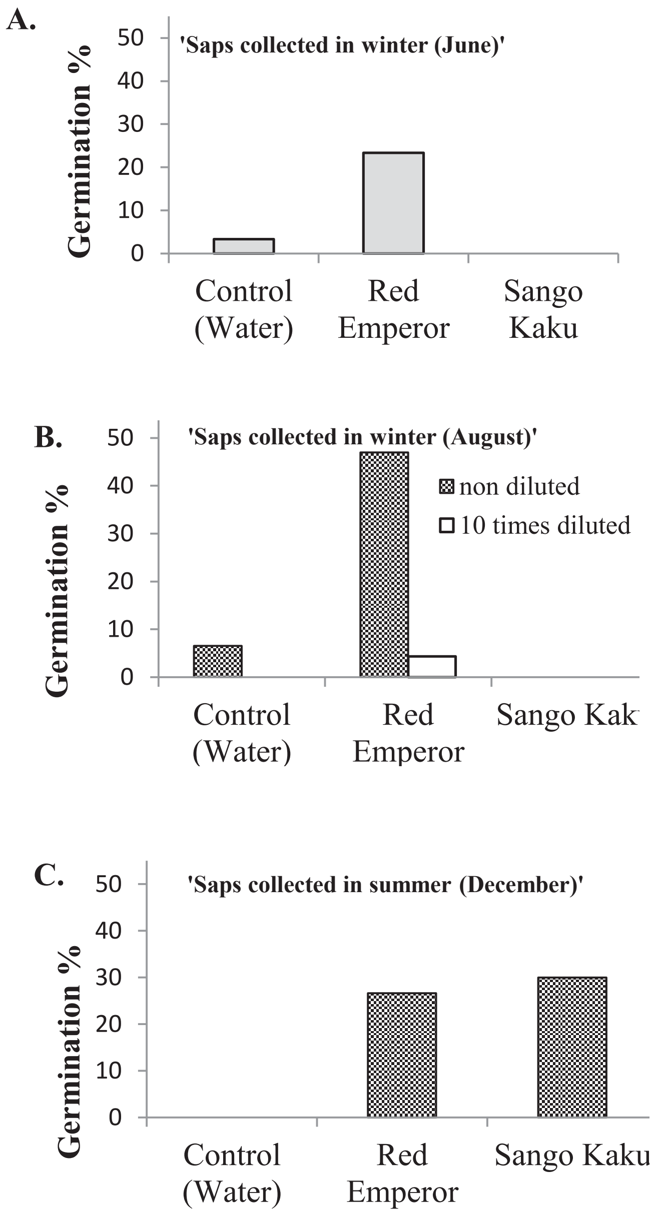


Figure 2.7 Germination of *O. minor* seeds as affected by shoot xylem sap of cultivars Red Emperor (low branched) and Sango Kaku (highly branched) of *Acer palmatum* Thunb. (Japanese maple) collected in mid-June, 2010 (A) and mid-August, 2013 (B) and late December 2010 (C). Water was used as a Control. Within Figure A, germination % was obtained with the sap diluted 5 times; data with non diluted sap was not obtained due to the contamination in a Petri dish containing *O. minor* seeds treated with non diluted sap.

Since Red Emperor is highly apical dominant with no or very few axillary shoots during the first season of regrowth (refer Figure 2.8), it may be suggested that SL may have inhibited the release of axillary buds. As suggested for *Zantedeschia*, SL may have interacted with CK on bud release. Since in *Acer*, 'Sango Kaku' (highly branched) contained higher concentration of CK compared to 'Red Emperor' (low branched) (Appendix IV), SL may have reduced the CK level ultimately enhancing inhibition of bud release. It is however not clear about the role of SL on the subsequent growth in *Acer*. Direct application of SL to the released buds within the highly branched cultivar may help to understand the effect of SL on subsequent growth. Recently, Ni et al. (2015) found GR24 (a synthetic strigolactone) inhibition axillary shoot length in one of the woody perennial plant, *Jatropha curcas*, using 3-week-old intact seedlings, suggesting that SL inhibits the subsequent growth in woody perennial. Since SL inhibited the axillary shoot length induced by either CK or GA_3, it was suggested SL may have interacted with CK and/or GA on subsequent growth. Although the formation of an axillary shoot (about 3 mm) appeared to be stimulated by GA_3 alone, the release of the bud may have been actually due to the endogenous CK, as GA_3 failed to stimulate any growth of the axillary bud in rootless seedlings of *Jatropha curcas*. Interestingly, on supplying CK basally via the base of the cut stem, GA applied to the buds produced a substantial growth axillary shoot (about 8mm). This suggests CK is necessary for bud release whereas GA enhance the subsequent growth of the released bud, as well as illustrating the importance of root-derived CK on bud release (also refer Section 1.4.3.1, Theory A). The roles of CK and GA on axillary bud release and/or subsequent growth have been further explored in Chapter 6 using pea stems.

Sango Kaku **Red Emperor**

Figure 2.8 Highly branched (Sango Kaku) and low branched (Red Emperor) cultivars of *Acer palmatum* Thunb. (Japanese maple). Pictures were by provided by Keith A. Funnell.

In apple, sap of the dwarfing ‘M9’ plants contained higher SLs than within the vigorous cultivar Royal Gala (Figure 2.9). Like in ‘Sango Kaku’ of *Acer*, the sap of cultivar Royal Gala did not stimulate germination. Since the diluted sap (Figure 2.9) did not increase the rate of any germination, existence of a seed germination inhibitor within ‘Royal Gala’ is considered less likely.

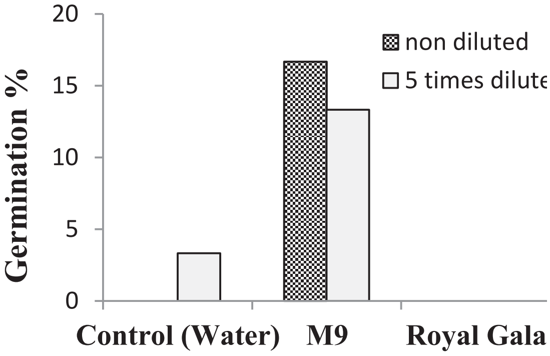


Figure 2.9 Germination of *O. minor* seeds as affected by shoot xylem sap of cultivars M9 (dwarfing growth) and Royal Gala (vigorous growth) of *Malus domestica* Borkh (Apple). Water was used as a Control. The graph represents results of two assays using non diluted or diluted 5 times. Control did not produce any germinated seeds when non diluted sap samples were tested whereas about 3% germination occurred in the Control in the assay that used diluted saps.

While drawing a connection between dwarfing, vigour and branch inhibition has yet to be fully explored, SLs produced in ‘M9’ roots might have reduced shoot branching of the ‘Royal Gala’ scion, as when M9 was used as a rootstock branching was reduced (van Hooijdonk et al., 2010; 2011). This explanation would be additional to the mechanism proposed by van Hooijdonk et al. (2011), in which reduced shoot branching of ‘Roya Gala’ when grafted over ‘M9’ rootstock, was due to reduced auxin transport to the roots resulting in less CKs synthesis and, hence, reduced transport of CKs to the shoot. From the current result, it is possible that SLs present in M9 rootstock may also reduce root CKs thereby resulting in less release of axillary buds. Perhaps, the effect of SLs and lower CKs on reducing axillary shoot number could be additive in inhibiting bud release and of importance in developing new rootstocks for apple. However, further determination of SLs and CK concentration in these two cultivars is required, taking into account the different stages of the annual growth cycle.

Kiwifruit sap of both ‘Hayward’ and ‘Hort16A’ stimulated germination to a similar level (Figure 2.10), suggesting the existence of SLs at similar levels in these cultivars, certainly at the stage of growth sampled.

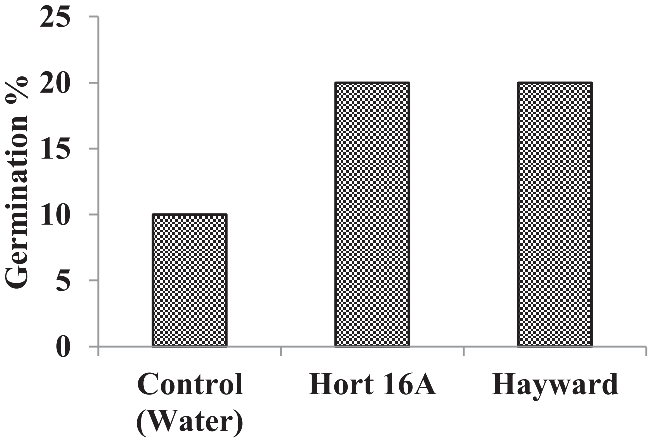


Figure 2.10 Germination of *O. minor* seeds as affected by shoot xylem sap of cultivars Hort 16A and Hayward of *Actinidia spp.* (Kiwifruit). Water was used as a control.

The existence of SLs in these cultivars also supports the idea that, despite extensive shoot growth of proleptic shoots (shoots of previous seasons), the lack of sylleptic (axillary or new shoots produced during the current season, often on proleptic shoots) branches arising from these two cultivars may partly be caused by SLs. However, it is acknowledged that hormonal integration between SLs and other hormones should be carefully studied so as to determine the underlying mechanism of shoot branching in kiwifruit plants. Since GA can cause formation of more sylleptic shoots in kiwifruit in the cultivar Hort 16A (Vattiprolu, 2012) indicating direct release of axillary buds, SL may have interacted with GA on bud release. However the author suggested that such shoots may be associated with the developmet of the released buds caused by the collaspse of apex due to GA application rather than the direct effect of GA on bud release. Along with this idea, in *Jatropha carcus*, the release by GA_3 may not be casual; the development of more sylleptic shoots may have been due to the extension of released buds.

Overall, simultaneous determination of SLs, CKs and GAs in woody perennials such as *Acer*, apple and kiwifruit is likely to be necessary to advance the understanding of the hormonal control of branching.

2.3.3 Radicle length

The radicle length described in this section pertains to germination assays already reported in the preceding sections. All seeds treated with concentrations of GR24 above 1 mg L^{-1} had short radicles compared to those at lower concentrations (Figure 2.10). In contrast, seeds at very low concentration of GR24 (e.g. 0.0001 mg L^{-1}) had long radicles compared to both the control and more concentrated solutions. Inhibition by higher concentration and stimulation by lower concentration of SLs on radicle length is supported by a similar effect of SLs on primary root length of *Arabidopsis* (Ruyter-Spira et al., 2011).

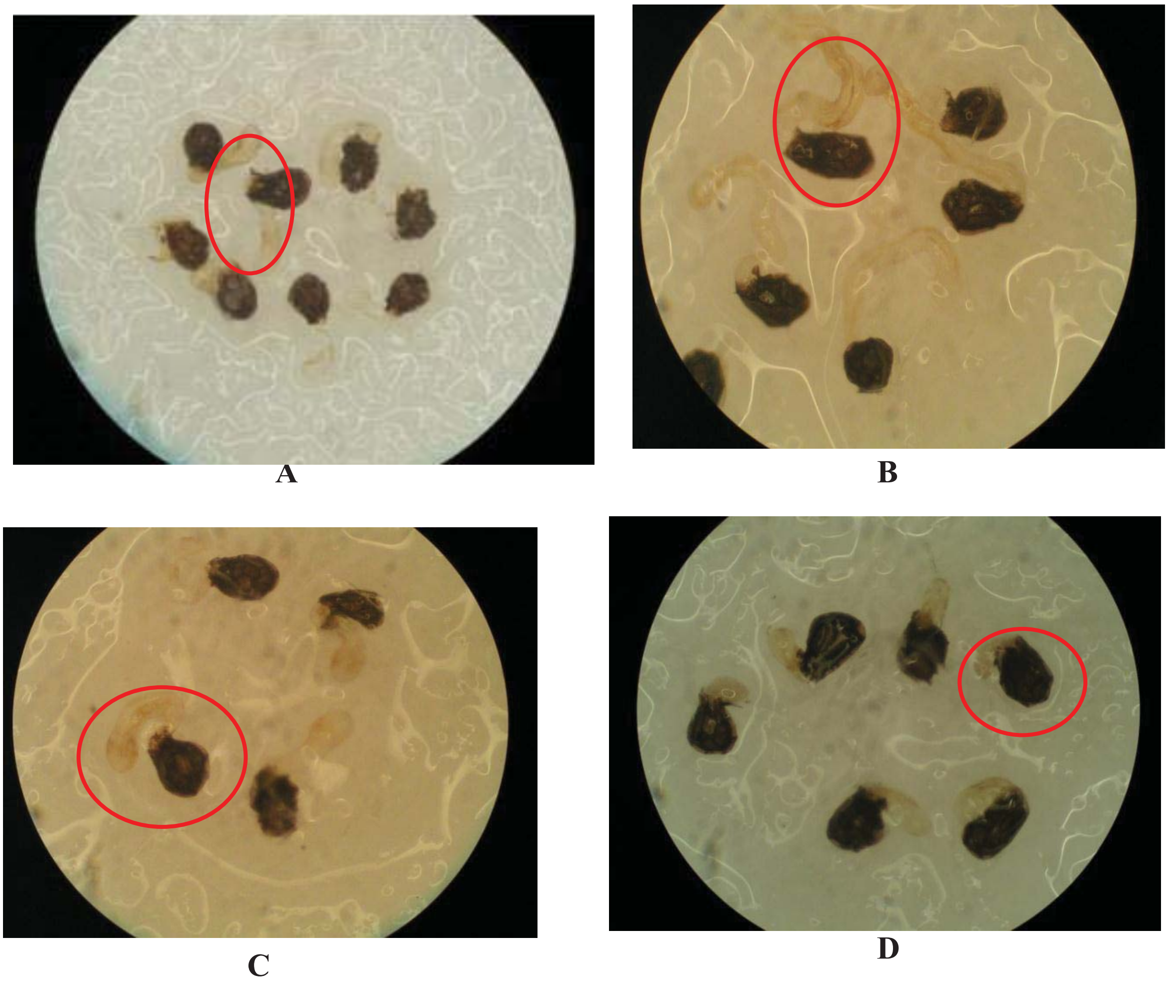


Figure 2.11 Radicle length of germinating seeds of *O. minor* as affected by different concentrations of GR24; (A) control (water) with medium radical length, (B) 0.00001 mg L^{-1} with long radicles, (C) 0.01 mg L^{-1} with medium length radicle, and (D) 100 mg L^{-1} with short radicles (red circle in each figure indicates a seed with a typical radicle for that treatment).

In *Zantedeschia*, radicle length of germinating seeds were medium to long when treated with guttation fluid collected during leaf emergence through to the flowering stage of the sample plants, except in 'GE45' during leaf emergence (Table 2.4). Hence, radicles were generally not affected by the guttation fluid at these stages. In contrast, when foliar senescence had commenced, the guttation fluid shortened the radicle length (Table 2.5). Likewise in samples collected from the *Petunia* mutants, undiluted sap produced seeds having short radicles (Table 2.6).

Table 2.4 Radicle length^{Z} of germinated *O. minor* seeds treated with guttation fluid of different *Zantedeschia* cultivars collected at different stages of the annual growth cycle.

***Zantedeschia* cultivars**	**Different stages of annual growth cycle**				
	Leaf Emergence	**Branching**	**Flowering**	**Early senescence**	**Late senescence**
Goldilocks	medium	medium	long	short	short
Best Gold	medium	medium	long	short	NA
GE45	short	medium	medium	short	NA

^{Z} Radicle length: short (equal or less than the length of the seed), long (more than twice as long as seed length) or, medium (radicle length in between short and long categories).
'NA' denotes non applicable

Table 2.5 Radicle length^{Z} of germinated *O. minor* seeds treated with guttation fluid of different *Zantedeschia* cultivars collected at early senescence either 10 times diluted or non diluted.

***Zantedeschia* cultivars**	**Guttation fluid collected at early senescence stage**	
	Non diluted	**10 times diluted**
Goldilocks	short	long
Best Gold	short	long
GE45	short	short + long

^{Z} Radicle length: short (equal or less than the length of the seed), long (more than twice as long as seed length) or, medium (radicle length in between short and long categories).

Table 2.6 Radicle length[Z] of germinated *O. minor* seeds treated either 10 times or non diluted root xylem sap of *Petunia*

Petunia (mutants and wild-type)	Non diluted	10 times diluted
dad1	short	medium
dad2	short	medium
dad3	short	medium
V26	-	short

[Z] Radicle length: short (equal or less than the length of the seed), long (more than twice as long as seed length) or, medium (radicle length in between short and long categories).

Short radicles in the undiluted sap of mutants and guttation fluid of *Zantedeschia* during the foliage senescence stage may be due to the presence of non-hormonal or hormonal substances inhibitory to development of radicles rather than supra-optimal concentration of SLs which also affected seed radicle length (see Section 2.3.1, Figure 2.2.) since the diluted samples of both *Petunia* (Figure 2.6) and *Zantedeschia* did not increase the number of germinated seeds (data not shown). Similar to seed germination, the most likely inhibitor of radicle length could be ABA. The probable presence of ABA both during the foliage senescence stage in *Zantedeschia* (refer Section 2.3.2.1) and within blue-flowered *Petunia* (refer Section 2.3.2.2, Ferrante et al., 2006) has already been mentioned. The hypothesis that ABA might have reduced the length of radicle is supported from evidence in other plant species. For example, in seeds of *Medicago truncatula,* an annual legume plant, ABA significantly reduced the radicle length (Gimeno-Gilles et al., 2009). However, there is no published evidence on the effect of ABA on radicle length of seeds of *O. minor*.

2.4 Conclusion

Results based on two years data of *Zantedeschia* strongly support hypothesis for the effect of SLs on reducing shoot branching, at least during the early stage of the annual growth cycle when leaves on the primary shoot had just started to emerge. Similarly a good correlation was found between SLs and branching in woody perennials (during winter and/or early spring). Overall branching of different horticultural crops appears to correlate with the concentration of SLs (GR24 equivalent) as determined by *O. minor* germination assays, but consideration has also to be given to the developmental stage of the plant.

These overall results indicate that the germination assay can be a powerful tool to detect and quantify the level of endogenous SLs. A limitation of this assay is that it is unable to identify specific forms of SLs related to branch inhibition. However the germination assay may still be useful in analyzing purification fractions of plant samples that contain only SLs. These fractions could then be further purified and analyzed using a branching-specific assay (Chapter 3) to separate SLs active in branching inhibition from those that may not be active. This approach would then provide a basis for chemical identification of the active compound(s) using liquid chromatography and mass spectrometry.

3 Development of methods to detect a specific branch inhibiting hormone

3.1 Introduction

After the discovery of strigolactones (SLs) as a major plant hormone influencing axillary bud inhibition, SLs were also found to be involved in many other physiological roles (refer Section 1.3, Chapter 1). Although SLs are widely accepted as affecting branching, the specific bioactive compound that inhibits axillary bud outgrowth is still to be confirmed (Foo and Reid, 2013), be it via influencing bud release or subsequent growth or both. One reason could be that since more than one form of SL is present in a single species (Xie et al., 2013), it is possible that only a few SLs, or even just one may affect axillary bud outgrowth. Another reason is that endogenous levels of specific SLs have not yet been quantified in relation to branching, thus currently there is a lack of information on correlation between endogenous level of such a bioactive compound and inhibition of branching (Foo & Reid, 2013). It is also possible that the endogenous branch inhibiting hormone is not a SL at all, though this seems unlikely.

In Chapter 2, the level of SLs present in plants was estimated by using a germination assay technique. In most plant genotypes studied, the level of SLs correlated with inhibition of branching (Section 2.3.2), suggesting SLs might influence this process. In *Zantedeschia*, there was a correlation between SLs level and branching during the early stage of the annual growth cycle when branches were not fully developed, but not after the branching stage (Section 2.3.2.1, Chapter 2). As this germination assay applies for all/most forms of SL, a limitation is that it cannot be used to analyse for a particular form of SL related with branching exists. In a recent study, nine different forms of natural SLs were found to have inhibitory activity (Boyer et al., 2012), but the main endogenous branching inhibitor(s) and/or precursors (Arite et al., 2009) are still unknown (Foo & Reid, 2013). It was suggested that the 'D14' gene that encodes α/β-hydrolase (Chapter 1, refer Figure 1.6), involved in SLs biosynthetic and transduction pathway, may act on SL to convert it into a bioactive compound in a biosynthetic pathway or signalling pathway (Boyer et al., 2012; Gong et al., 2012), but this is yet to be confirmed. Hence, the lack of knowledge about the complete pathway of SLs in controlling branching (Foo & Reid, 2013) highlights the fact that there may be other compounds that have a direct role in bud inhibition.

In this chapter, a physiological approach was taken in an attempt to identify a bioactive compound causally related to branching inhibition. This approach included the development of a branching-specific assay in combination with liquid chromatography (LC), to test for branching activity of purified SL containing fractions. If successful, ultimately, a particular fraction that has biological activity can be further analysed using LC/MS to detect specific compounds present in that fraction. To date, many SL-related branching assays have been conducted using seedlings of *Pisum sativum* L. (pea) (Gomez-Roldan et al., 2008; Braun et al., 2011; Boyer et al., 2012). This plant species has a simple morphological structure, providing easy physical access to axillary buds for direct exogenous application of hormones (Boyer et al., 2012). However, a limitation of direct bud application is that the application process is very tedious and time consuming, as hormone needs to be applied to each bud at one or two-day intervals, which would be prohibitive if possibly hundreds of chromatographic fractions were being assessed for branch inhibition activity. The other drawback of this method is that apart from SLs, compounds like ethanol (50%) and acetone (0.1%) are also invariably applied as solvents, thus possibly affecting the physiological response. Hence, a simple, rapid, highly sensitive, biological assay was required for detecting fractions with biological activity. As a biological assay determines a tissue's ability to respond to a chemical stimulus that is directly aligned with hormonal sensitivity (Weyers et al., 1995), when developed, this branching assay should help in identifying a particular fraction of plant sample (e.g. xylem sap) that has inhibition activity, prior to identification of the specific bioactive compound by mass spectrometry.

SLs are mainly synthesized in roots, and move from root to shoot through xylem sap (Kohlen, 2011), as almost all SLs detected so far had been derived from root exudates. However, there is an evidence of SL synthesizing gene expression within stems

(Drummond et al., 2011) suggesting SL may also be synthesized in shoots, but SL synthesis in shoots has yet to be confirmed. It has been hypothesized that SLs found in root exudates are the biologically active molecules in shoots (Boyer et al., 2012). Thus, it could also be assumed that either a derivative of SL, or a specific form of SL, may be the bioactive compound. This study aimed to identify a particular fraction that has biological activity, through application of a branching assay, and further analysing this fraction through LC/MS, in order to identify a bioactive compound from root xylem sap of two varieties of *Petunia ×hybrida* L. (*Petunia*), V26 and dad3, having different branching phenotypes. These two varieties differed in the level of SL as shown by the germination assay experiment (Chapter 2) and, in addition, dad3 is a mutant that lacks a SL synthesis gene (Napoli, 1996). Given the pre-existing knowledge, *Petunia* was selected for inclusion within this experiment. The wild-type V26, which is less branched, contained high levels of SLs as compared to the highly branched dad3 (Chapter 2, Section 2.3.3). In addition, the guttation fluid of *Zantedeschia* cultivars was also analysed, but due to inconsistencies only some experimental results are presented for *Zantedeschia.*

In this study it was hypothesized that the wild-type *Petunia* ‘V26’ contains a branching inhibitor, which may be a SL or its derivatives. As presented here, the first experiment was mainly focussed on developing a branching assay, and the second experiment for analysing root xylem sap using both biological assays and LC/MS.

3.2 Materials and methods

3.2.1 Experiment One - Dose response of a branching assay of pea seedlings to GR24

In contrast to the biological assay utilised in Chapter 2 which was based on germination rather than axillary shoot development, in this experiment the synthetic strigolactone ‘GR24’ was used to develop a branching-specific assay. ‘GR24’ has been widely used as a standard for studying the involvement of SLs in shoot branch control, as it has been shown to reduce axillary shoot length in pea plants (Gomez-Roldan et al., 2008) and number of shoots in *Arabidopsis* (Umehara et al., 2008).

3.2.1.1 Plant material and growing conditions

Seeds of pea (*Pisum sativum* L.) variety (var) Ashton, which phenotypically expresses a high level of apical dominance, were germinated in darkness within rolls of moist germination paper (Figure 3.1 A) at 21°C for 3 days. Germinated pea seedlings (Figure 3.1B) were then transferred to a plastic container half filled with fine, moist, sand, grown at 23°C with a 16-h photoperiod in a growth cabinet (CV 30, SKOPE Industries Pvt. Ltd., New Zealand) and harvested after approximately 4 days for use in the bioassay. At this stage the seedlings had formed three elongated internodes (also refer Figure 3.8).

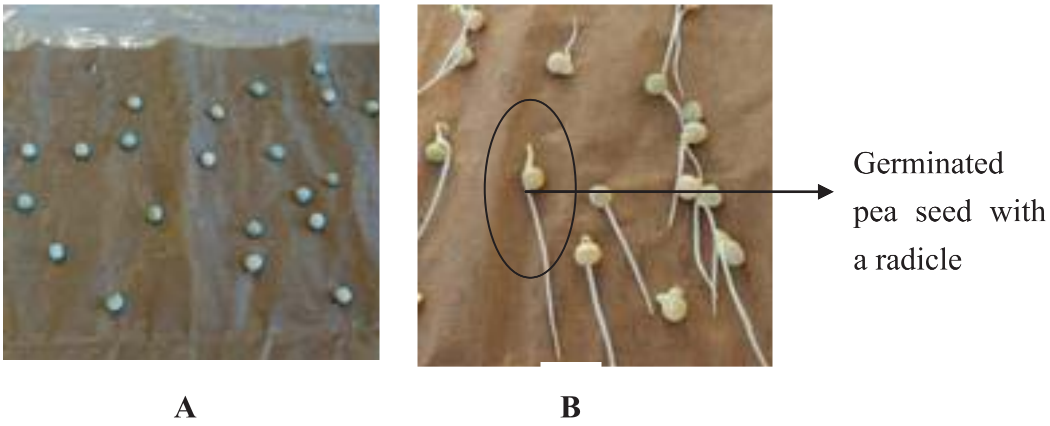


Figure 3.1 Seeds of pea var Ashton spread over moist germination paper (A) and germinated pea seeds with radicles (B). For germination, seeds were wrapped within the paper and allowed to germinate in darkness for 3 days.

3.2.1.2 Growth condition for pea seedlings and GR24 application

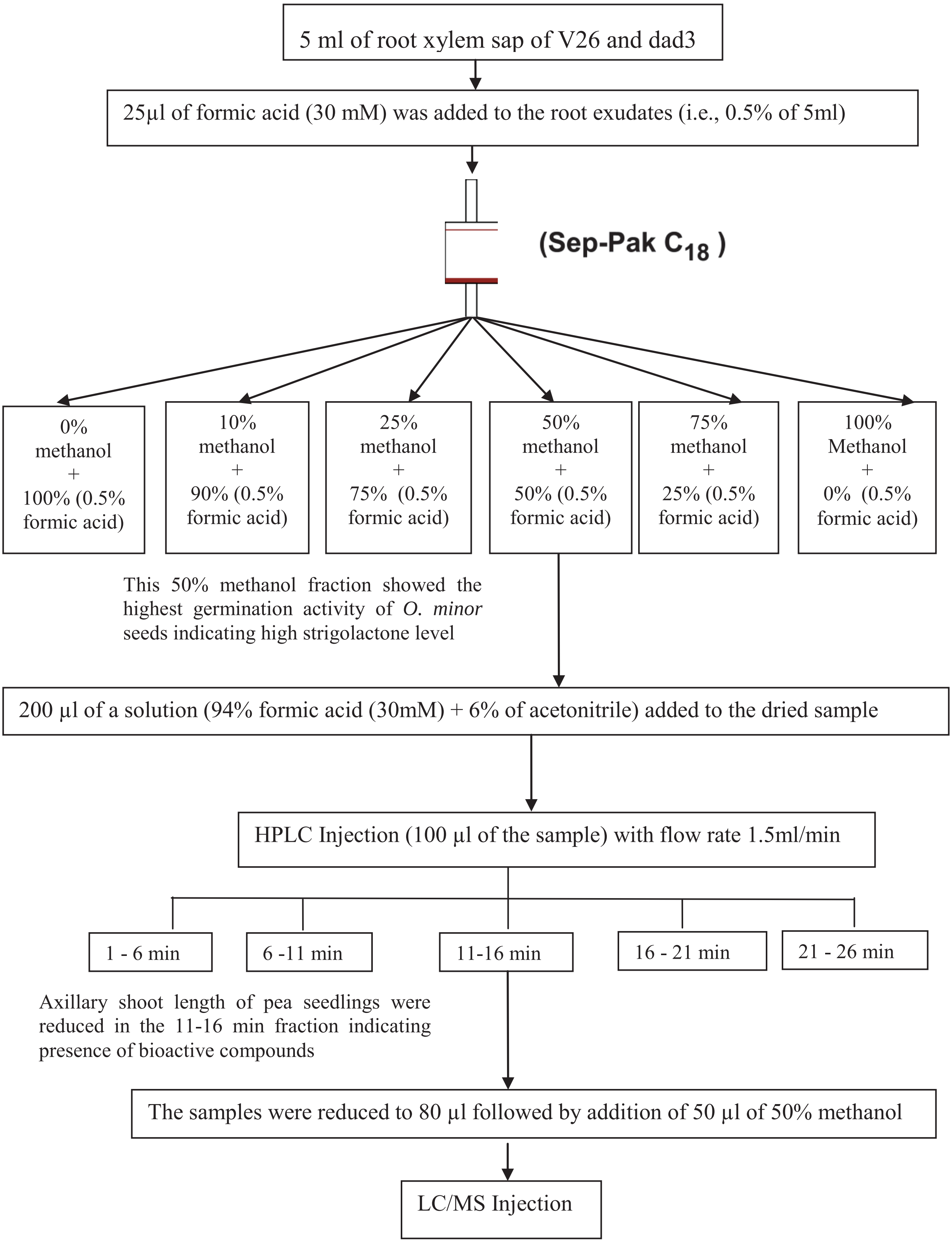
Decapitation of pea seedlings was performed in order to stimulate axillary shoot growth (Thimann & Skoog, 1933; Brewer et al., 2009). Seven-day-old (3 days under germination condition and 4 days after transplantation) pea seedlings were decapitated and immediately placed horizontally in 9 cm diameter glass Petri dishes filled with 20 ml of one of the treatment concentrations of GR24 solution. Before decapitation, radicles were removed from the cotyledons in order to minimize space and solution requirements. Using decapitated and de-rooted pea seedlings lying in a solution, allowed easy access of hormones to the buds of these seedlings, both through direct contact and vascular supply. Each Petri dish was covered with a glass Petri dish of the same size and sealed with cellulose tape in order to minimize loss of solution due to evapo-transpiration.

3.2.1.3 Treatments and experimental design

Stock solution of 100 mg L^{-1} GR24 (Chiralix, Nijmegen, The Netherlands) was prepared and diluted to different treatment concentrations as described in Section 2.2.1 (Chapter 2). There were five treatments that included 1, 0.1, 0.01 and 0.001 mg L^{-1} GR24; distilled water was used as the control. A Petri dish with seven pea seedlings represented one replication, and each treatment was replicated three times in a completely randomized design.

3.2.2 Experiment Two - Bioassays and LC/MS analysis to detect a branch inhibiting hormone

In this experiment, bioassays (germination and branching assay), liquid chromatography (LC) and mass spectrometry (MS) were utilized for attempting to detect a branch inhibiting hormone within *Petunia* and *Zantedeschia*. A summative outline of all the sequential methods applied for samples from *Petunia* is presented in Figure 3.2.



This 50% methanol fraction showed the highest germination activity of *O. minor* seeds indicating high strigolactone level

Axillary shoot length of pea seedlings were reduced in the 11-16 min fraction indicating presence of bioactive compounds

Figure 3.2 Sequential steps of methods applied to detect bioactive compound(s) that inhibit bud outgrowth using *Petunia* samples.

The same method was applied for analysing guttation fluid of *Zantedeschia.* Within *Zantedeschia,* the tested cultivars included both highly branched (Goldilocks) and low branched (Best Gold and GE45) cultivars.

3.2.2.1 Preliminary purification of plant samples using Sep-Pak® C_{18} columns

The preliminary purification procedure followed that of van Hooijdonk (2009) for apple; samples of *Petunia* root xylem sap and guttation fluid of *Zantedeschia* were purified using Sep-Pak® C_{18} columns (Waters Corp., Massachusetts, USA). Each Sep-Pak® column was sequentially preconditioned with 100, 75, 50, 25, 10, and 0 % methanol/0.5% formic acid. To 5 ml of each sample, 25 µl of formic acid (30 mM) was added, and then adsorbed onto one of the preconditioned Sep-Pak columns. The columns were then eluted sequentially with 8 mls of 0% methanol/0.5% formic acid, followed by 10, 25, 50, 75 and 100% methanol/0.5% formic acid solutions. The fractions were subsequently completely dried in a vacuum concentrator (miVAC Quattro concentrator, Genevac Ltd., UK). Three separate runs were conducted for use within a germination assay (Section 3.2.2.2), branching assay (Section 3.2.2.4) and LC/MS (3.2.2.5).

3.2.2.2 Separation of fractions having SLs detected by the *O. minor* germination assay

Five mls of distilled water was added to each dried sample and agitated vigorously using a shaker (IKA NS3, Selangor, Malaysia). A germination assay was performed as described in Chapter 2 (refer Section 2.2.1). Germination of 90-100 preconditioned seeds of *O. minor* was tested on each Sep-Pak fraction of *Petunia* and *Zantedeschia*.

3.2.2.3 HPLC purification of the selected Sep-Pak fraction for branching assay

The dried 50% methanol-eluted fraction derived from *Petunia* root xylem sap as well as *Zantedeschia* guttation fluid were taken for further purification, as this fraction had produced more germinated seeds of *O. minor* (refer Section 3.3.1), being indicative of a high SL level. To each of the dried samples, 200 µl of 94% formic acid (30 mM) and 6% acetonitrile solution was added. After being filtered through a Phenex filter (0.2 µm)

(Phenomenex, North Shore City, New Zealand), 100 µl of each sample was injected into a Dionex Ultimate 3000 LC system (Germering, Germany). The flow rate was 1.5 ml/min and the column temperature was set to 30°C. Solvents were A = Formic acid (30 mM) and B = acetonitrile, and the gradient was: 93% A, 0-3 min; 93% A, 3-20 min; 20% A, 20-25 min; 93% A, 25-26 min. The samples were collected every five minutes, resulting in collection intervals of; 1-6 minutes, 6-11 minutes, 11-16 minutes, 16-21 minutes and, 21-26 minutes. All fractions were dried in a vacuum concentrator (miVac Quattro concentrator).

3.2.2.4 Branching assay on HPLC fractions and experimental design

Before performing the branching assay, 20 ml of (0.01M) phosphate buffer was added to each dried HPLC fraction and agitated. The branching assay was performed as described in Section 3.2.1 but utilizing nine-day-old pea seedlings (3 days under germination condition and 6 days after transplantation) rather than seven-day-old (3 days under germination condition and 4 days after transplantation). Within the current experiment, after decapitation, nine-day-old seedlings produced more than one shoot (normally two shoots) at node 2, whereas seven-day-old seedlings produced only one shoot.

Unlike for GR24 treatment (see Section 3.2.1.3), each seedling was treated as one replication (i.e., seven seedlings represented seven replications). All ten HPLC fractions of dad3 and V26 were applied as treatments, in a completely randomized design. A phosphate buffer (0.01M) was used as the control. Likewise, another set of branching assay was performed that included HPLC fractions of *Zantedeschia* cultivars.

3.2.2.5 Preparation of samples for LC/MS

Based on the results of the branching assay (refer Section 3.3.3), the HPLC fraction of dad3 and V26 collected during the 11-16 minute collection interval was selected for LC/MS analysis. The length of axillary shoots of the pea seedlings were significantly inhibited in this fraction (Table 3.1) interpreted as indicating the presence of a branch inhibitor.

Fractions of V26 and dad3 sap (7 ml out of 8 ml) (1 ml being used to test whether or not this volume of sample being enough for detecting compound/s of interest through LC/MS), collected at 11-16 minutes following the preceding purification procedure (refer “sample purification through HPLC” above), were dried using a rotary evaporator (Rotavapor-R, Buchi Laboratories, Switzerland). Each fraction was dried to approximately 80 µl, with 50 µl of 50% methanol added to create a final volume of each sample of 130 µl, which was stored at -20°C until analysed. Unfortunately, the branching assay results using *Zantedeschia* samples did not help identify a fraction that had bud out-growth inhibition activity. So, based on the results obtained within *Petunia*, the HPLC fraction of *Zantedeschia* collected in the 11-16 minute collection interval was analysed through LC/MS.

3.2.2.6 Analysis of HPLC fraction by LC/MS

While performing analysis through LC/MS, the contributions of staff within Plant & Food Research NZ, especially Tony McGhie, in facilitating the analysis is highly acknowledged. The method followed was that as described in McGhie et al. (2011), with slight modification. For sample analysis using LC/MS, the system comprised a Dionex Ultimate 3000 Rapid Separation LC system and a microOTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray ion (ESI) source. The LC system contained a SRX 3300 solvent rack/degasser, HPR-3300RS binary pump, WPS-3000RS thermostated autosampler, and a TCC-3000RS thermostated column compartment. The analytical column was a Zorbax^{TM} SB-C18 2.1 x 100 mm, 1.8 µm (Agilent, Melbourne, Australia) maintained at 50°C and operated in gradient mode at a flow of 300 µl/min. Solvents were 100% MeOH and 0.5% formic acid represented by A and B respectively. The gradient was 5% A, 95% B, 0-0.5 minutes, linear gradient to 30% A, 0.5-8 minutes; 30% A, 8-11 minutes; 75% A, 11-12 minutes; 100% A, 12-13.2 minutes and 5% A, 13.2-17.5 min.

High resolution mass spectrometry (HRMS) was used for identifying branching inhibitor/s within the HPLC fraction (11-16 minutes) of dad3 and V26. The elemental composition of compounds of interest (refer Section 3.3.4) were calculated from the accurate mass data. The synthetic SL ‘GR24’ (1 or 10 µM) was used as a standard to optimize mass spectrometry values where the temperature was 200°C, N_2 flow at the rate of 8 L/min for drying, N_2 for nebulizing was set at 1.5 bar, endplate offset of 500V, mass range of 60-1000 Da at 1 scans/s and a capillary voltage of 4000 (McGhie et al. 2011). Positive ion mode electrospray was used.

Within the base peak chromatogram (BPC) traces of GR24 (1 or 10 µM), a peak that appeared at 14 minutes retention time (RT) (Figure 3.3) was GR24, whereas the other peaks (Figure 3.3) were actually the background compounds or contaminants. The peak at 14 minutes RT was verified as being GR24 as it had three extracted chromatograms having ionic masses *m/z* 299.0946, *m/z* 321.0767 and *m/z* with molecular ions $(M + H)^+$, $(M + Na)^+$ and $(2M + Na)^+$, respectively (Figure 3.4), that correspond to the molecular mass of GR24 (i.e., 298). In addition to the GR24 molecules, the LC/MS allowed detection of a daughter fragment of GR24 having a 'D' ring of *m/z* 97 (Figure 3.4), further confirming that the peak identity was GR24 as the 'D' ring is a common structure for all forms of SLs (refer Section 1.5.6, Figure 1.10, Chapter 1). Following the LC/MS of GR24, the analysis of *Petunia* and *Zantedeschia* samples were carried out. The injection volume for plant samples and the standard (GR24) was 2 µl.

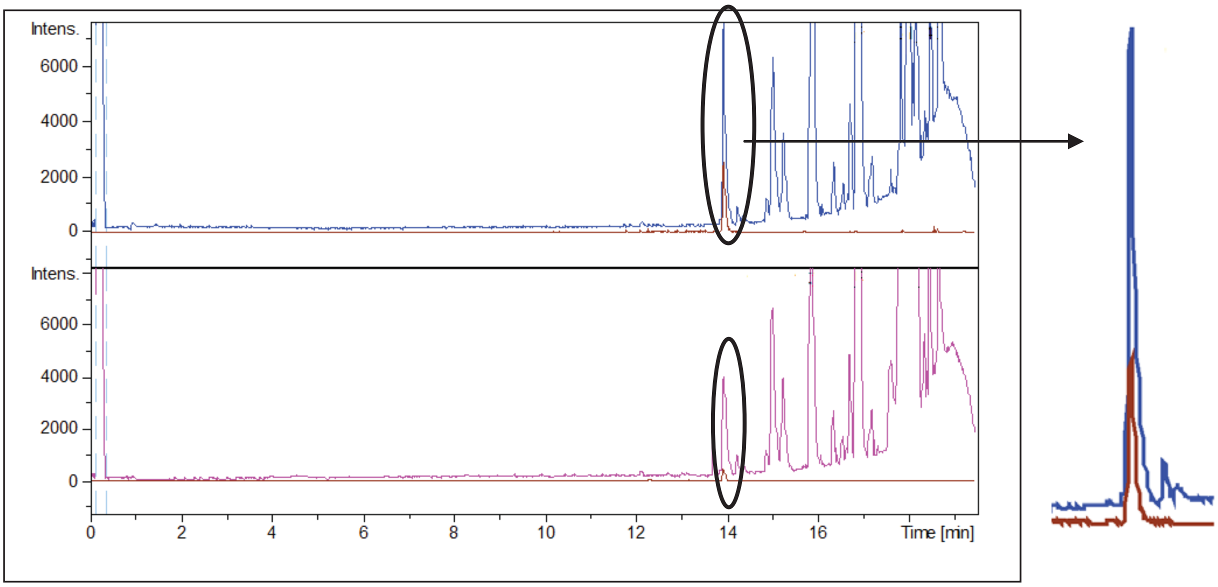


Figure 3.3 Base peak chromatograms (BPC) of GR24 (10 µM, top, blue traces) [magnified] or (1 µM, bottom, pink traces). The peak detected within each chromatogram trace (i.e., top or bottom) at 14 minutes retention time represents GR24, whereas the other peaks are the contaminant (or background) compounds. Brown traces within GR24 10 µM or 1 µM are the extraction ion chromatograms (EIC) for *m/z* 299.0914.

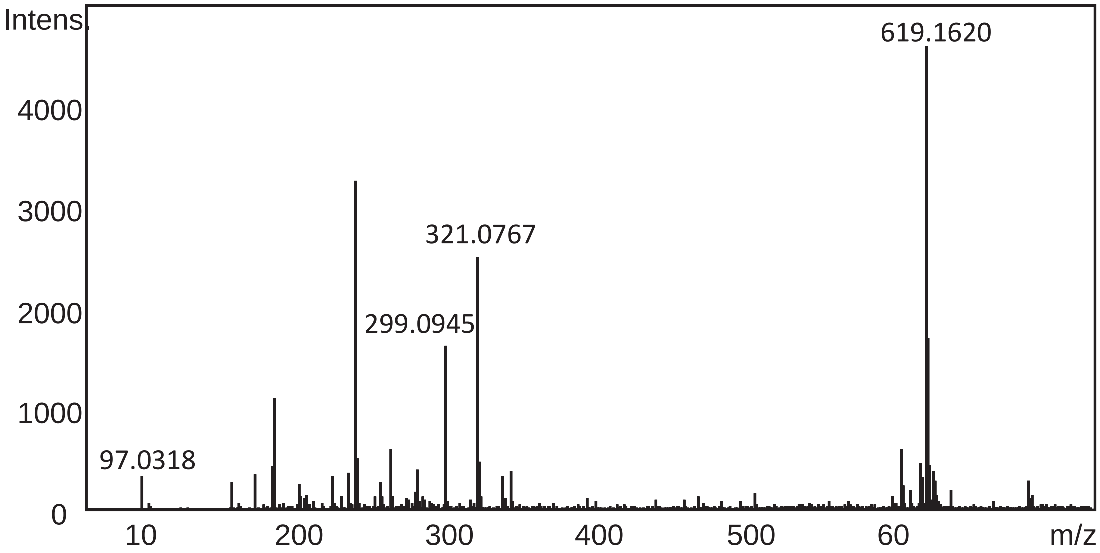


Figure 3.4 Mass chromatograms of a compound detected at 14 minutes retention time within base peak chromatograms (BPC) of GR24 (10 µM), see Figure 3.3. Only four ions with *m/z* 97.0318 $(M + H)^+$, 299.0945 $(M + H)^+$, 321.0767 $(M + Na)^+$ and 619.1620 $(2M + Na)^+$ derived from GR24, whereas the other ions are derived from the contaminant (or background) compounds. Out of four ions, the ion with *m/z* 97.0318 corresponds to the daughter fragment of GR24 for the D ring moiety, and the other three to the GR24 molecule (GR24 molecular mass is 298)**.**

3.2.3 Data collection and analysis

Nodes of the pea seedlings were numbered acropetally with the node above the cotyledon named as node 1 (refer Figure 3.9). In Experiment One, about 98% of seedlings produced only one axillary shoot at node 2. So for consistency, the seedlings that produced two shoots at node 2 (e.g. seedlings treated with 0.001 and 0.01 mgL^{-1} GR24) were not included. However, in Experiment Two, seedlings produced either one or two shoots at node 2. In addition to the shoot at node 2, there was substantial growth (about 1 to 4 mm) of the axillary shoot at node 1 (refer Table 3.1) in some of the seedlings. When this occurred, the total length of the shoots at node 1 and also at node 2 was considered as a measure of growth (Braun et al., 2011). Data (axillary shoot length and number) were collected daily, for up to ten days Experiment One) or just once, 10 days (Experiment Two) after commencement of treatment application. Data were analysed using the GLM procedure of SAS 9.2 (SAS Institute Inc., NC, USA). Mean separation was done using the least significant difference (LSD) at $P = 0.05$.

3.3 Results and discussion

3.3.1 Effect of GR24 on axillary bud release and shoot length

When applied to decapitated pea seedlings laid horizontally within a Petri dish, GR24 reduced the number of buds released at Day One ($P < 0.01$) (Figure 3.5). However by Day Ten only GR24 at 1 mgL^{-1} significantly ($P < 0.05$) reduced the number of buds released, although there was a strong general trend for increasing GR24 concentration to decrease the number of buds released (Figure 3.5).

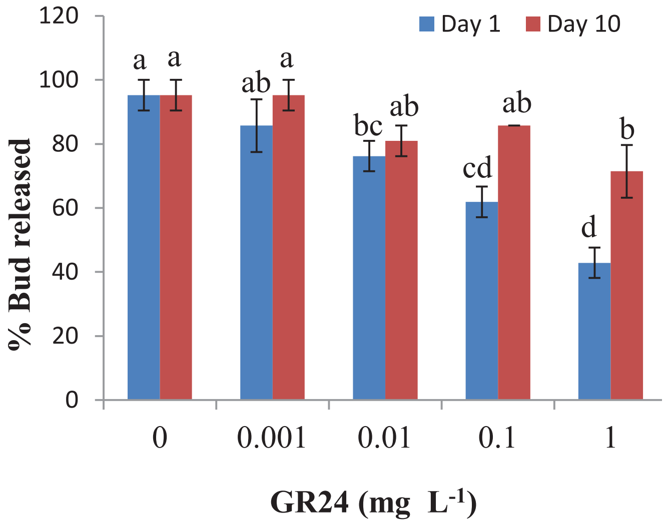


Figure 3.5 Percentage of buds released on Day One and Day Ten at node 2 of decapitated pea seedlings (seven days old), var Ashton, as affected by different concentrations of GR24. Seedlings were treated with the GR24 solutions immediately after decapitation. Nodes were numbered acropetally, with node 1 being the first above the cotyledons. For each parameter, means with the same letters do not differ significantly at $P \leq 0.05$ based on LSD. Vertical bars represent standard errors (n=3).

Compared to the control, the bud release in GR24 (1 mgL^{-1})-treated seedling was about 45% less at Day One, but reduced to 25% less at Day Ten. About 20% of buds were released in between Day One and Day Five, but with no further release subsequently

(Figure 3.6), suggesting that GR24 inhibits as well as delays the release. With lower concentration of GR24 (0.1 and 0.01 mgL^{-1}), at Day Ten the bud release was not significantly different from that in the control, however the release was delayed by one to four days (Figure 3.6). For example, on Day One, about four buds (i.e., about 57%) were released in 0.1 mgL^{-1} GR24 treated seedlings, which increased to about six (about 86%) by Day 4 (Figure 3.6). Supporting the delay in release by GR24, Crawford et al. (2010) mentioned that 10% buds were released later in basally applied GR24-treated *Arabidopsis* nodal segments, as compared to the control. However, in their experiment, the number of days by which bud release was delayed, was not reported.

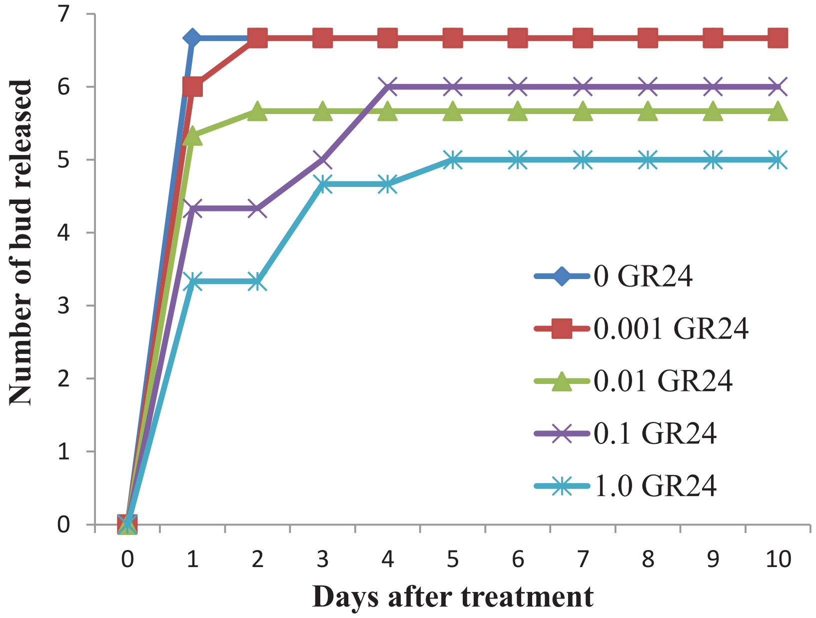


Figure 3.6 Number of buds released from Day Zero to Day Ten at node 2 of decapitated pea seedlings (seven days old), var Ashton, as affected by different concentrations of GR24. Seedlings were treated with the GR24 solutions (mgL^{-1}) immediately after decapitation. Nodes were numbered acropetally, with node 1 being the first above the cotyledons. Numbers of buds recorded were out of a total of seven seedlings.

In the current experiment, the reduced number of released buds with GR24 at Day Ten (Figure 3.5) suggests that SL inhibits decapitation-induced bud release, particularly at the highest GR24 concentration. In contrast, SL applied basally to the nodal segments of

Arabidopsis failed to inhibit the release (Crawford et al., 2010), although the concentration of GR24 was higher (about 1.5 mgL^{-1}) than that used in the current experiment. As a rapid signal was proposed to induce the initial trigger of bud release after decapitation (refer Section 1.4.4, Chapter 1), it is highly likely that within the experiment by Crawford et al. (2010), the buds were released before they responded to basally supplied GR24. When GR24 was applied basally, it is also possible that the buds did not receive adequate SL to overcome the effect of decapitation-induced bud release. After decapitation, CK levels increased by 3 times within 3 hours, and about 10 times within 6 hours, within nodal segments of pea plants (Tanaka et al., 2006), suggesting that CK releases buds after decapitation. Recently, it was found that sucrose induced bud release within two hours since sucrose was found to increase by 2.5 times at node 2 of pea plants 24 hours after decapitation (Mason et al., 2014). Hence in the current experiment, increased levels of cytokinin and/or sucrose may have reduced the inhibition effect of SL on bud release. However, the recent finding (Mason et al., 2014) suggests that sucrose is more important than cytokinin as the decapitation-induced initial trigger for bud release. Increased cytokinin and/or sucrose level in shoots after decapitation may also explain why the lower concentration of GR24 was less effective in inhibiting bud release (Figure 3.5). Although in the current experiment, endogenous CK or sucrose was not measured, it is likely that GR24 (1 mgL^{-1}) may have interacted with endogenous CK or sucrose in influencing bud release. Further attempts to examine the effect of GR24 over the number of buds released, as well as its interaction with CK in un-decapitated pea stems, have been described in Chapter 6. However, for any future experiments, it would also be interesting to explore the interaction between SL and sucrose on bud release, both in decapitated and un-decapitated plants.

The length of axillary shoots at node 2 of decapitated pea seedlings was reduced ($P < 0.001$) by higher concentrations (0.1 and 1 mgL^{-1}) of GR24 at both Day Three and Day Ten (Figure 3.7, 3.9). Compared to the control, higher conencentrations of GR24 reduced the shoot length by two times and by three times at Day Three and Day Ten respectively (Figure 3.7). The growth rate observed for 10 days in terms of shoot length was reduced with increasing concentration of GR24 (Figure 3.8).

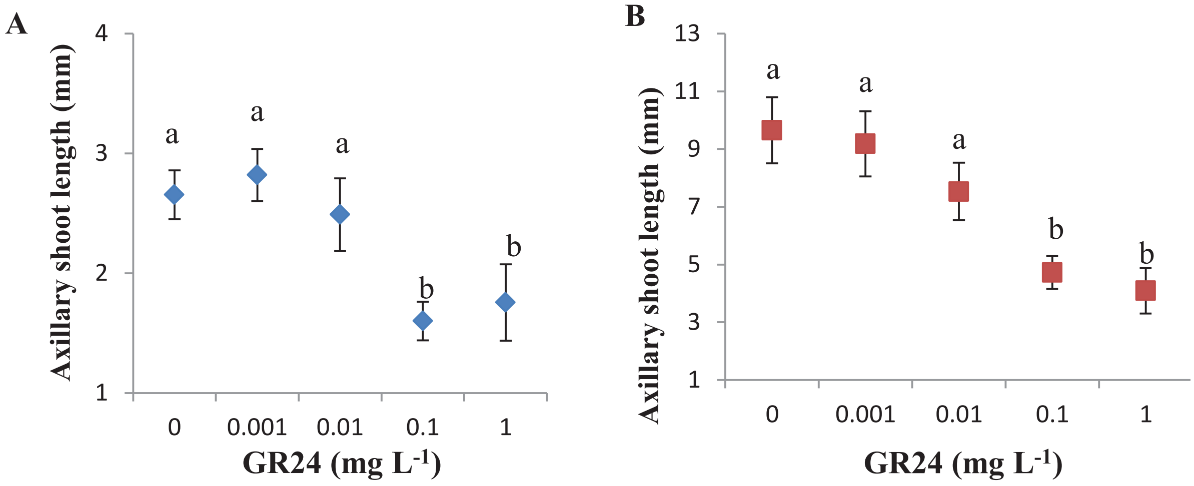


Figure 3.7 Effect of different concentration of GR24 on the length of the axillary shoot at node 2 of decapitated pea seedlings (seven days old), var Ashton, observed at 3 days (A) and 10 days (B) after treatment. Seedlings were treated with the GR24 solutions immediately after decapitation. Nodes were numbered acropetally, with node 1 being the first above the cotyledons. Vertical bars represent standard errors (n=2 or 3). In each graph, mean values with the same letters do not differ significantly at $P \leq 0.05$ based on LSD.

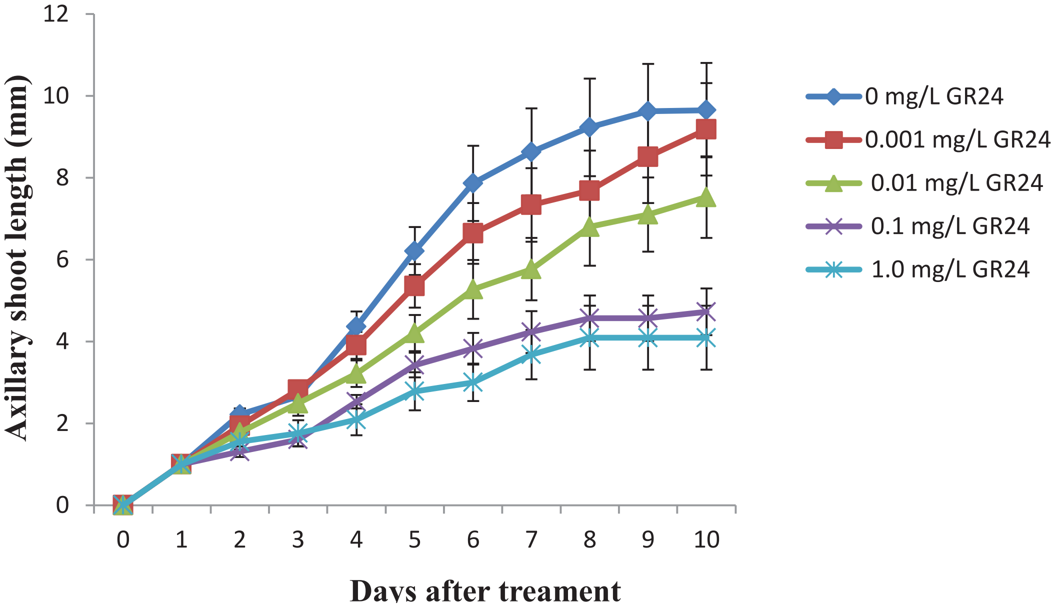


Figure 3.8 Axillary shoot length at node 2 of decapitated pea seedlings (seven days old), var Ashton, as affected by different concentrations of GR24 observed from Day 0 to Day 10 after treatment. Seedlings were treated with the GR24 solutions immediately after decapitation. Nodes were numbered acropetally, with node 1 being the first above the cotyledons. Vertical bars represent standard errors (n=2 or 3).

Reduced shoot length with GR24 suggests that SL inhibits the subsequent growth of the buds in decapitated plants. Brewer et al. (2009) and Dun et al. (2013) also found inhibition of bud growth with directly applied GR24 to the bud of decapitated pea seedlings. However, they made no clear distinction between bud release and the subsequent growth, since all they reported was mean length. In the experiment reported by Brewer et al. (2009), since GR24 was applied to the buds of a certain length (about 0.4 mm), the physiological stage of the buds during hormonal application was not clear, but release of such buds prior to GR24 application is highly likely.

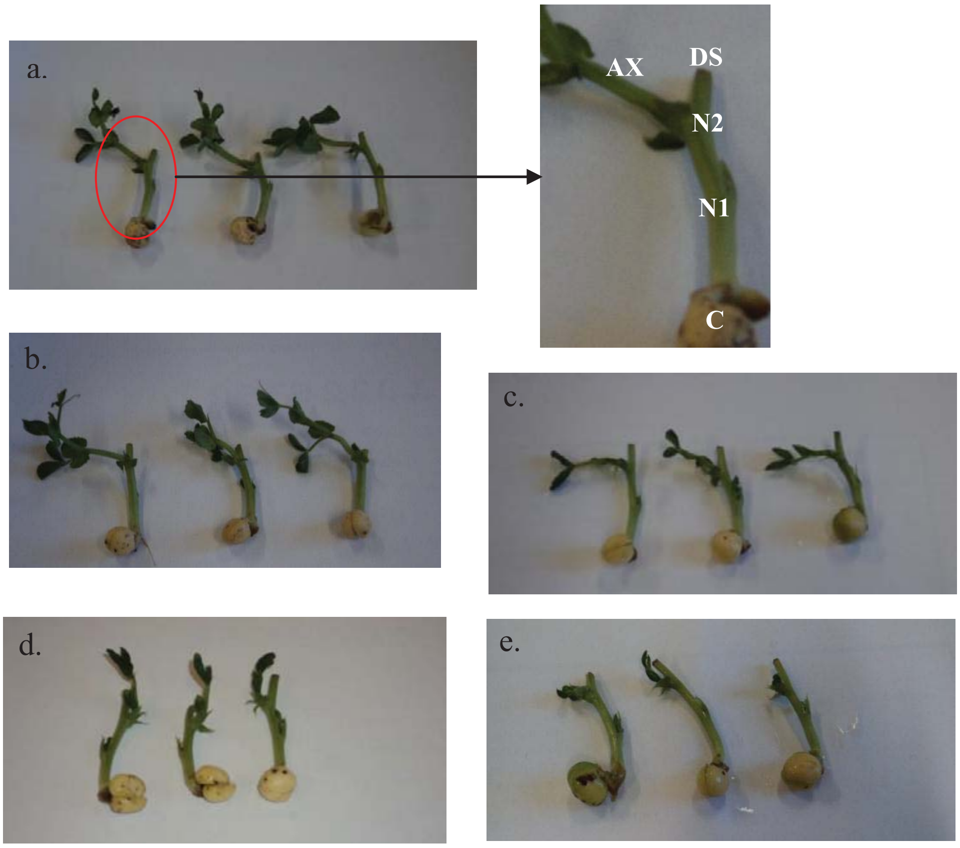


Figure 3.9 Axillary shoot growth of decapitated pea seedlings (seven days old), var Ashton, 10 days after decapitation following treatment with; (a) 0 (control) with magnified view of the part within red oval, (b) 0.001, (c) 0.01, (d) 0.1 and (e) 1 mgL^{-1} GR24. Seedlings were treated with the GR24 solutions immediately after decapitation. N – node (values indicate node number), AX - axillary shoot, C – cotyledon and, DS – Decapitated stump.

3.3.2 Strigolactones (SLs) in different Sep-Pak fractions

For the low branching *Petunia* phenotype V26, the fraction eluted with 50% methanol produced the maximum germination activity (i.e. about 70% germination, being about 45% greater than in 0% methanol) of *O. minor* seeds, suggesting that SLs are mainly present in this fraction (Figure 3.10). Similarly the samples from the low branching phenotypes of *Zantedeschia* ('GE45' and 'Best Gold') also showed the highest germination activity in the fraction that contained 50% methanol (Figure 3.10), further supporting the fraction eluted with 50% methanol has high level of SLs. However, analyzing this fraction through the LC/MS for identifying the presence of SL may be worth testing. Identifying SLs using LC/MS was not carried out at the current time of point as the focus of this study was to first isolate the fraction that has branching inhibition effect (refer Section 3.3.3 and 3.3.4). For the samples from the dad3 mutant of *Petunia*, germination activity was highly reduced in all the fractions, suggesting no or reduced SLs. Lack of germination in the fractions particularly the fractions eluted with 10% or 100% methanol may be due to the presence of germination inhibitors.

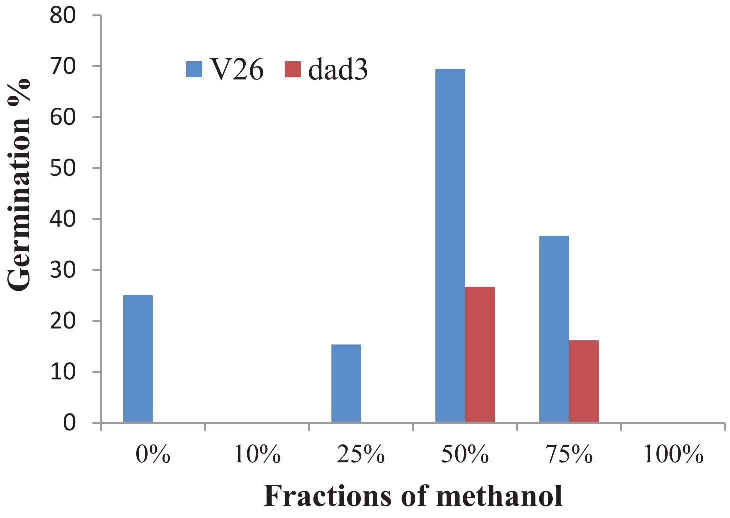


Figure 3.10 Germination percentage of *O. minor* seeds as affected by different Sep-Pak methanol fractions of *Petunia* (V26 and dad3).

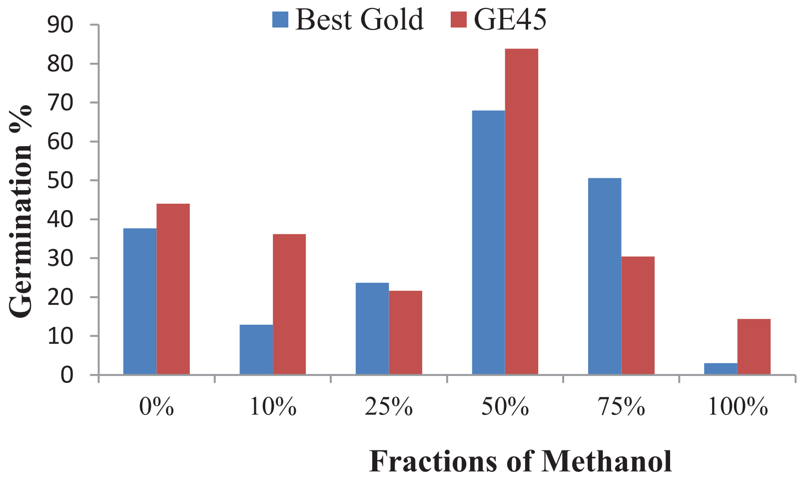


Figure 3.11 Germination percentage of *O. minor* seeds as affected by different Sep-Pak methanol fractions of the less branched *Zantedeschia* cultivars Best Gold and GE45.

With these data, a high concentration of SL within the fraction eluted with 50% methanol is in fact reported for the first time in samples of *Petunia* and *Zantedeschia*. Consistency in the results in these two species suggests that SL (perhaps related to branching) eluted with 50% methanol may exist in other species as well. So, although identification of SL within the fraction was not undertaken, this fraction was useful to investigate whether SL or its derivates present in this fraction inhibits branching (bud release or subsequent growth) (also see Section 3.2.2.3).

The fractions eluted with 10% or 100% methanol did not produce any germinated seeds of *O. minor*; it could be suggested that these fractions might contain inhibitors. As noted in non diluted sap of *Petunia* particularly for the wild-type V26, the zero germination (Figure 2.6, Chapter 2), was probably due to the presence of a germination inhibitor (refer Section 2.3.2.2 and 2.3.3, Chapter 2).

3.3.3 Axillary shoot length in different HPLC fractions

Axillary shoot length at node 2 of decapitated pea seedlings was significantly ($P < 0.05$) affected by fraction collected between 11 to 16 minutes from samples of the wild-type *Petunia* V26 (Table 3.1). The shoot length within this fraction was reduced by more than half compared to the control. In contrast, the shoot length of pea seedlings at node 2 treated with the rest of the fractions from V26, and all dad3 fractions, were not significantly different from that of the control (Phosphate buffer 0.01M). Reduced shoot length derived from the fraction 11-16 minutes of V26 suggests the presence of a bioactive inhibitor in this fraction that specifically inhibits axillary shoot length. As this fraction was derived from the Sep-Pak 50% methanol fraction that contained SLs (Section 3.3.2), it was hypothesized that the bioactive compound was either a SL itself or derivative/s.

Table 3.1 Length of axillary shoots (±SE) at each of two nodes of decapitated seedlings (nine days old) of pea var Ashton, as affected by different HPLC fractions derived from 'dad3' and 'V26' *Petunia*, obtained 10 days after treatment commenced.

Treatments	**Shoot length (mm)**			**Number of shoots**	
	Shoot at node 1	**^{Z}Shoot at node 2**	**^{Z}Total shoots at node 1 & 2**	**Node 2**	**Node 1 & 2**
Control*	3.00 ±1.00	17.13 ±2.34 ab	19.38 ±2.97 a	1.75 ±0.25	2.50 ±0.38
dad3 (1-6min)	3.20 ±1.50	17.38 ± 2.17 ab	19.38 ±2.15 a	1.75 ±0.16	2.38 ±0.32
dad3 (6-11min)	2.17 ±0.60	21.14 ± 1.55 a	23.00 ±1.23 a	2.00 ±0.22	2.86 ±0.26
dad3 (11-16min)	2.50 ±0.67	19.29 ±2.83 ab	21.43 ±2.91 a	1.86 ±0.14	2.71 ±0.18
dad3 (16-21min)	2.00 ±0.44	14.88 ±1.38 b	16.63 ±1.22 ab	2.00 ±0.00	2.88 ±0.13
dad3 (21-26min)	1.60 ±0.40	18.75 ±1.60 ab	19.75 ±1.35 a	1.88 ±0.13	2.50 ±0.27
V26 (1-6min)	3.43 ±1.09	16.29 ±3.11 ab	19.71 ±3.57 a	1.71 ±0.18	2.71 ±0.18
V26 (6-11min)	4.38 ±1.36	18.00 ±1.95 ab	22.38 ±2.37 a	1.75 ±0.16	2.88 ±0.13
V26 (11-16min)	2.63 ±0.63	8.63 ±2.06 c	11.25 ±2.05 b	1.38 ±0.18	2.38 ±0.18
V26 (16-21min)	3.25 ±1.35	18.75 ±3.08 ab	22.00 ±2.77 a	1.75 ±0.16	2.75 ±0.16
V26 (21-26min)	3.57 ±0.84	19.25 ±1.59 ab	22.38 ±1.66 a	1.88 ±0.13	2.75 ±0.16
P value	0.569	0.023	0.026	0.404	0.714

^{z} Means with same letters within a column do not differ significantly at $P \leq 0.05$ based on LSD
* Phosphate buffer (PB) (0.01M) was used as the control.

Unlike pea seedlings tested in the branching assay with GR24, there was substantial growth (about 1 to 5 mm) of the shoot at node 1. Considering the total shoot length of node 1 and node 2 combined, interestingly the V26 fraction (11-16 minutes) significantly reduced the shoot length compared to the control (Table 3.1). However, at node 1, the axillary shoot length arising from treatment with this fraction was not significantly different from the control (Table 3.1), which might be due to less access of the inhibitor to the bud at node 1 compared to the bud at node 2.

By Day Ten, unlike with GR24 which inhibited bud release (Figure 3.5), none of the fractions repressed the number of shoots of the decapitated seedlings at both nodes, i.e. node 1 and node 2 combined, or node 2 alone (Table 3.1). However, the lack of inhibition over release may be due to the low concentration of inhibitor within the fraction.

3.3.4 Detection of unknown compounds in V26

Four unknown compounds were detected in the HPLC fraction (11-16 minutes) of the samples from the wild-type *Petunia* V26 that were not present in dad3, making them candidates for closer examination as potential inhibitor compounds. Among these compounds, two were detected between 9-10 minutes and 12-13 minutes, respectively, and the other two at 14-16 minutes (Figure 3.12). Since the sample of ‘V26’ was derived from the HPLC fraction that had shown reduced branching activity (refer Section 3.3.3), these compounds are possibly the bioactive compounds.

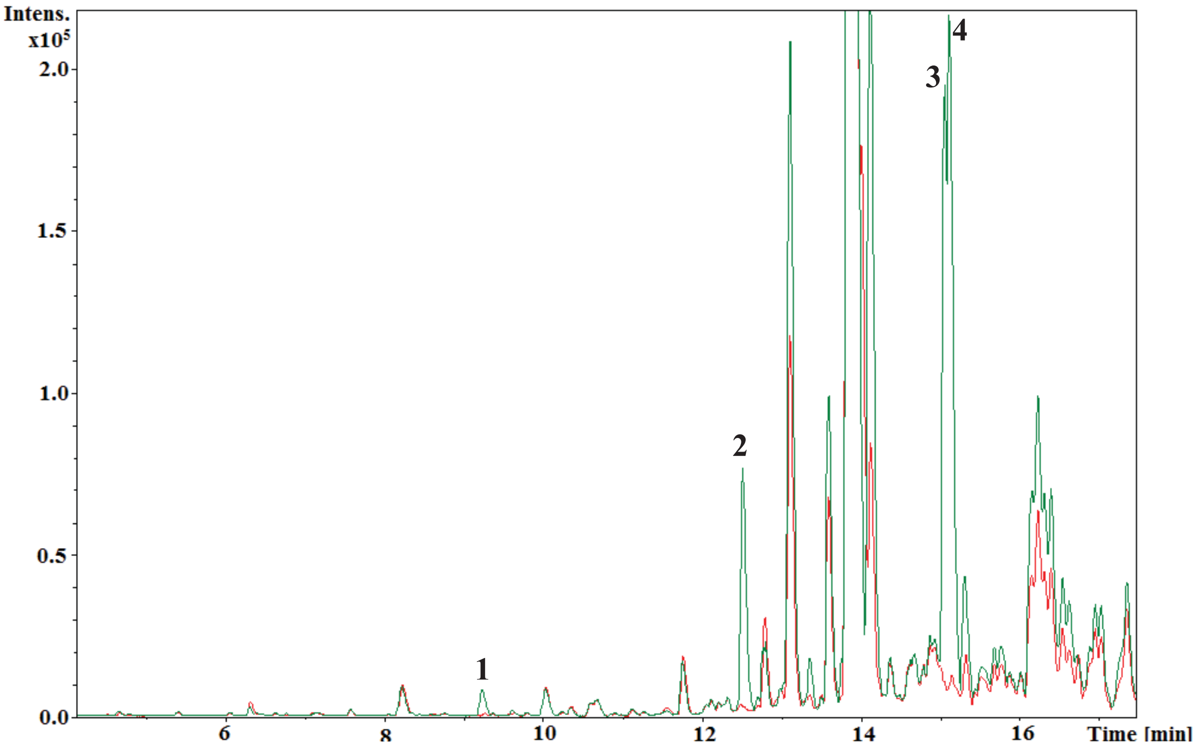


Figure 3.12 LC/MS traces or base peak chromatogram (BPC) of HPLC fractions derived from either *Petunia* V26 or dad3 root xylem sap collected between 11-16 minutes. Green coloured peaks represent LC/MS trace of V26 (low branched) and red peaks represent dad3 (highly branched). Four distinct peaks were observed, indicated by the numbers (1-4), in V26 but not in dad3. Among these peaks, two were detected in between 9-10 minutes and 12-13 minutes and the other two at 14-16 minutes.

SL was not evident within LC/MS traces of V26 and dad3 based on the fact that the daughter fragment ionic mass with D-ring moiety was not detected, although this may be due to lack of SL fragmentation. Even though SL is commonly detected through LC/MS using MRM (multiple reaction monitoring) mode (Xie et al., 2010), due to unavailability of internal standards and inaccessibility of MRM mode within the MS system used in this experiment, this method was not attempted. In fact, the objective of the experiment was to determine bioactive compound/s (refer Section 3.1) rather than SL itself, and it was recognised that such compound/s may or may not be SLs. So, high resolution mass spectrometry (HRMS) mode, which is used for qualitative and quantitative analysis of different unknown compounds without a need of internal standards (Aceña et al., 2015), was adopted (refer Section 3.2.2.6). In the current experiment, the LC/MS system with HRMS mode, accompanied with comparative sample analysis between high and low branched *Petunia*, enabled detection of compounds (refer Figure 3.12) that could be the branching inhibitor(s). Unfortunately, due to limitation of resources, the identity of the four compounds detected in V26 has not been determined. To verify whether the compounds were not contaminants, a blank run was made. As no corresponding peaks were found in the blank, it was therefore confirmed that all four compounds came from the V26 sample.

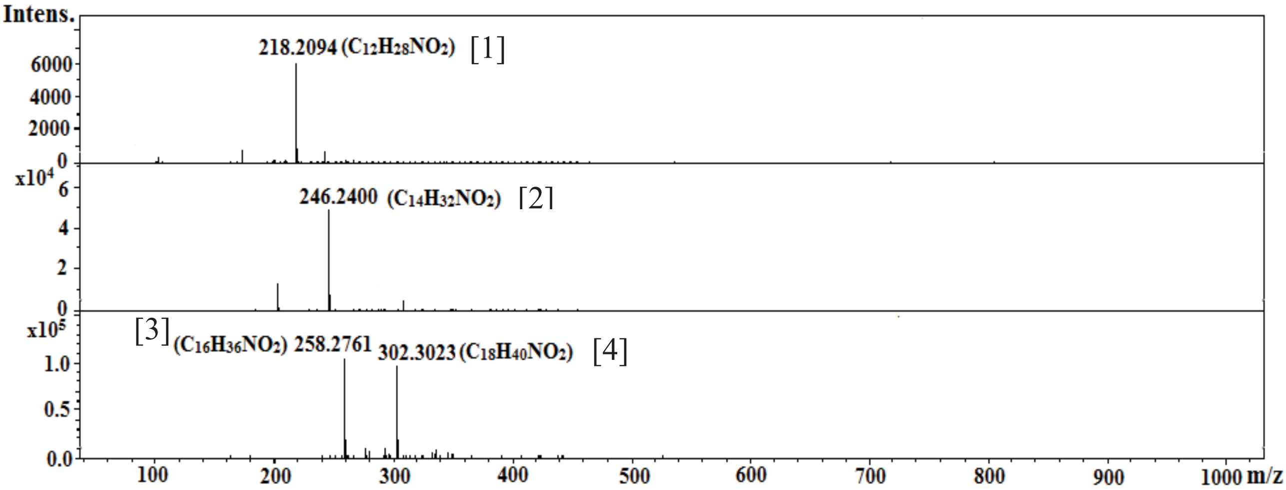


Figure 3.13 Mass spectrum of four compounds derived from the sample from V26 ([1] –[4]) (refer Figure 3.12), with their respective elemental compositions within small brackets and *m/z* values. MS conditions: positive mode; capillary voltage, −4000 V; end plate offset, −500 V; source temperature, 200°C; drying gas flow, 4 L/min.

Although the compounds could not be chemically identified, interestingly through the mass spectrometric analysis, it was found that the elemental composition of each unidentified compound contains N in addition to C, H and O (Figure 3.13). In contrast however, the SL-related compounds such as SL precursors (e.g. carlactone, refer Figure 1.6), or different forms of SL discovered (Chapter 1, Figure 1.10) so far, do not contain N. It is possible that the compounds detected may be SL downstream products that contain N or SL-conjugates like in other hormones such as auxin that conjugates with amimo acids (eg. IAA-aspartate, IAA-Alanine). It is also possible that such SL-conjugates may be actually a branching inhibitior. For example, the hormone Jasmonate which does not contain any N compound in itself but in association with Isoleucine the compound becomes bioactive (Staswick & Tiryaki, 2004). The presence of N in association with SL is in fact the first time reported within this study and needs further investigation.

3.4 Conclusion

In summary, externally applied SL can inhibit both the decapitation-induced bud release and the subsequent growth of axillary shoots. The integration of LC/MS techniques and bioassays, including germination and branching specific assays, has been attempted in order to identify a bioactive compound. Despite failure to chemically identify a bioactive compound that may have inhibited the axillary bud outgrowth, the development of methods can be considered successful to some extent. It is expected that with further improvement of the overall method and use of samples from highly branched and low branched plant types/cultivars of different species may help to discover the chemical identity of a bioactive inhibitor which may or may not be SL.

4 Transport of strigolactones in guttation fluid of *Zantedeschia*

4.1 Introduction

In Chapter 2, it was demonstrated that strigolactones (SLs) are present in the guttation fluid of different cultivars of *Zantedeschia* K. Spreng. Other hormones such as auxins (Aloni et al., 2003) and abscisic acid (ABA) (Thompson et al., 2007) have been reported as present in the guttation fluid of *Arabidopsis* and tomato (*Solanum lycopersicum),* adding further support to the probability that SLs would be present within guttation fluid. The presence of SLs in guttation fluid, which is actually the xylem sap (Grunwald et al., 2003), suggests that this hormone is transported from the root to the shoot via xylem vessels. Supporting this hypothesis, SLs are mainly synthesized in roots (Xie et al., 2010) and, as reported for other root produced hormones like cytokinins (Kulaeva, 1962) and ABA (Zhang & Davies, 1987), SLs are delivered to the shoot system via flow of the xylem sap (Kohlen, 2011).

Analysis of SLs in the guttation fluid of *Zantedeschia* has shown that 'Goldilocks', a highly branched cultivar, had a lower concentration of SLs as compared to the low branched cultivars Best Gold and GE45, at the leaf emergence stage of the annual growth cycle (Chapter 2). This leads to the suggestion that SLs could have a role in inhibiting branching in this crop. It remained unclear however, what if any relationship might exist at other stages of the annual growth cycle, as 'Goldilocks' also showed a high concentration of SLs at a stage when branches were visibly evident. It was previously hypothesized (Chapter 2) that any relationship between SLs and branching, might be lacking after branches have been developed. As an alternative hypothesis, the varying concentration of SLs at different growth cycle stages in different cultivars may also be due to the varied volume of guttation fluid (or xylem sap). Supporting this hypothesis, Beck & Wagner (1994) found a negative correlation between volume of xylem sap and concentration of a hormone present in the sap. If so, then this may ultimately lead to differences in concentration of SLs within the guttation fluid, as noted in the earlier experiments with *Zantedeschia* (refer Table 2.2, Chapter 2), but the total amount delivered to the shoot may not vary.

Guttation fluid appears as droplets mainly at leaf tips through hydathodes (Grunwald et al., 2003). Guttation occurs when the air is humid, especially during darkness at night and early morning (Komarnytsky et al., 2000) when stomata are closed and transpiration is suppressed, resulting in a build-up in root pressure (i.e. a positive hydrostatic pressure) (Vančura et al., 1977). Hence, guttation and transpiration often happen alternately within a 24 hour period. There are different factors such as weather, stress, growth stage and cultivar (Singh et al., 2009) that affect root pressure, ultimately affecting the amount of exudate or guttation fluid. Among those factors however, root pressure is mainly affected by relative humidity and water content of the growing medium (Singh & Singh, 2013). High relative humidity and high water content of the growing medium is favorable for more guttation (Ivanoff, 1963), suggesting these factors enhance root pressure. Since plants grown in a medium that received a regular supply of water produced guttation some hours earlier than those grown under water withheld (Singh et al., 2009), it is highly likely that plants that were well watered produced a greater total volume of guttation than those that were water withheld. In their experiment, guttation commenced late in the afternoon in well watered plants, but not until the early morning of the following day in water withheld plants. Despite no details being presented by Singh et al. (2009) on the actual transpiration rate of well watered plants, the appearance of guttation fluid in the late afternoon suggests that guttation might have occurred when transpiration was either zero or minimal. With transpiration and guttation normally occurring at alternate time periods within the diurnal period, it can be interpreted that the lag period between transpiration stopping and guttation starting was longer for water withheld compared to well watered plants. Building upon this logic therefore, in ‘Experiment One’ of the current chapter, varied volume of guttation fluid was created by withholding water supply to the medium for some days before guttation fluid was collected. In addition, the relative water content (RWC) of the shoot, which is indicative of leaf/shoot water status (Smart & Bingham, 1974), was measured in order to demonstrate the capacity of a water withheld and well watered shoot to hold water. Additionally, as explored within this chapter the logic was also that the guttation or the water 'spill-over' out of the shoot is the result of water saturation in the shoot (also refer Figure 4.11 'C'). Within the current chapter therefore, 'Experiment Two' was designed to determine whether or not the concentration of SLs transported from root to shoot differs in the guttation fluid, and whether it may vary in terms of total volume.

The experiments reported in the current chapter were based two assumptions. One assumption is that there is a constant synthesis of SLs in roots during daylight and darkness. In general, plant hormones including SLs have most commonly been analysed from the xylem exudate extracted/collected during the light period (daylight). Additionally however, SLs have also been detected in the guttation fluid (collection during the natural dark period) (refer Section 2.2.3.2.1 and 2.3.2.1, Chapter 2). Together this therefore supports the assumption that the process of hormonal synthesis is a continous process occuring during both light and dark periods. The second assumption is that the SLs are transported from root to shoot (see Figure 4.11), and the fact that SLs are synthesized in roots and transported to the shoot has been described earlier in this section.

Hence, the aim of the work described in this chapter was to test the hypothesis that the:

1. greater volume of guttation fluid in plants was due to a reduced lag period between transpiration and guttation and,

2. concentration of SLs is not affected by the volume of guttation fluid, and thus provides a reliable estimate of the amount of SLs being exported to the shoot system.

To address the aim, the research objectives explored in the two experiments were, therefore, to:

- quantify the changes in concentration of SLs with changes in the volume of guttation produced,

- determine the transpiration rate and guttation rate in water withheld and well watered plants and,
- quantify the lag period before guttation started and after transpiration stopped in both water withheld and well watered plants.

4.2 Materials and methods

4.2.1 General growing conditions and treatment methodology

In general, the growing system and environmental conditions utilised for cultivation of plants of *Zantedeschia* followed those described in Subbaraj et al. (2010). Each polythene planter bag (‘PB5’ equivalent to 3 L of growing medium) was filled with a bark-based growing medium (Daltons; New Zealand) (see Appendix II) and fertilizers containing 150 kg m^{-3} of dolomite (Ravensdown Fertilizers, New Zealand), 2 kg m^{-3} of 8-9 month Osmocote® (16N–3.5P–10K, Scotts International B.V., Nijverheidsweg, The Netherlands) and 1 kg m^{-3} of 3-4 month Osmocote® (15N–4.8P–10.8K). The glasshouse was maintained at a minimum temperature of 15 °C and vented at 19 °C. The experiments were conducted during mid summer in December/January.

During cultivation, i.e. prior to experimentation, automatic irrigation supplied 50-60 ml of water per plant per day through capillary matting on drained benches. In addition, plants received an overhead irrigation immediately after planting, and continued once a week, in order to reduce any increase in salinity within the surface of the growing medium.

For plants in different treatments, altering the water status of the growing medium was used to achieve different volumes of guttation fluid per plant. Preliminary trials established that the volume of guttation fluid produced by *Zantedeschia* plants could be reduced by an increase in the number of days plants were restricted in their water supply (see Appendix V). In the main experiments presented in this chapter, differing water status of the growing medium was created by withholding water to the medium for a differing numbers of days prior to the collection of guttation fluid. Hence, when the treatment regimes were applied, the irrigation system noted above was slightly modified (refer Irrigation Schedule treatments). Throughout this chapter, plants that received water supply once daily until water saturation level of the medium was achieved, were referred to as ‘well watered plants’, while those which were subjected to restricted periods of watering and consequent reduced water status of the medium, were referred to as ‘water withheld plants’.

4.2.2 Experiment One- Determination of lag period between transpiration and guttation

4.2.2.1 Plant material

Flowering sized tubers of the *Zantedeschia* cultivar Goldilocks were used as planting material for this experiment. Treatments were applied (refer Section 4.2.2.3) when plants were at the stage of leaf emergence, when between one and two leaves per primary shoot first became evident (refer Figure 4.1). At this stage of development, no roots were evident from the base of the planter bag and, therefore, no physical root damage was encountered when plants were moved as part of applying each treatment.

4.2.2.2 Treatments and experimental layout

Treatments comprised two periods of water withheld; water withheld for 0 days i.e., well watered, and stressed for 3 days of no water application i.e., water withheld. In each treatment, the number of plants allocated for the measurement of transpiration/guttation rate, water content of the medium and, relative water content of the leaves were three, three and nine, respectively.

4.2.2.3 Irrigation schedule treatment

To implement the treatments, three days prior to the collection of guttation fluid, plants were shifted to benches with dry (i.e. unwatered) capillary matting, so as to assure no water uptake from the base of the plant. For plants in the ‘water withheld’ treatment, from this point in time automatic irrigation stopped, and plants did not receive water for 3 days before collection of guttation fluid commenced. In contrast, plants within the ‘well watered’ treatment continued to be supplied with overhead irrigation once a day, and one additional overhead irrigation in the afternoon of the day guttation fluid collection commenced.

4.2.2.4 Transpiration and guttation rate

For describing potential differences in transpiration rate between the well watered and water withheld treatments, transpiration rate of each plant was calculated by recording weight loss over time. As such, three plants from each treatment were set on an individual weighing balance. The planter bag containing each plant was covered in a plastic bag in order to avoid water loss due to evaporation (Figure 4.1). The weight of the planter bag with a plant was recorded every hour for 24 hours, starting from 10 am through until 10 am of the next day, and weight loss calculated (i.e. Initial weight – Final weight) from these measurements. This value of weight loss therefore, equated to the total transpiraton per plant over the 24 hour period.

For determining the guttation rate of monitored plants, the amount of guttation fluid was measured every three hours. The distal half of an individual leaf of each plant was covered with a plastic zip-lock bag for collecting guttation fluid (Figure 4.1B) soon after transpiration was determined to have stopped, but before the first appearance of water droplets (guttation fluid) at the leaf tip. The purpose of covering the distal half of the leaf was for proper positioning of the leaf that helps drop the guttation directly into the plastic bag without any leakage. In contrast, if covering the whole leaf, there was a chance of fluid leakage through the mouth of the plastic bag. Collection continued until no further droplets appeared at the leaf tip. At three hour intervals the plastic bag was replaced with a new bag, and a 10 ml syringe with a needle was used to collect the accumulated guttation fluid from each bag, with the contents stored at -20 °C until analysed.

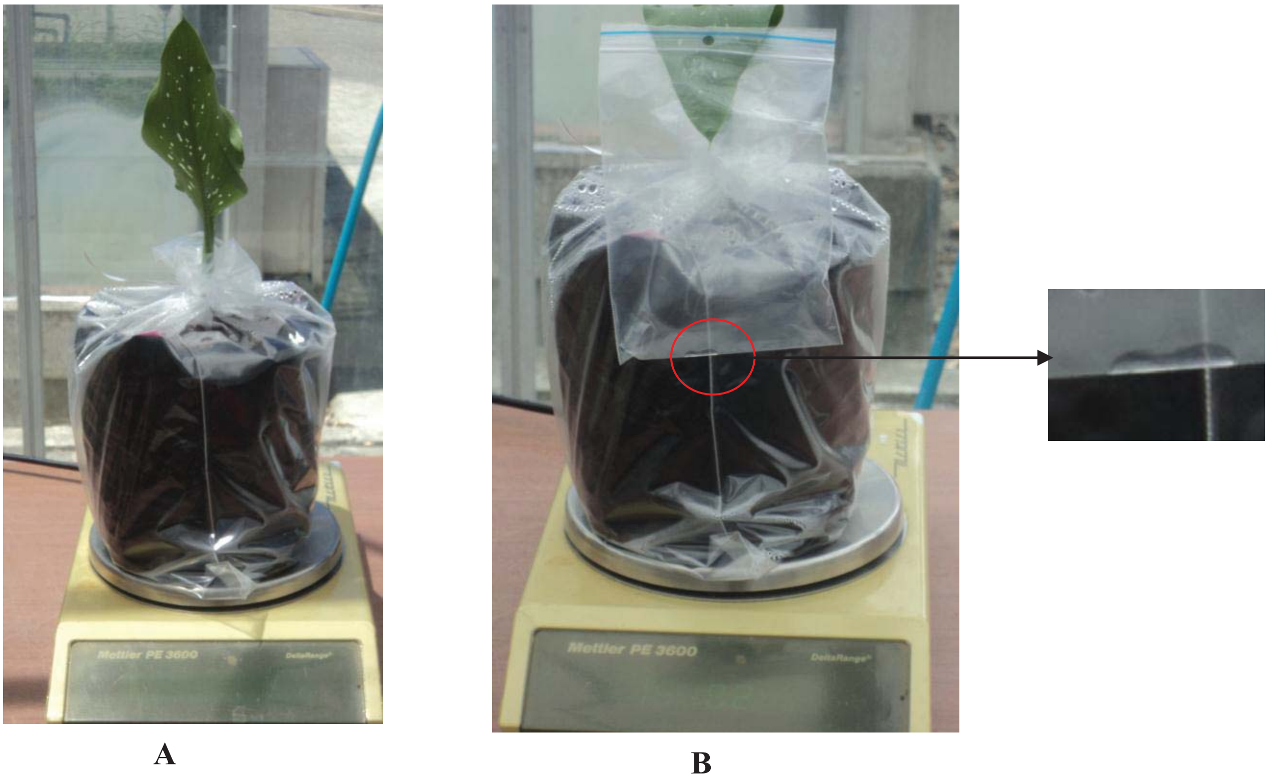


Figure 4.1 Illustration of methodology for use of weighing balance in calculating transpirational water loss from plants of *Zantedeschia*. The planter bag was sealed within a plastic bag to eliminate water loss due to evaporation (A). Collection of guttation fluid from the *Zantedeschia* plant using a zip-lock plastic bag. The bag was positioned to cover the distal half of the leaf for collecting guttation fluid (B). The magnified image within the red circle illustrates the accumulated guttation fluid during one such period.

4.2.2.5 Water content of the growing medium

So as to quantitatively describe the water status of the medium, the water content of the growing medium was measured gavimetrically (Gardner, 1986) at three times (i.e., 11:15 am, 7:20 pm and 5:30 am) over the 24 hour period of sampling guttation fluid. Samples were taken from the same medium of three plants at each time of sampling in both treatments. During sample collection, 40-50 g of growing medium was scooped out about 3 cm below the medium surface and weighed immediately. The medium was dried in an oven at 70 °C for 4 days, with the water content (%) of the medium calculated following the equation:

$$\text{Water content of the medium (\%)} = [(FW_m - DW_m) / DW_m] \times 100$$

where, FW_m = Fresh weight of the medium and

DW_m = Dry weight of the medium

4.2.2.6 Relative water content (RWC)

So as to quantitatively describe the comparative treatment effect on plant water status, the relative water content of the plants were measured. Nine plants in each treatment were allocated for the measurement of RWC of the leaves. Fresh weight, turgid weight, and dry weight, of leaf disks were used to calculate the RWC of plants within each treatment as described by Barrs & Weatherley (1962), using the equation:

$$RWC = [(FW_L - DW_L) / (TW_L - DW_L)] \times 100$$

where, FW_L = Fresh weight of leaf disks,

DW_L = Dry Weight of leaf disks and,

TW_L = Turgid Weight of leaf disks.

The RWC was measured from the leaf samples taken at three times (i.e. 10:30 am, 6:30 pm and 4:30 am) during the 24 hour period of sampling of guttation fluid. At each time, unlike measurement of water content of the growing medium, samples were derived from three additional plants (or single plant replicates), as the method was destructive. Eight leaf disks were taken as subsamples from leaves of a single plant replicate, weighed, and dipped in distilled water for four hours in the dark. Subsequently the turgid weight of leaf disks was recorded, and weights were recorded again after drying in a oven at 70 °C for 4 days.

4.2.2.7 Measurement of leaf area

Leaf area was considered for determining the amount of guttation fluid per unit leaf area. Leaves of each plant subjected to obtaining transpiration and guttation rates had their lamina area measured using a leaf area meter (LI-3100, LI-COR Inc, USA).

4.2.3 Experiment Two- Relationship between concentration of SLs and guttation volume

4.2.3.1 Plant materials

Flowering-sized tubers of *Zantedeschia* ‘Goldilocks’ and ‘Best Gold’ were used as planting material for this experiment. As described for Experiment One, the experiment was started when plants were at leaf emergence of the primary shoots (e.g. refer Figure 4.1A).

4.2.3.2 Treatments and experimental layout

In this experiment, treatments comprised three periods of water withheld (0 or well watered, 1 or 2 days water withheld) and two cultivars (‘Best Gold’ and ‘Goldilocks’; Figure 4.2). There were a total of five plants (each plant represents one replication) of each cultivar in each water withheld treatment regime. Two additional plants of ‘Goldilocks’ and ‘Best Gold’ were allocated in each water withheld treatment regime for obtaining samples from the growing medium to measure water content (refer Section 4.2.2.4).

4.2.3.3 Irrigation schedule treatment

For the treatment regime described as either 2 days or 1 day water withheld, all supply of water was stopped 2 days and 1 day before sample collection, respectively. Well watered plants were irrigated once daily, being increased to twice on the day sample collection commenced (i.e. morning and afternoon). In order to avoid water accumulation in the capillary matting on which the plants were placed, automatic irrigation was stopped 10 days before the sample collection. Plants were overhead irrigated once daily after the automatic irrigation was stopped, in the days prior to application of the three periods of water withheld.

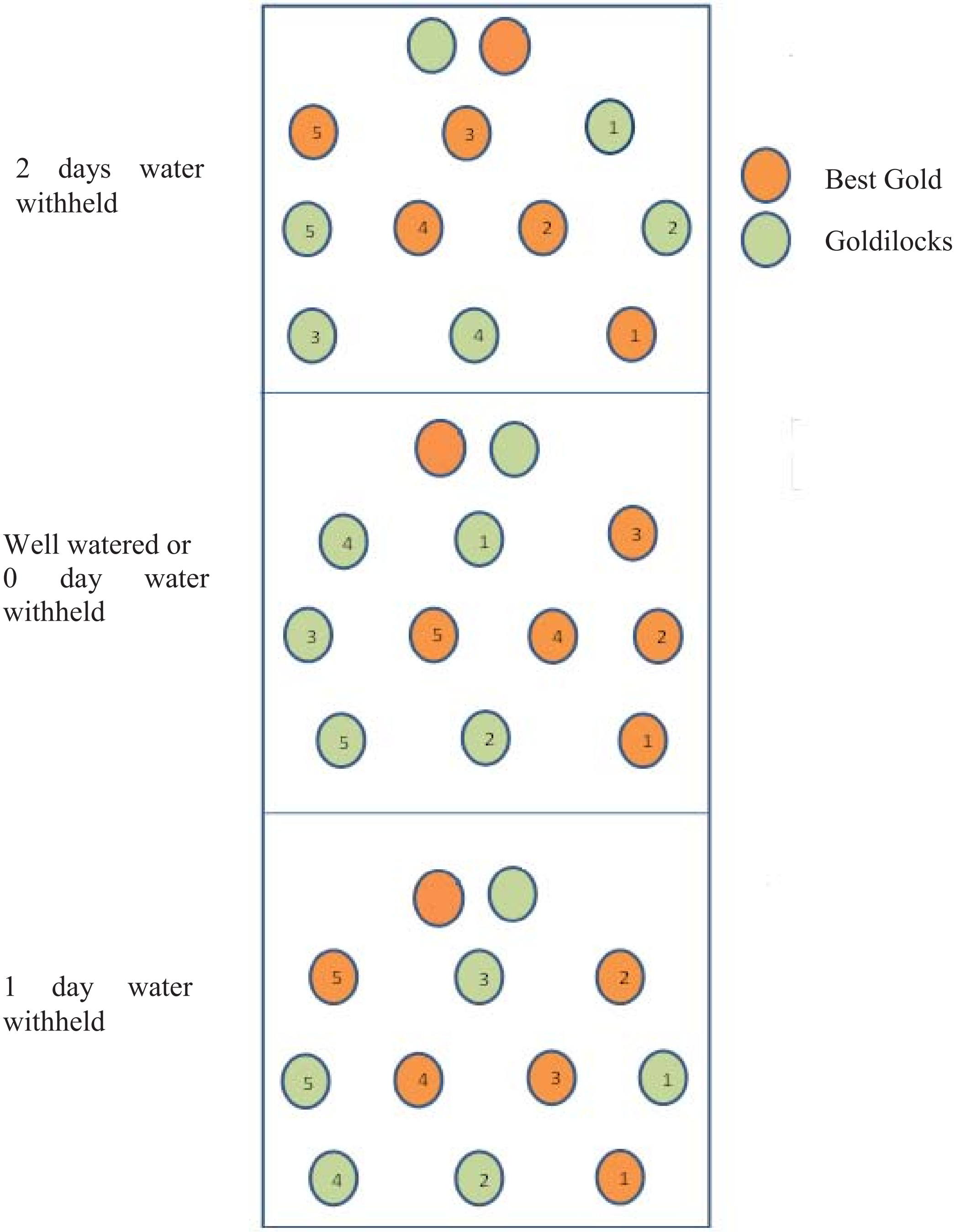


Figure 4.2 Physical arrangement of plants of *Zantedeschia* 'Best Gold' and 'Goldilocks' in terms of the three treatments of differing periods of water withheld (i.e. 0, 1 and 2 days). Two plants of both cultivars, as indicated by the spheres without a number, were allocated to each period of water withheld for obtaining samples from the medium to measure water content.

4.2.3.4 Germination assay

Guttation fluid collected from each plant of ‘Goldilocks’ and ‘Best Gold’ grown under each of the three water withheld treatments (refer Section 4.2.2.3) were evaluated for comparative concentration of SLs using the germination assay of *Orobanche minor*. The method of conducting the germination assay followed that as described in Chapter 2 (refer Section 2.2.1). Each treatment had five replications represented by five Petri dishes with each Petri dish containing 90 seeds.

4.2.3.5 Variables recorded and data analysis

The frequency of germination of seeds of *O. minor* were observed using a binocular microscope (see Section 2.2.2.2, Chapter 2 for detail). The leaf area of an individual leaf were recorded from each plant of ‘Goldilocks’ and ‘Best Gold’. Water content of the medium under three periods of water withheld was obtained as described in Section 4.2.2.5. In addition, for both cultivars, the amount of guttation fluid exudated by an individual leaf of each plant was measured.

Data were analysed by a two-way ANOVA, using the GLM procedure of Minitab 16 (Minitab Inc., State College PA, USA). Comparison between the means was made at $P \leq 0.05$ using Duncan’s mean range test (DMRT) method. Correlation analysis was performed using the Regression method of Minitab 16. Residual plots were checked for normality and constant variance through Minitab 16.

4.3 Results

Experiment One- Determination of lag period between transpiration and guttation

4.3.1 Transpiration and guttation rate of *Zantedeschia* plants

Plants under the well watered treatment possessed a slightly higher transpiration rate as compared to water withheld plants (upto 0 to 8μl/cm^2 difference), but the rate did not differ significantly at most times of sampling (Figure 4.3). For each water status, transpiration rate increased from 10 to 40 μl/hr/cm^2 leaf area until 1:00 pm and reduced to about 20 μl/hr/cm^2 leaf area in between 2:00 pm and 3:00 pm. Interestingly, the transpiration rate subsequently increased again to 30 μl/hr/cm^2 leaf area until 4:00 pm, whereupon it progressively decreased to finally reach zero between 9:00 pm and 10:00 pm (Figure 4.3). Transpiration did not occur during the night time in either water withheld or well watered plants.

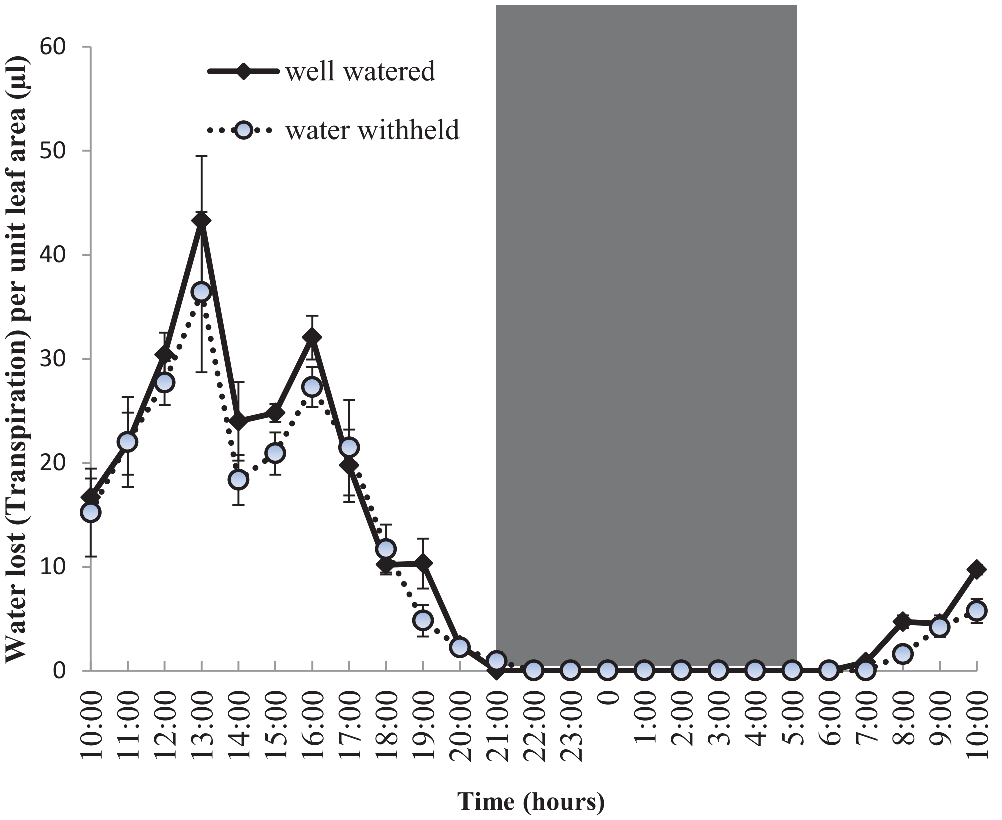


Figure 4.3 Transpiration (Water lost) per cm^2 leaf (µl/cm^2) at different hours of a day after midnight in plants of *Zantedeschia* 'Goldilocks' that were either water withhelded or well watered. Well watered plants received irrigation once daily prior to the experiment and twice on the day of experiment during the morning and evening, whereas water withhelded plants did not receive irrigation 3 days prior to the collection of guttation fluid. Shaded area within the graph represents the dark period. Vertical bars represent standard errors (n=3).

In the current experiment, guttation occurred during the night and early morning (refer Figure 4.4). There was no guttation during late morning (after 8:00 am) until the dark period commenced, as no visual droplets on leaf tips was observed during that period. The first guttation appeared at 10:00 pm (or 22:00) in one of the well watered plants, i.e. soon after transpiration had completely stopped at 9:00 pm. Maximum guttation (1.5

µl/3 hr/cm^2 leaf area) occurred between 11:00 pm and 2:00 am, but with no significant difference compared to the guttation rate between 2:00 am and 5:00 am. Guttation rate averaged 0.5 µl/3 hr/cm^2 leaf area between 5:00 am to 8:00 am, and no droplets appeared after 8:00 am (Figure 4.4). Guttation fluid first appeared at 3:00 am from one of the water withheld plants, and this was limited to a tiny single drop which was too little to measure.

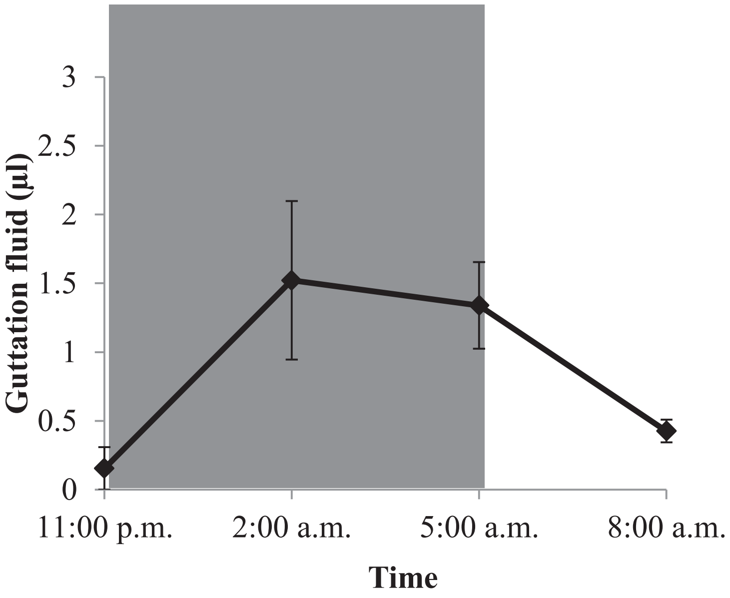


Figure 4.4 Guttation fluid per unit leaf area (µl/cm^2) collected every three hours from leaves of well watered plants of *Zantedeschia* 'Goldilocks'. Water withhelded plants did not produce guttation fluid except a single drop from one of the water withheld plant. Shaded area within the graph represents the dark period. Vertical bars represent standard errors (n=3).

4.3.2 Measures of water content

At each time of observation, water content of the medium was higher ($P < 0.05$) (about double during the evening just after an additional irrigation) in well watered plants as compared to water withheld plants (Figure 4.5). The water content remained constant (about 13%) throughout the period of observation in water withheld plants. However, in well watered plants, as compared to the morning, the water content of the medium increased by 7% during the evening, so that the water content was about double that evident in water withheld plants (Figure 4.5).

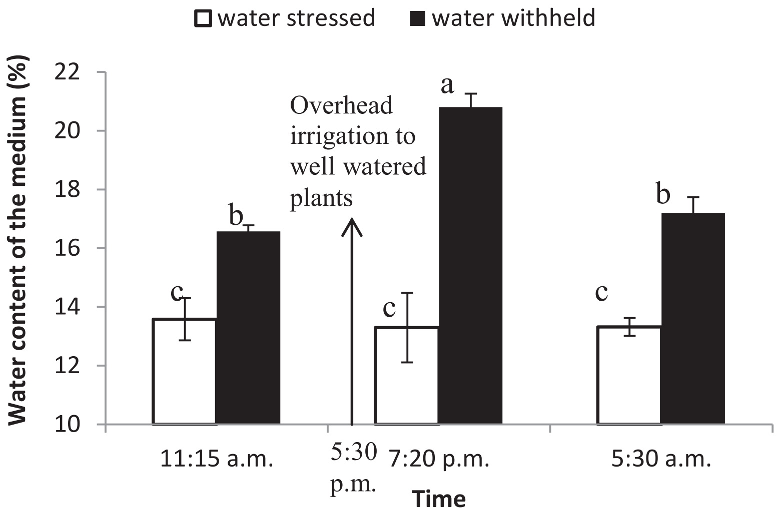


Figure 4.5 Water content (%) of medium of plants of *Zantedeschia* 'Goldilocks' that were either water withheld or well watered, at three measurement times of a 24 hr period. Means with same letters do not differ significantly at $P \leq 0.05$ based on DMRT. Bars represent standard error (n=3)

The relative water content (RWC) of leaves of well watered plants was greater ($P < 0.05$) (by about 3%) than water withheld plants in the evening, but not in late and early mornings (Figure 4.6). In both well watered and water withheld plants, the RWC tended to increase during the course of the experiment in both water withheld and well watered plants, however, about an hour after irrigation (i.e. at 6:30 pm) the RWC in well watered plants was significantly greater (by about 2-3%) than in water withheld plants ($P < 0.05$, Figure 4.6).

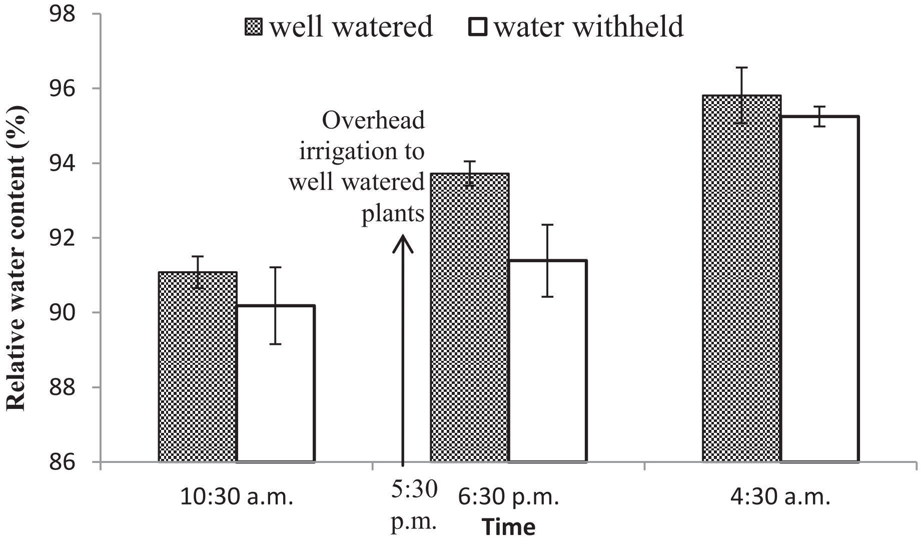


Figure 4.6 Relative water content (RWC) of leaves of 'Goldilocks' plants that were either water withhelded or well watered at different times of a 24 hour period. Means with same letters do not differ significantly at $P \leq 0.05$ based on DMRT. Bars represent standard error (n=3)

Experiment Two- Relationship between concentration of SLs and guttation volume

4.3.3 Guttation fluid and water content of the medium

Unlike in Experiment One, guttation was evident within the plants at all water levels (ie., 0, 1 or 2 days of water withheld). The volume of guttation fluid ($P < 0.01$) and the water content of the medium ($P < 0.001$) was significantly greater (by about one and half times) in well watered plants than water withheld plants (Table 4.1). However, the volume of guttation fluid did not vary ($P = 0.103$) between the cultivars despite the growing medium for 'Best Gold' containing more water ($P < 0.05$) than that of 'Goldilocks'. That was, approximately 21.84% water content in the medium of 'Best Gold' compared to about 19.84 % in 'Goldilocks'.

4.1 Amount of guttation fluid (µl/cm^{2}leaf) collected over overnight and water content of the medium (%) of two cultivars of *Zantedeschia* after different durations of water withheld.

Days of water withheld	Guttation fluid per unit leaf area (µl/cm^2)	Water content of the medium (%)
0	15.29 ± 0.86 a^Z	23.57 ± 0.60 a
1	9.77 ± 1.27 b	19.54 ± 1.09 b
2	10.26 ± 1.04 b	19.39 ± 0.70 b

^{Z} Means within each column with same letters do not differ significantly at $P \leq 0.05$ based on DMRT.

4.3.4 Strigolactones in *Zantedeschia* cultivars

As evident in Chapter 2 (refer Section 2.3.2), germination percentage could readily be interpreted as indicating the relative concentration of SLs as being either high or low. In the current experiment, the low branched 'Best Gold' produced about one and half fold more ($P < 0.0001$) SLs than the highly branched 'Goldilocks' (Figure 4.7). However, importantly, the days of water withheld did not affect ($P = 0.79$) the concentration of SLs.

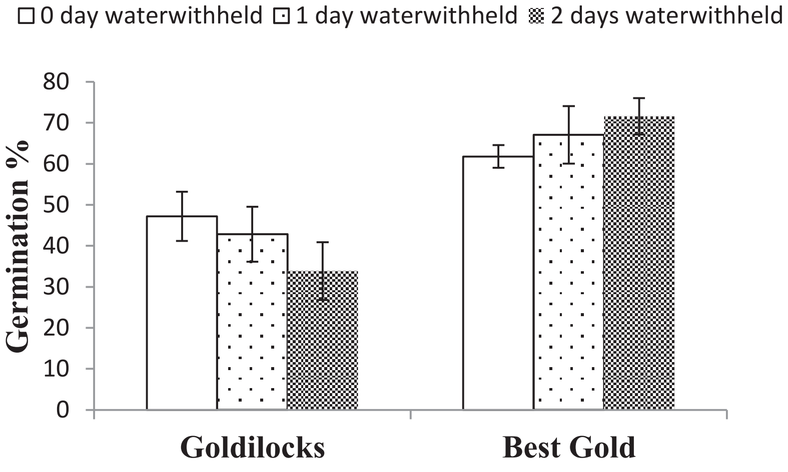


Figure 4.7 Germination of *O. minor* seeds treated with guttation fluid of highly branched (Goldilocks) and low branched (Best Gold) *Zantedeschia* cultivars at three different levels of water withheld i.e., 0, 1 or 2 days of water withheld. Means with different letters between two cultivars differ significantly at $P \leq 0.05$ based on DMRT. Vertical bars represent standard errors of means between each level of water withheld within each cultivar.

4.3.5 Correlation between strigolactone content and volume of guttation fluid

For all treatments (i.e., 0, 1 or 2 days water withheld), the correlation between the volume of guttation fluid per unit leaf area and concentration of SLs (i.e. germination percentage), in both cultivars, was statistically insignificant with a P *and* R^2 value of 0.407 and 0.058 respectively for 'Best Gold', and 0.278 and 0.090 respectively for 'Goldilocks' (Figure 4.8).

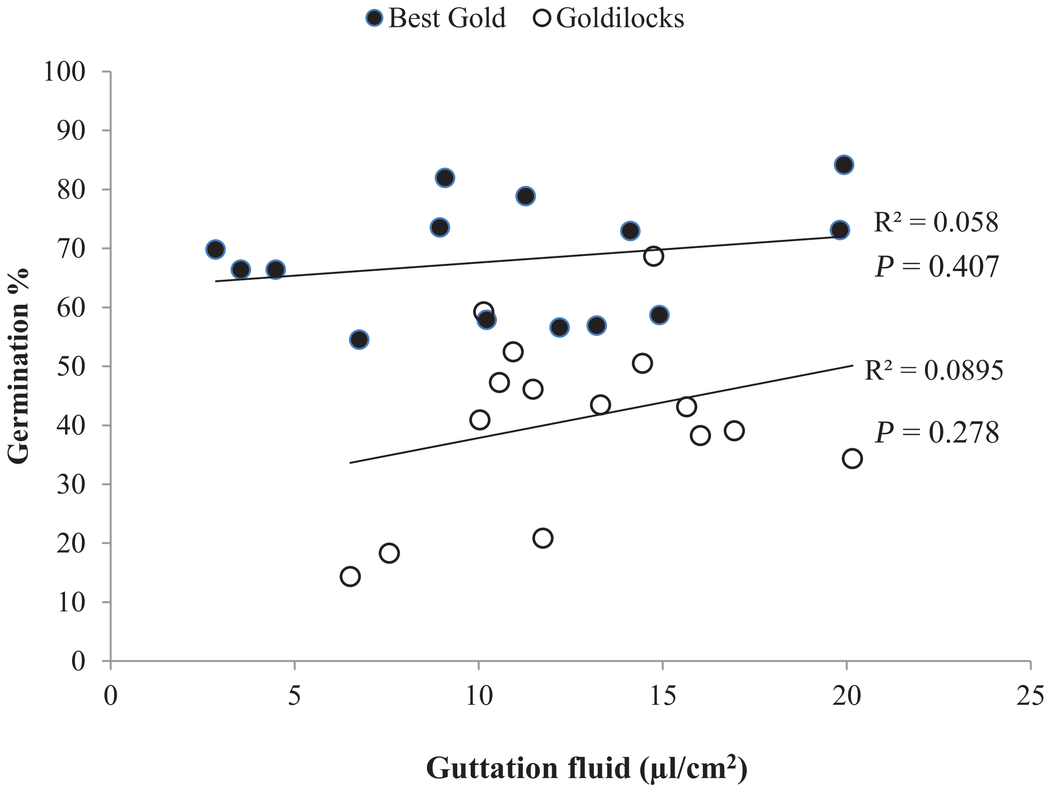


Figure 4.8 Correlation analysis between germination percentage of *O. minor* seeds and amount of guttation fluid (µl/cm^2) for individual plants of the highly branched Goldilocks and low branched Best Gold *Zantedecshia* cultivars.

4.3.6 Correlation between guttation fluid and leaf area

The volume of guttation fluid increased with increased leaf area ($P < 0.0001$ to $P < 0.05$) in plants of both 'Goldilocks' and 'Best Gold' when grown under different levels of water withheld. For both cultivars at all water withheld levels, the correlation 'R^2' value was above 70% (except for 'Goldilocks' after one day of stress), and the probability levels ranged between $P < 0.017$ and $P < 0.001$ (Figure 4.9).

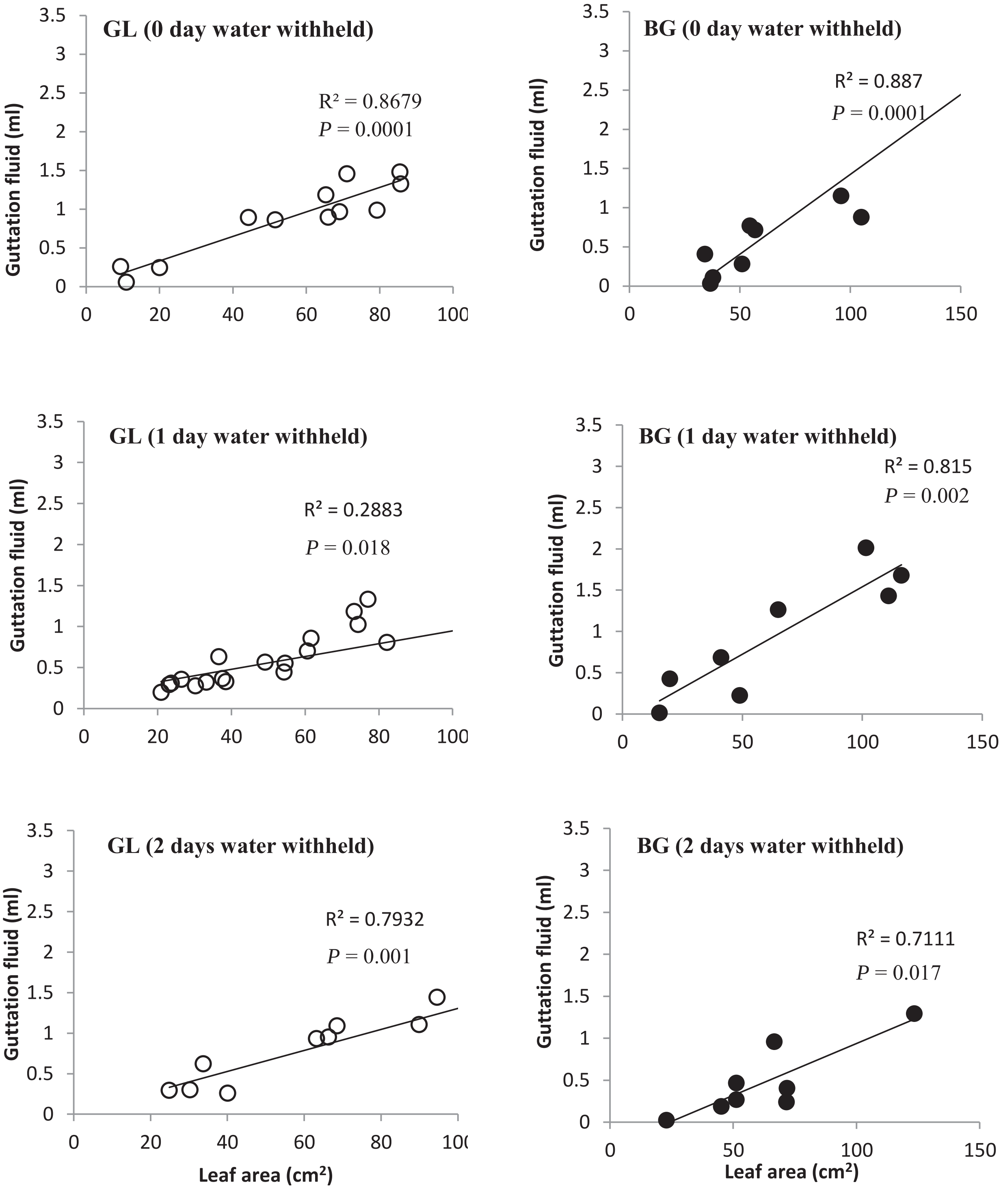


Figure 4.9 Correlation analysis between amount of guttation fluid (ml) per leaf and leaf area per leaf (cm^2) at different levels of water withheld in two *Zantedecshia* cultivars Goldilocks (GL) and Best Gold (BG).

4.4 Discussion

4.4.1 Guttation in *Zantedeschia*

Experiment One simply determined that in well-watered plants the guttation rate was fairly constant between 11:00 pm and 5:00 pm (Figure 4.4). Experiment One also determined the water withheld conditions that just prevented guttation (refer Section 4.3.1). Thus in Experiment Two, water withheld conditions could be imposed that varied the amount of guttation, to determine the effect, if any, on concentration of SL in the fluid.

Compared to root exudate or xylem sap extraction by either vacuum or increased pressure (Section 2.2.3.2, Chapter 2), the use of guttation fluid has several advantages, including the provision of clean samples (free of debris) and sampling being non-destuctive. However under the assumption that roots are the primary source of synthesis of SLs (see Section 4.1), the main advantage is that the concentration of SLs in the guttation fluid seems to give a true representation of the concentration of hormone delivered from the root to the shoot. The evidence supporting the interpretation for it providing a true representation is given in the following sections.

In many different species, guttation occurs via; leaf tips, margins, as well as the abaxial and adaxial surfaces of the leaf lamina (Chen & Chen, 2005; Lersten & Curtis, 1982) (Figure 4.10B). However, in the experiment within this chapter, guttation fluid was collected by encapsulating only the lower half of leaves. This therefore collected guttation fluid mainly from leaf tips (refer Figure 4.1B), from where it first appeared (Figure 4.10A). In fact, the volume of the guttation fluid increased with increase in individual leaf area (Figure 4.9) which supports the hypothesis that *Zantedeschia* do not guttate from the margins and leaf lamina, i.e. only from the tip. That means, guttation fluid if exudated via leaf margins (as in Figure 4.10B) or any other leaf parts, the plastic bag covering only the distal half part of a leaf (see Figure 4.1B) would not have collected the total volume of guttation fluid. Collection of guttation from leaf tips and the correlation between the volume of guttation and the leaf area therefore, confirms that the total amount of guttation fluid collected within this experiment is truly representative of that produced by the plant. Thus, the method (refer Section 4.2.2.4) applied for collecting guttation fluid within *Zantedeschia* appears to be highly useful. Guttation exudation through leaf tips suggests that the hydathodes (see Section 4.1) may have been present in leaf tips at least in the leaves of *Zantedeschia*. In fact, to confirm the location of hydathodes within the leaf, microscopic observation of the hydathodes throughout an entire leaf would be useful, albeit this was not done in the current thesis.

A **B**

Figure 4.10 Exudation of guttation fluid; (A) at the tip of a leaf of a *Zantedeschia* plant (present work) and, (B) around the margin of leaves of an unidentified plant (Koning, 1994).

4.4.2 Guttation fluid and strigolactones concentration

In Chapter 2, the difference in concentration of SLs between highly branched and low branched cultivars of *Zantedeschia* was consistently observed in two separate experiments during the stage before the branches were visibly evident within the annual growth cycle. In the experiments within this chapter, as noted in those previous experiments (Chapter 2, Section 2.3.1), the concentration of SLs was higher in the plants of ‘Best Gold’ as compared to ‘Goldilocks’ (Figure 4.7). Looking at the concentration of SLs in each plant of each cultivar, with the exception of one plant, all replicate plants of ‘Goldilocks’ contained less SLs than those of ‘Best Gold’ (Figure 4.8); hence strongly supporting the hypothesis that the high frequency of branching in ‘Goldilocks’ was due to less SLs.

The focus of this chapter was to differentiate whether the difference in SLs between these high and low branched cultivars was due to the volume of guttation, or the genotype, as these two factors may influence the concentration of hormones present in the guttation fluid (Singh et al., 2009). In both cultivars studied, the concentration of SLs and the volume of guttation fluid did not correlate with each other (Figure 4.8). The lack of effect of volume of xylem sap on hormonal concentration is in contrast with the results reported by Beck & Wagner (1994), who found reduced concentration of cytokinin with an increase in volume of xylem sap in *Utrica* sp. However, in their study, cytokinin was measured from the xylem sap extracted via the cut stem, using an external pressure within a root chamber. Exposing plants to excessive external pressure produced a higher volume of xylem sap, which contained a lower concentration of the sap constituents (Berger et al., 1994). Enhancing the exudation rate from the roots with externally applied pressure can increase the volume of exudates or the xylem sap, but may affect the concentration of xylem sap constituents, including plant hormones of cut stem plants under this stressed state (Goodger et al., 2005). Reduced sap constituents with increased xylem flux is because of the inability of plants to increase the synthesis of elements including plant hormones with an increase in exudation rate or flow rate. In contrast, collection of guttation fluid undertaken under natural conditions of plant growth (refer Section 4.2.2.4) does not impose any stress to the plants, and the collection is continuous without interrupting the natural rate of synthesis and transport of hormones to the xylem sap. On the other hand, the guttation rate is about 10-40 times less than the transpiration rate (Figure 4.3 and 4.4) and, when applying external pressure to a cut stem, the exudation rate can be increased to be equivalent to the natural transpiration rate (Beck, 1996). This suggests that the exudation rate during guttation is normally lower than that achieved with external pressure when using a cut stem. The difference in the rate of exudation between natural guttation and when external pressure in a cut stem is applied, is also supported by Dieffenbach et al. (1980) who found 3-4 times greater rate of exudation in applied root pressure exudates than the guttation rate in barley plants.

Despite being unable to obtain a measurable amount of guttation from water withheld plants in Experiment One, the appearance of guttation in at least one of the water withheld plants 4 hours later than those in well watered plants (refer Section 4.3.1), suggests that the lag period between transpiration stopping and guttation starting was affected by the water status of the medium. Thus in Experiment Two, using a milder form of water withheld (Section 4.2.2.3), differences in the volume of guttation were probably due to differences in the lag period before guttation spill-over (see Figure 4.11) occurred. Hence, early commencement of guttation in well watered plants results in more guttation, as compared to water withheld plants (Table 4.1) (Singh et al., 2013). In order to clarify the likely cause for the reduced volume of guttation fluid in water withheld plants, the dynamics of xylem sap flow and transport of SLs within the guttation fluid of *Zantedeschia*, has been described in detail in Section 4.4.3.

Hence, the lack of correlation between the concentration of SLs and the volume of guttation suggests that the difference in SL concentration evident between ‘Goldilocks’ and ‘Best Gold’ was not due to different volumes of guttation; but rather due to difference in synthesis of endogenous SLs between these two genotypes. Extrapolating across other genotypes therefore, it is hypothesized that those which are highly branched produce less SLs during the critical stage of development, whereas low branched genotypes produce higher concentration of SLs.

4.4.3 Dynamics of xylem sap flow and transport of SLs

In Chapter 2, the presence of SLs in the guttation fluid of *Zantedeschia* has been demonstrated, adding the potential use of guttation fluid for hormonal analysis where possible. The experiments within this chapter were designed to determine the dynamics of xylem sap in *Zantedeschia* plants in order to identify whether or not the sap in the form of guttation fluid collected during the dark (Figure 4.4) actually represents the concentration of plant hormones within the plant in each cultivar. As there was no guttation during the day time (Section 4.3.1, see Figure 4.11A), the major/or all hormone exudation must have occurred during the night through guttation. In *Zantedeschia*, when transpiration is completely suppressed, guttation became evident by the visible appearance of water droplets at leaf tips (Figure 4.10A). Such guttation is a result of root pressure building up as transpiration rate decreases and this pressure pushes water from root to shoot through xylem vessels (reviewed in Singh & Singh 2013). In well watered plants, due to higher water content of the medium (Figure 4.5), root pressure builds up faster than in water withheld plants. Increased root pressure being built-up increases the movement of xylem sap from roots up to the shoot (Figure

4.11B). Once the water stream in xylem vessels, or the xylem sap in the shoot, is almost full (>95%) (as indicated by leaf water status, refer Figure 4.6, also refer Figure 4.11C), exudation occurs via hydathodes.

In water withheld plants, due to low water content in the medium (Figure 4.5), the “filling up” of the xylem stream within the shoot consumes comparatively more time (see Figure 4.6 for leaf RWC) than in well watered plants, due to the slow build up of root pressure. As a result, the appearance of guttation in water withheld plants occurs chronologically later (see Section 4.3.1) than in well watered plants. In different plants, either water withheld or well watered, stopping of guttation occurred nearly at the same time (8:00-8:30 am, Figure 4.4, plus based on visual observation in Experiment 2 within this Chapter) but the start of guttation varied (see Section 4.3.1); ultimately leading to the varied volume of guttation fluid. That means, early guttation in well watered plants resulted in increased accumulation of guttation fluid than in water withheld plants. This guttation can be thought of as a ‘spill over’ (refer Figure 4.11C), where the concentration of hormone in the fluid represents a true measure of the concentration throughout the total shoot xylem sap.

Regarding transport of plant hormones in the xylem sap, it is well known that transpiration causes transport of plant hormones from roots to the shoot (Grunwald et al., 2003). Since 3 days of water withholding did not affect the transpiration rate (Figure 4.3), in both water withheld and well watered plants, the transport of SLs from root to shoot might also be expected to be similar during transpiration. After transpiration has stopped, the hormones may keep accumulating within the xylem sap until ‘spill-over’ occurs in the form of guttation, but since this time period is small and the total volume of water is large, the effect on SL concentration is likely to be small. It is possible therefore that the SL concentration may vary within the exudation for the first few minutes/hours and that exuded later, due to the lag period between transpiration stopping and guttation starting. In *Urtica dioica* L. in which transpiration at night is minimal, the root exudates acquired under applied pressure contained a higher concentration of cytokinin in the morning than in the afternoon (Beck, 1996), which suggests that the hormone accumulates in the xylem sap when transpiration is suppressed/reduced. However, in the experiments within this thesis, only the total guttation fluid accumulated once guttation had stopped for the 24 hour was considered for SLs analysis. So in the current experiments the chances of time dependant changes

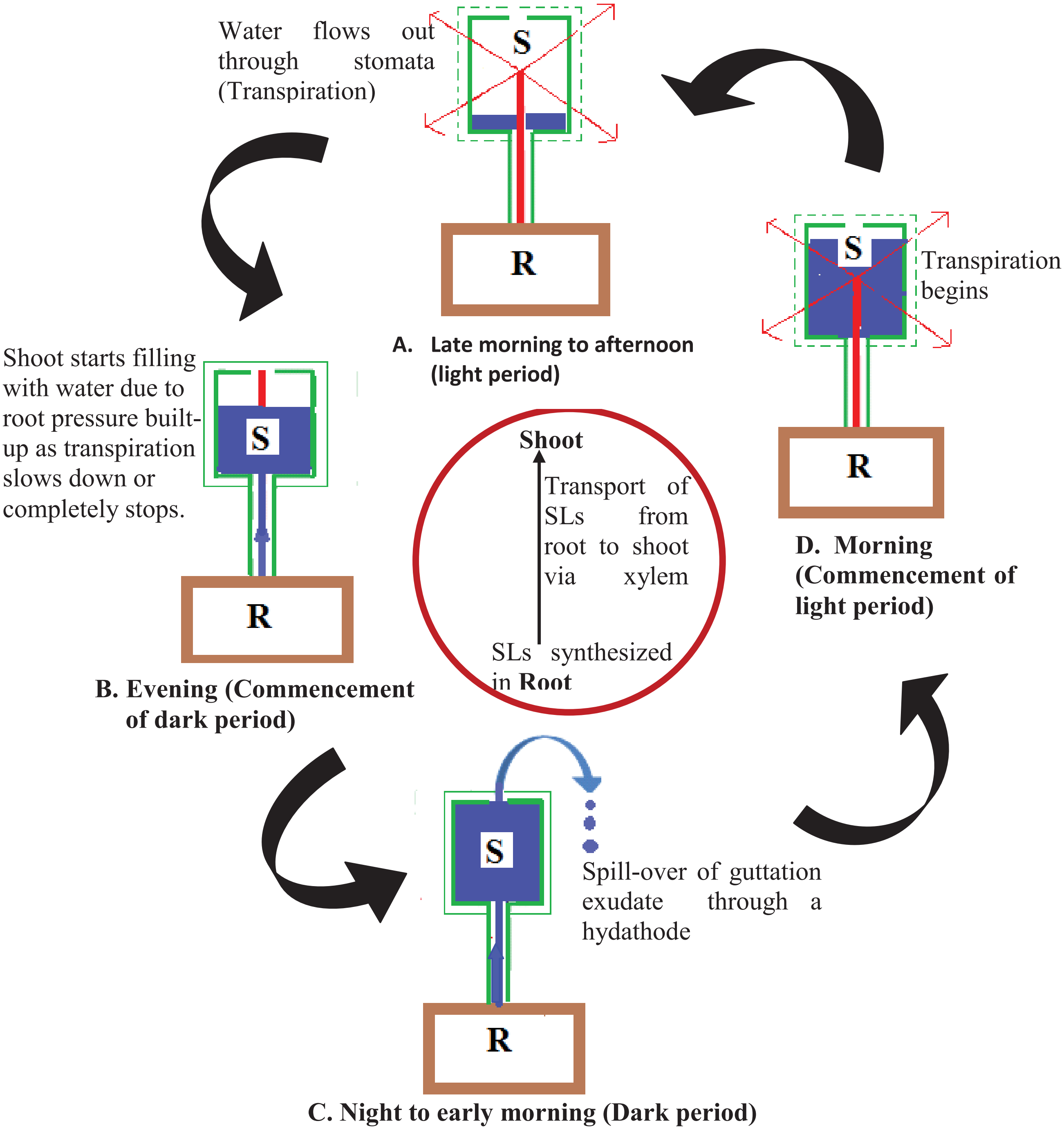


Figure 4.11 Dynamics of xylem sap flow during day and night or period of light and dark (Stages A-D) and transport of SLs from root (R) to shoot (S) (within a red circle). Red arrows indicate water coming out through stomata during transpiration and the blue arrows indicate root exudates due to root pressure. Green solid line represents stomata closure whereas green dotted line indicates open stomata.

in hormonal concentration between the two cultivars is unlikely. In future, it would be useful to determine if the rate of biosynthesis of SLs differed through a 24 hour day, as the assumption made in this work was that the biosynthetic rate was constant (see Section 4.1)

4.5 Conclusion

In summary, the difference in concentration of SLs between ‘Goldilocks’ and ‘Best Gold’ was due to the difference in potential of producing SLs between these two cultivars, and not due to differences in volume of guttation fluid.

5 Interaction between strigolactone and cytokinin in shoot branching of *Zantedeschia* grown *in vitro*

5.1 Introduction

Endogenous hormonal signals controlling lateral bud outgrowth involve a network of hormonal interactions (Cline, 1991; Sae Shimizu-Sato & Mori, 2001). While the interaction between different plant hormones such as auxin, cytokinins, and gibberellins is well established (refer Chapter 1, Section 1.4.3.1) recent work has suggested that interactions between strigolactones (SLs) and cytokinins (CKs) may also be very important, as both of these hormones appear to be involved in branching (Shimizu-Sato et al., 2009; Umehara et al., 2008). Based on the fact that apically derived auxin positively regulates SL biosynthesis (Foo et al., 2005) but negatively regulates CK (Ferguson & Beveridge, 2009), interaction between SL and CK might occur in branching inhibition. External application of SL inhibited both decapitation-induced (Brewer et al., 2009) or CK-induced (Dun et al., 2012) axillary shoot growth, further supporting an interaction between SL and CK in axillary bud outgrowth. However, clear evidence about the stage of bud outgrowth (bud release or subsequent growth, refer Section 1.2, Chapter 1) at which these two hormones interact, is still lacking.

In *Pisum sativum* (pea), SL reduced CK-promoted axillary shoot length (Dun et al., 2012), suggesting interaction between these two hormones after bud release, rather than bud release *per se*. Higher levels of SLs was observed in low branched cultivars of horticultural species including *Zantedeschia* (Chapter 2); supporting the hypothesis for a role of SLs in inhibition of bud release and subsequent shoot growth during the process of shoot branching. The current study therefore, focused on interaction between these two hormones in terms of the number of bud released, as represented by the number of axillary shoots in *Zantedeschia* plants grown *in vitro*.

As with axillary shoot development, the role of plant hormones particularly CKs and auxin and the balance between these hormones in the formation and development of adventitious buds is well known (Charrière et al., 1999). Hence, the role of SL and its interaction with CK on the formation of adventitious shoots is also reported in this chapter. In fact, this study might be the first to demonstrate a role for SL in adventitious bud/shoot formation. On the other hand, CKs are well known for stimulating adventitious bud/shoot formation; hence as explored within this chapter it was of interest whether SL influences adventitious shoots as well as axillary shoots and, if so, how SL interacts with CK.

In *Zantedeschia*, the highly branched cultivar Goldilocks was found to have low SLs early within the annual growth cycle compared to the low branched cultivar Best Gold (Chapters 2 and 4), suggesting SLs might have influence on inhibition of shoot branching, particulary on the number of buds released. In contrast, the CK, 6-benzylaminopurine (BAP), has been reported to stimulate branching in *Zantedeschia* plants grown *in vitro* (Naor, Kigel, & Ziv, 2005; Ngamau, 2001) and *in vivo* (Subbaraj et al., 2010). As reported by Subbaraj et al. (2010), the effect of CK in stimulating branching was more prominent early in the annual growth cycle of ‘Best Gold’. Hence, it is possible that there is an interaction between SL and CK in *Zantedeschia* plants in shoot branching, at least at an early stage in the annual growth cycle. Thus, in the experiment presented in this chapter, it was hypothesized that SL might act antagonistically with CK in development of axillary shoots in *Zantedeschia* plants.

This chapter includes a series of studies focussed on the interaction between SL and CK in different genotypes of *Zantedeschia* having different branching phenotypes, using an *in vitro* technique. It also covers development of the method for studying SL in shoot branching *in vitro* and identifying optimum levels of SL and CK for interaction studies. Most of the studies conducted on other species like pea, *Arabidopsis* and *Oryzae sativa* (rice), included applying ‘GR24’ (a synthetic SL) either directly to buds and/or via a vascular supply in intact plants grown *in vivo* (Gomez-Roldan et al., 2008), including

SL supply via hydroponics (Boyer et al., 2012; Umehara et al., 2008). However, methods such as direct bud application, adopted with pea, are not possible in species like *Zantedeschia* that do not have easily accessible axillary buds. Hence application via an *in vitro* technique may be an alternative method as utilised in studies by Liang et al. (2010) and Ward et al. (2013) for other plant species to study SL effects on branching. In addition, due to the high market price of GR24 (Liang et al., 2010), the smaller quantities required *in vitro* techniques should be a more cost effective method than the commonly used direct spraying of hormones *in vivo*.

The synthetic SL, GR24, has normally been used at concentrations between 0.15 (0.5 μM) and 3 mgL^{-1} (10 μM) (Boyer et al., 2012; Umehara et al., 2010) when applied in other plant species grown *in vivo,* with maximum branch inhibition occurring around 0.3 mgL^{-1} (1μM). Therefore, concentrations of GR24 ranging from 0.1 to 1 (mgL^{-1}) were considered likely to result in inhibition of axillary shoot growth *in vivo*, in the initial studies presented in this chapter. For CKs, different cultivars of *Zantedeschia* grown *in vitro* have responded to a range of BAP concentrations with maximum effect on root growth observed at 3 mgL^{-1} (13.3 μM), the highest concentration evaluated (D'Arth et al., 2002) . However, for analysing the response of shoot growth to BAP by *Zantedeschia* plants *in vitro*, it was acknowledged that the optimum level of CK might differ, since sensitivity of plants to respond to hormones is highly dependent on type of tissue and plant species (Weyers & Paterson, 2001). In some plant species, such as *Vigna unguiculata* (cowpea), the optimum level of BAP for maximum shoot growth was 1 mgL^{-1} (Aasim et al., 2009). Hence it was considered worth conducting experiments to identify optimum levels of both BAP and GR24 for subsequent interaction studies between these two hormones with *Zantedeschia* grown *in vitro*.

The objectives of the experiments in this chapter were to:

- quantitatively describe the concentration response to a synthetic SL ‘GR24’ as well as BAP on the number of axillary shoots in *Zantedeschia grown in vitro*
- examine the interaction between SL and CK in affecting axillary shoot number of *Zantedeschia*
- determine the effect of GR24 and BAP on the number of adventitious shoots and quantify the interaction of SL and CK on adventitious shoot formation.

5.2 Materials and methods

5.2.1 **General methods**

All experiments were conducted at The New Zealand Institute for Plant & Food Research Ltd. (PFR), Palmerston North, New Zealand. Stem cuttings and/or embryos of *Zantedeschia* were grown *in vitro* in a base medium consisting of half strength macro & full strength micro salts of Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), LS vitamins (Linsmaier & Skoog, 1965), 7.5 g/L agar (Davis) and 3% (87.6 mM) sucrose supplemented with IBA (0.05 mgL^{-1}) and gibberellic acid (0.1 mgL^{-1}). The pH of the medium was adjusted to 5.7 with either 0.1N NaOH or 0.1N HCl, prior to autoclaving at 121°C and 103 kPa for 15 min. Media was cooled to about 50°C after autoclaving, then 50 ml of media was placed into each culture vessel (disposable plastic; Alto Packaging, Hamilton, New Zealand). The growth room utilised cool white tubes that provided 30 ± 5 μmol m^2 s^{-s} photosynthetic photon flux density (PPFD) for a 16 h photoperiod, and the temperature was 25 ± 1°C.

For those experiments utilising GR24 (i.e. Experiment One and Three) the stock solution of 100 mgL^{-1} GR24 was prepared as described previously (refer Section 2.2.2.1) and sterilized by filtration using 0.2 µM Ministart® filters (Goettingen, Germany). The desired volume of this stock solution was added to the autoclaved base medium after cooling to 50°C, so as to prepare the final concentrations of GR24 in the medium.

The main focus of the study was to understand the interaction between SL and CK on shoot branching. Hence, the optimum concentrations of these two hormones to be used for the interaction study were determined by applying GR24 and BAP on the highly branched cultivars (Experiment One - Section 5.2.2) and the low branched cultivar (Experiment Two – 5.2.3), respectively.

5.2.2 Experiment One - Concentration response relationship of *Zantedeschia* to GR24

5.2.2.1 Plant material

From cultures previously established *in vitro*, stem cuttings of four highly branched cultivars; Goldilocks, GE49, GE4120 and GE20/4 (Green Harvest Pacific, Auckland, New Zealand) of *Zantedeschia* were used as source material. Stem cuttings included the stem apex (Figure 5.1) and swollen stem base with one or two nodes, but with no roots. Stem cuttings of these cultivars were sub-cultured every four weeks for eight weeks before use. Despite being reported as naturally branching, pilot studies had indicated that these cultivars did not branch when grown *in vitro* in the absence of CK, so 0.3 mgL^{-1} BAP was added to the base medium (see Section 5.2.1).

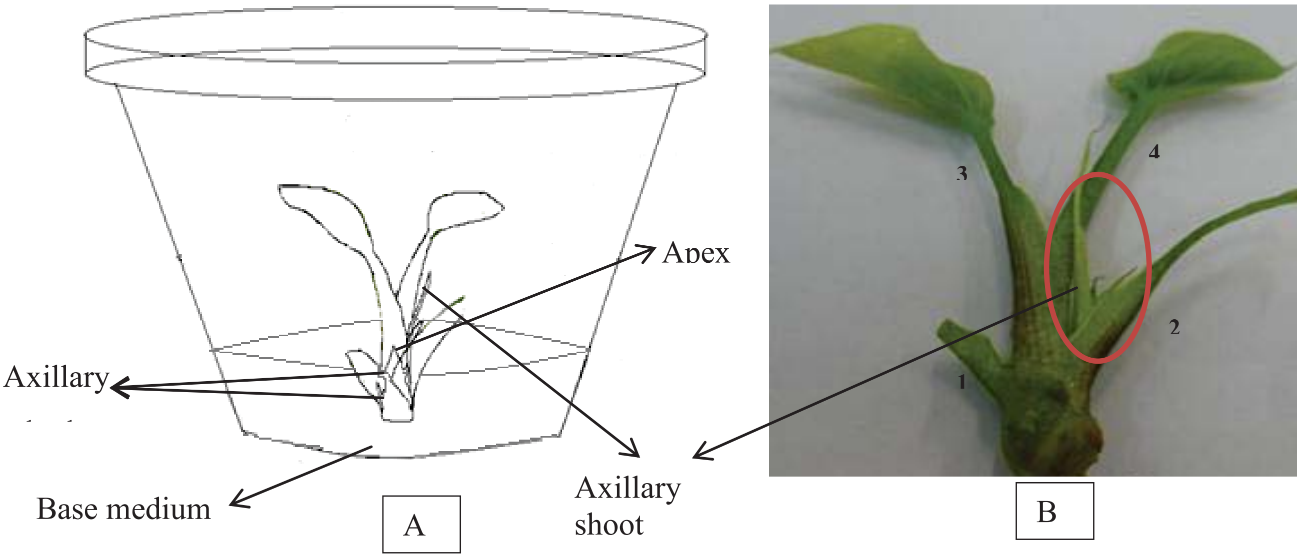


Figure 5.1 Leaf emergence and axillary shoot development on *Zantedeschia* grown *in vitro*. Shoot within figure (A) represents a diagrammatic representation of the primary shoot in figure (B) inserted into the media within a culture vessel. Structures labelled 1-4 represent leaves, with 3-4 presenting newly emerged leaves. Axillary shoot development occurring at node 2 is highlighted within the red elipse.

5.2.2.2 Treatments and experimental design

Treatments comprised a 4 × 4 factorial arrangement of each of the four cultivars and four concentrations (0, 0.01, 0.1 and 1 mgL^{-1}) of GR24. The experiment was conducted as a completely randomized design (CRD) with each of the sixteen treatments having three replications, represented by three culture vessels containing eight stem cuttings per vessel.

5.2.3 Experiment Two - Concentration response relationship of *Zantedeschia* to BAP

5.2.3.1 Plant material

With 'Best Gold' believed to be apomictic and therefore genetically uniform, stem cuttings used for this experiment were derived from shoots that emerged from embryos of 'Best Gold' seeds. Seeds were first surface sterilized by placing them in 30% Janola (1.2% Sodium hypochlorite) and shaken overnight for uniform mixing of seeds with sodium hypochlorite. Seeds were then thoroughly washed three times with sterilized water under a laminar flow hood and the seed coat removed using a scalpel. Seeds were then dipped in ethanol for 30 seconds, immediately followed by rinsing with sterilized water. Sterilized seeds were cut in half, the embryo dissected out and transferred onto the base media. Prior to conducting the experiments, in order to produce more shoots from each embryo, the base media was ammended with BAP at 0.3 mgL^{-1} (also refer Appendix VI).

5.2.3.2 Treatments and experimental design

Four different concentrations of BAP (0, 0.3, 0.9 and 3 mgL^{-1}) were tested on shoots of 'Best Gold' in a completely randomized design. Four culture vessels as replicates of each treatment were used and each culture vessel contained five stem cuttings of 'Best Gold'.

5.2.4 Experiment Three - Interaction between cytokinin and strigolactone

5.2.4.1 Plant material

‘Goldilocks’ (highly branched) and ‘Best Gold’ (low branched) were used, as these two cultivars not only differed in their phenotypic expression of branching, but also had been investigated in previous experiments focussed on quantitative analysis of SL content (Chapter 2). In addition, ‘GE49’ was also included within this experiment in order to confirm the results from Experiment One. Despite the difference in source of materials of highly and low branched cultivars, they were phenotypically uniform according to their genotype. The stem cuttings of ‘Goldilocks’ and ‘GE49’ were derived from multiple subcultures of shoots, whereas those of ‘Best Gold’ were derived from shoots developed from what is assumed to be genetically pure seeds. The stem cuttings of ‘Best Gold’ were generated from subculturing the shoots arising from the embryos as described in Section 5.2.3.1, except the basal medium did not contain BAP.

5.2.4.2 Treatments and experimental design

The treatments comprised a 3 × 3 × 2 factorial arrangement of each of the three cultivars, three concentrations (0, 0.3 and 0.9 mgL^{-1}) of BAP and two concentrations (0 and 0.1 mgL^{-1}) of GR24. The experiment was conducted as a CRD with eighteen treatments each with four replications, represented by four culture vessels with eight stem cuttings per vessel. The concentrations of BAP and GR24 were selected based on the results of Experiment One and Two.

5.2.5 Variables recorded and data analysis

As described in more detail below, the following variables were recorded:

Experiment One: number of axillary and adventitious shoots;
Experiment Two: number of axillary and adventitious shoots, root length and number;
Experiment Three: number of axillary and adventitious shoots, and number of nodes on the primary shoot.

The number of axillary and adventitious shoots were counted at weekly intervals for eight weeks, with the number of nodes counted after four weeks and eight weeks. In

Zantedeschia, unlike axillary shoots which develop within leaf axils (Figure 5.1) adventitious shoots develop from the stem/tuber tissue (Figure 5.7). The length of primary roots was measured and the primary root number counted after eight weeks of cultivation on treatment media.

Data were analysed by ANOVA (two-way analysis in Experiment One and three-way analysis in Experiment Three) using the GLM procedure of Minitab 16 (Minitab Inc., State College PA, USA). Mean separation were conducted at $P \leq 0.05$ (unless otherwise stated) using the Duncan's multiple range test method (DMRT). When there were significant interactions, only the interaction effect was discussed. When there were significant main effects with no interaction effect, only the main effects were presented. Residual plots were checked for normality and constant variance through Minitab 16. If data were not normally distributed, square root transformations were carried out before further analysis. In Experiment One, the slopes of fitted lines for each concentration of GR24 were calculated and analysed using GenStat 14.2 (VSN International Ltd, Hemel Hempstead, UK).

5.3 Results

5.3.1 Experiment One - Concentration response relationship of *Zantedeschia* to GR24

5.3.1.1 Axillary shoot number

The interaction effect between 'cultivars' and the presence of 'GR24' in the medium, on the mean number of axillary shoots, was insignificant, both after four weeks ($P = 0.523$) and eight weeks ($P = 0.404$). In contrast, the main effect for 'GR24' was significant at both times of measurement, but especially after four weeks ($P < 0.0001$) compared to eight weeks ($P < 0.05$). The main effect of 'cultivars' was insignificant at both times of measurement (four weeks, $P = 0.960$; eight weeks, $P = 0.240$).

Compared with the control, the lowest concentration (0.01 mg L^{-1}) of GR24 had no influence over the number of axillary shoots (Figure 5.2), whereas higher concentrations of GR24 (0.1 and 1.0 mg L^{-1}) significantly reduced (by about half compared to the

Control) the mean number of axillary shoots after both four and eight weeks cultivation (Figure 5.2).

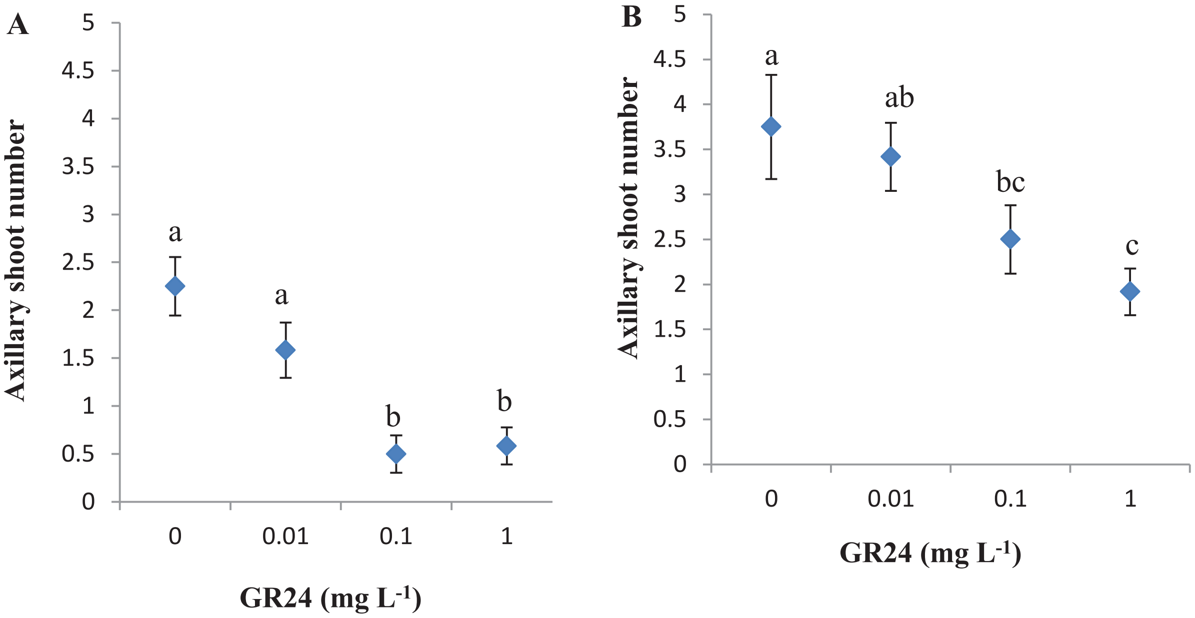


Figure 5.2 Influence of different concentrations of GR24 on number of axillary shoots per culture vessel (eight plants in each vessel) of highly branched cultivars (GE49, GE4120, Goldilocks and GE20/4) of *Zantedeschia* grown *in vitro* observed four weeks (A) and eight weeks (B) after treatment. Bars represent standard errors (n=3). Mean values within each graph with the same letter do not differ significantly at $P \leq 0.05$ based on DMRT.

The rate of increase in the number of axillary shoots over time, for different concentrations of GR24, for the first four weeks differed from that for the last four weeks (Figure 5.3, 5.4). During the first four weeks the rate of increase in the number of axillary shoots also differed between the different concentrations of GR24 ($P < 0.0001$) with the maximum evident for the control (Figure 5.4) and lowest for plants in the 0.1 mgL^{-1} treatment. However differences in the rate of increase between 0.1 and 1 mg L^{-1} of GR24 were not significant (Figure 5.4). For 4-8 weeks, the differences between the different concentrations of GR24 were higher at the higher concentrations of GR24 compared with the control ($P < 0.05$; Figure 5.4).

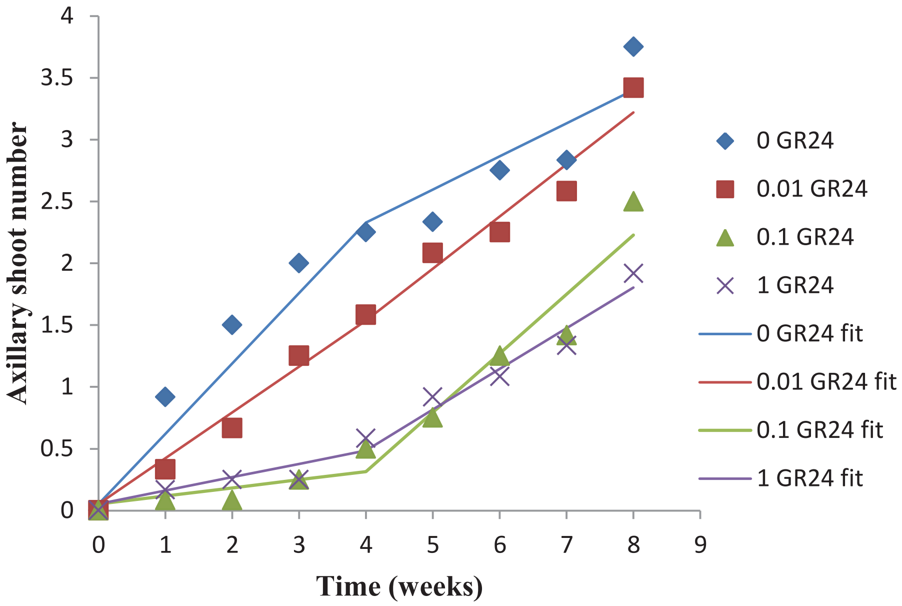


Figure 5.3 Influence of different concentrations of GR24 on the number of axillary shoots per culture vessel (eight plants in each vessel) of highly branched cultivars of *Zantedeschia* grown *in vitro* observed at weekly intervals up to eight weeks after the treatment. Fitted lines for each concentration of GR24 are separate for 0-4 weeks and 4-8 weeks.

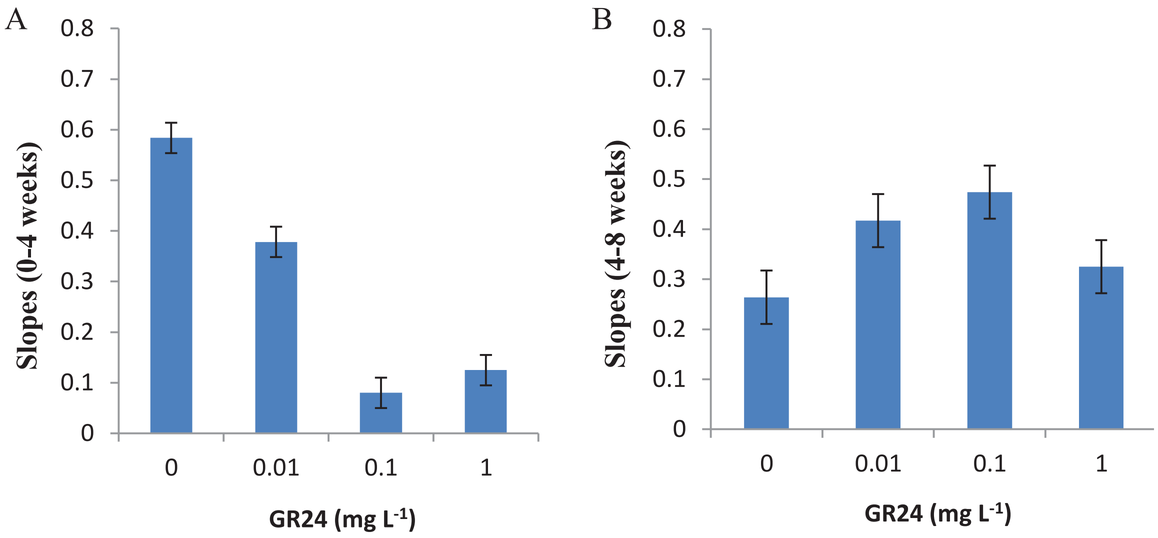


Figure 5.4 Slopes of fitted lines for the effect of different concentrations of GR24 on increase in axillary shoot numbers obtained from 0-4 weeks (A) and 4-8 weeks (B). Bars represent standard errors (n=3).

5.3.1.2 Adventitious shoots

The interaction effect between 'cultivars' and the presence of 'GR24' in the medium for adventitious shoot formation was significant ($P < 0.05$) at eight weeks, but not significant ($P = 0.613$) at four weeks. There were significantly fewer adventitious shoots produced at 0.01 mg L^{-1} of GR24 with 'Goldilocks', but a higher number of such shoots at the same concentration of GR24 with 'GE4120'. However, effects of higher concentrations of GR24 (0.1 and 1 mg L^{-1}) were not statistically evident for all cultivars studied, both at early and later weeks of assessment (Table 5.1). Although the analysis did show an interaction effect on the number of adventitious shoots after eight weeks, there seemed to be no consistency in response with regard to the formation of adventitious buds in response to different concentrations of GR24 for different cultivars.

Table 5.1 Effects of different concentrations of GR24 on number of adventitious shoots (±SE) per culture vessel (eight plants in each vessel) of four cultivars of *Zantedeschia* grown *in vitro* observed at four weeks and eight weeks after GR24 application.

Cultivars	**GR24(mg L^{-1})**	**Week 4**	**Week 8**
GE20/4	**0**	2.33 ±1.86 a[Z]	3.33 ±2.85 a[Z]
	0.01	1.33±0.88 a	3.33 ±1.2 a
	0.1	1.67±1.20 a	3.33 ±2.03 a
	1	2.00±0.58 a	7.00 ±1.53 a
P value		**0.94**	**0.51**
GE4120	**0**	1.33 ± 0.67 a	1.67 ±0.33 b
	0.01	2.67 ±0.33 a	5.33 ±0.88 a
	0.1	1.33 ±0.88 a	2.33 ±1.2 b
	1	0.68 ±0.68 a	2.33 ±0.88 b
P value		**0.27**	**0.07**
GE49	**0**	3.00 ±0.58 a	4.33 ±0.88 a
	0.01	3.67 ±0.33 a	5.00 ±0.58a
	0.1	2.67 ±1.2 ab	4.33 ±1.86 a
	1	0.68 ±0.33 b	1.33 ±0.88 a
P value		**0.07**	**0.19**
Goldilocks	**0**	3.00 ±1.00 a	7.33 ±1.86 a
	0.01	1.67 ±0.33 a	1.67 ±0.33 b
	0.1	2.33 ±1.20 a	6.00 ±1.00 ab
	1	2.67 ±1.45 a	4.67 ±1.76 ab
P value		**0.84**	**0.09**
<u>Main effect</u>			
Cultivars		**0.40**	**0.26**
GR24		**0.52**	**0.99**
Culitvars × GR24		**0.61**	**0.03**

[Z] For each cultivar and time of assessment, means with same letters do not differ significantly at $P \leq 0.05$ based on DMRT.

5.3.2 Experiment Two - Concentration response relationship of *Zantedeschia* to BAP

5.3.2.1 Influence BAP on axillary shoot number

The number of axillary shoots of 'Best Gold' was significantly ($P < 0.0001$) affected by BAP. An increase in axillary shoot number occurred with increase in concentration of BAP, with a maximum evident (by about two fold more than 0 or 0.3 mg L^{-1}) at 0.9 mg L^{-1} (Figure 5.5). A further increase in BAP concentration to 3.0 mg L^{-1} however, did not increase the number of axillary shoots (Figure 5.5).

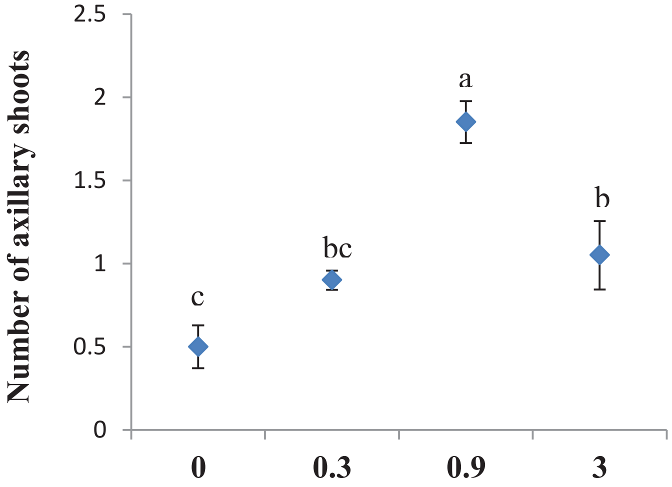


Figure 5.5 Influence of BAP concentration on number of axillary shoots per plant of low branched *Zantedeschia* cultivar Best Gold grown *in vitro* observed at eight weeks after treatment. Vertical bars represent standard errors (n=4).

5.3.2.2 Effect of BAP on adventitious shoot number and primary roots

BAP at 0.3 and 0.9 mgL^{-1} significantly increased the mean number of adventitious shoots with no further increase at 3.0 mg L^{-1}. All concentrations of BAP, including 3.0 mg L^{-1}, reduced total root length and mean root number significantly compared to the control (Figures 5.6).

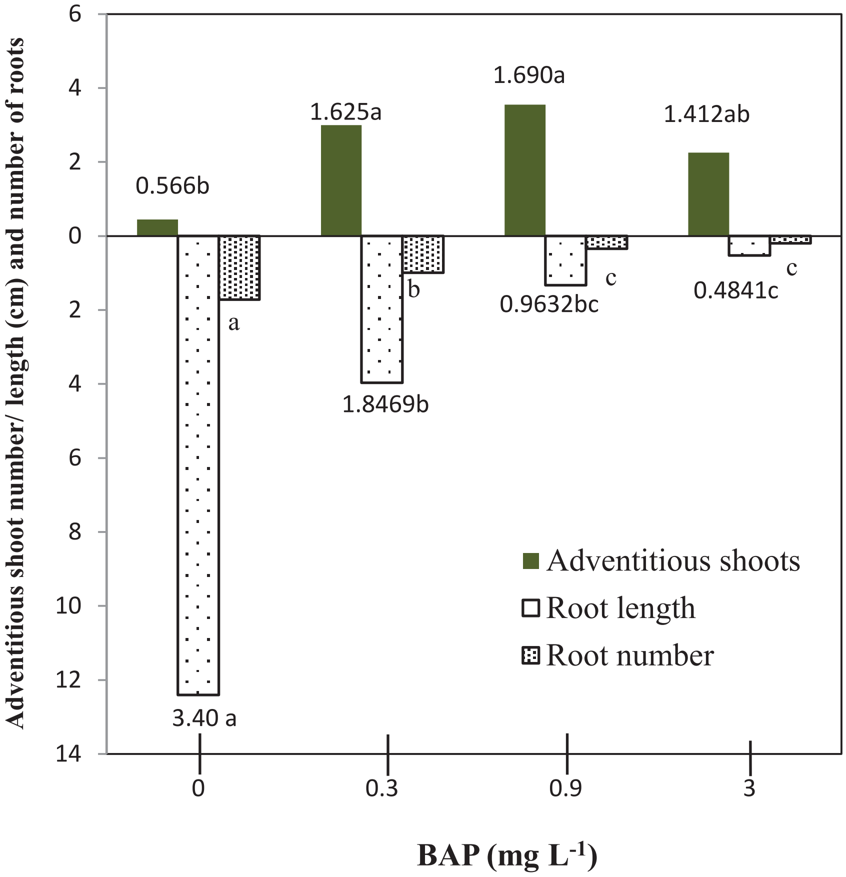
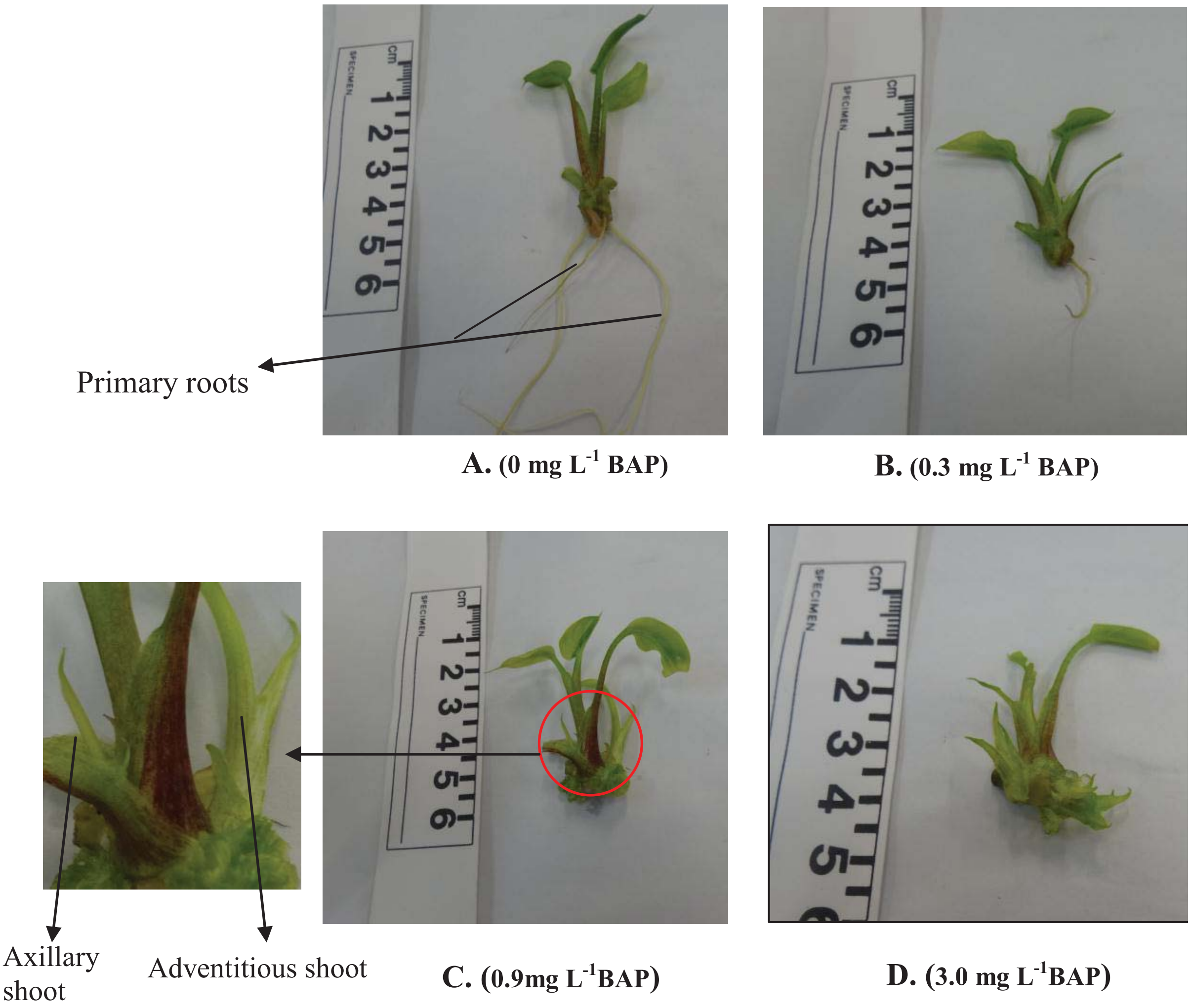


Figure 5.6 Influence of BAP concentration on number of adventitious shoots, root number and total root length per plant of *Zantedeschia* 'Best Gold' grown *in vitro* eight weeks after treatment commenced. For number of adventitious shoots and root length, the number associated with each column presents the square root mean value, with y-axis presenting back-transformed values so as to enable interpretation. Means with same letters of same growth parameter do not differ significantly at $P \leq 0.05$, based on DMRT.



A. (0 mg L^{-1} BAP)

B. (0.3 mg L^{-1} BAP)

C. (0.9mg L^{-1}BAP)

D. (3.0 mg L^{-1}BAP)

Figure 5.7 Shoots of *Zantedeschia* 'Best Gold' grown *in vitro* that received (A) 0 mgL^{-1}, (B) 0.3 mgL^{-1}, (C) 0.9 mgL^{-1} and (D) 3 mgL^{-1} of BAP observed eight weeks after treatment. A part of shoot within red circle in Figure C is magnified showing both axillary and adventitious shoots.

5.3.3 Experiment Three - Interaction between cytokinin and strigolactone

5.3.3.1 Axillary shoot numbers

There was no three-way interaction effect between 'cultivars', 'BAP' and 'GR24' on number of axillary shoots in week 4 and week 8 (Table 5.2). However, in week 4, the interaction between cultivars and hormones (BAP or GR24) was significant. Interaction between BAP and GR24 was also significant after both periods of assessment, but more so after eight weeks ($P < 0.001$) than after four weeks ($P < 0.05$; Table 5.2).

Table 5.2 Statistical main and interaction effects between cultivars and hormones as represented by *P* values for the number of axillary shoots in *Zantedeschia* grown *in vitro* observed in week four and week eight.

Variables	***P value***	
	Week 4	**Week 8**
Main Effects		
Cultivars	<0.001	<0.001
BAP	<0.001	<0.001
GR24	<0.001	0.002
Interaction Effects		
Cultivars × BAP	0.007	0.292
Cultivars × GR24	0.027	0.318
BAP × GR24	0.012	<0.001
Cultivars × BAP × GR24	0.697	0.830

The highly branched cultivars typically responded to BAP at low concentration (0.3 mg L^{-1}) and did not produce more axillary shoots at the higher concentration (0.9 mg L^{-1}) (Figure 5.8 B & C). In contrast, Best Gold' continued to respond to 0.9 BAP mg L^{-1} at 4 weeks, and slightly at 8 weeks (Figure 5.8A). In all cultivars studied fewer or no axillary shoots were produced in the absence of BAP, and the effect of GR24 in reducing axillary shoot numbers was only visible when BAP was present.

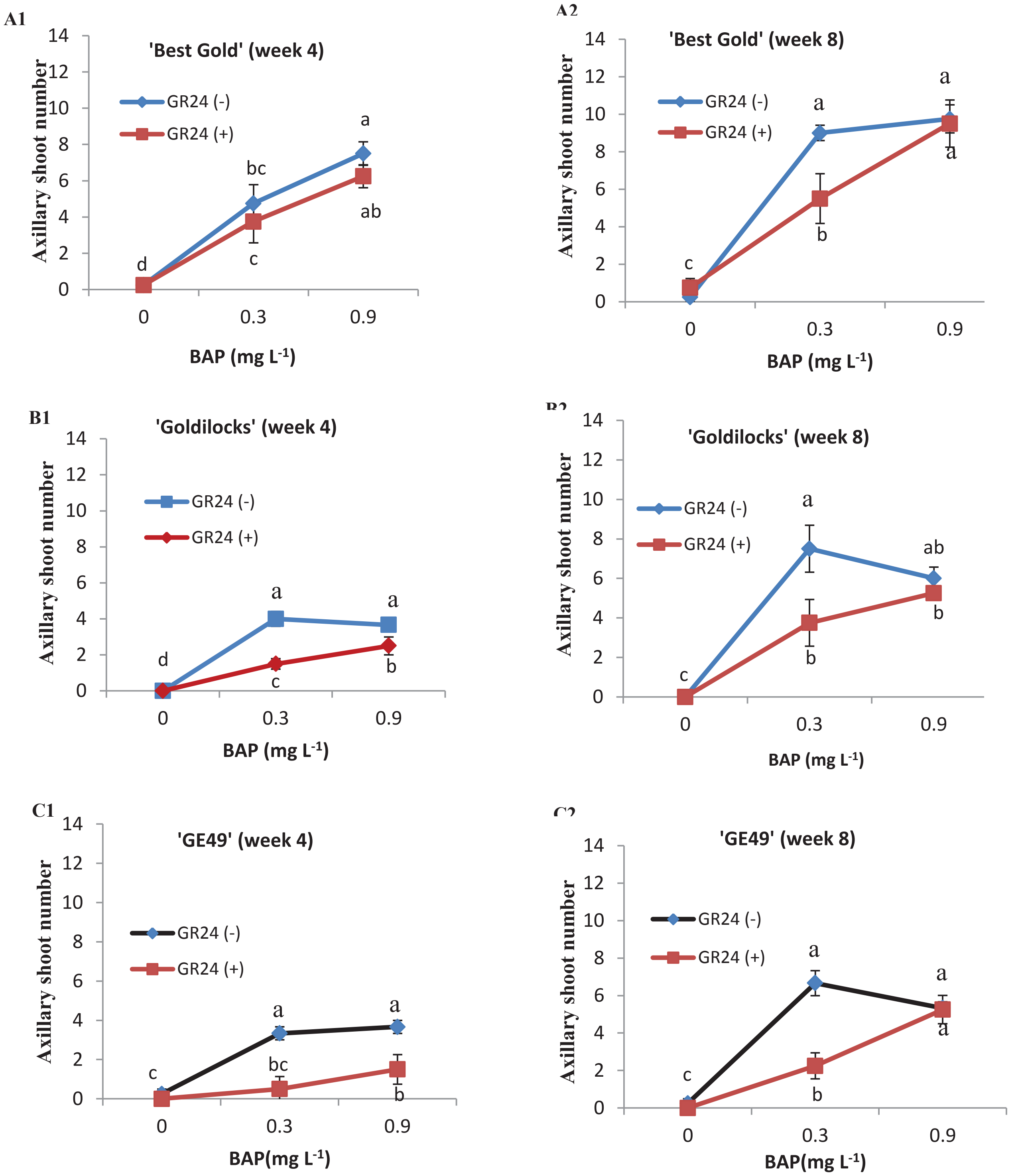


Figure 5.8 Number of axillary shoots per culture vessel (eight plants in each vessel) in three cultivars (A) Best Gold, (B) Goldilocks and (C) GE49 of *Zantedeschia* grown *in vitro* supplied with GR24 (±)(in mg L^{-1}) and/or BAP (±) observed at four (1) and eight (2) weeks after treatment. The vertical bars indicate SE of the mean (n=4), means within each graph with same letters do not differ significantly at $P \leq 0.05$ based on DMRT.

As illustrated within Figure 5.9, in week 4, GR24 greatly reduced (by about 70%) axillary shoot number stimulated by the lower concentration of BAP in highly branched cultivars (also refer Figure 5.8B1 & C1), but not in the low branched cultivar (Figure 5.8A1). However, by week 8, GR24 significantly reduced the number of axillary shoots produced at 0.3 mg L^{-1} of BAP, but not 0.9 mg L^{-1} of BAP (Figure 5.8), in all cultivars studied.

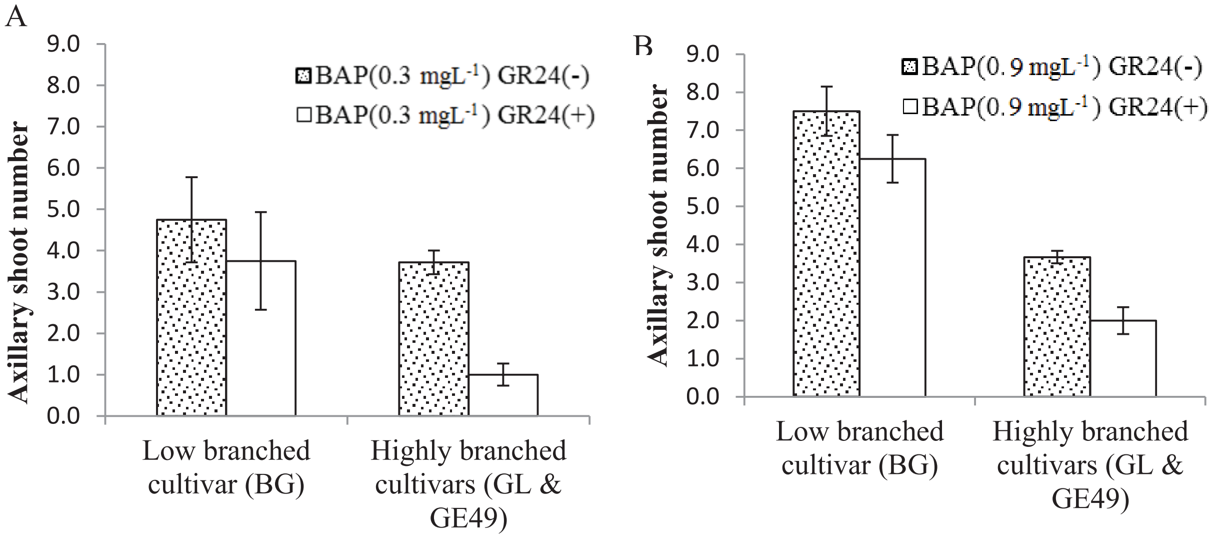


Figure 5.9 Number of axillary shoot per culture vessel (eight plants in each vessel) in low and highly branched cultivars of *Zantedeschia* grown *in vitro* supplied with GR24 (±) and 0.3 mg L^{-1} BAP (A), and GR24 (±) and 0.9 mg L^{-1} of BAP (B) observed four weeks after treatment. The vertical bars indicate SE of the mean (n=4).

5.3.3.2 Adventitious shoot number

At week 4, there were no interaction effects among 'cultivars', 'BAP' and 'GR24' on the number of adventitious shoots produced. Among these three treatment factors, only the main effect 'BAP' significantly increased the adventitious shoot number ($P < 0.001$), but with only a difference of 0.5 (in average) in the number of shoots between BAP treated and untreated shoots (Figure 5.10).

Table 5.3 Statistical main and interaction effects between cultivars and hormones as represented by *P* values for the number of adventitious shoots in *Zantedeschia* grown *in vitro* observed in week 4 and week 8.

Variables	*P value*	
	Week 4	**Week 8**
Main Effects		
Cultivars	0.077	<0.001
BAP	<0.001	<0.001
GR24	0.266	0.008
Interaction Effects		
Cultivars × BAP	0.543	<0.001
Cultivars × GR24	0.969	0.028
BAP × GR24	0.336	0.082
Cultivars × BAP × GR24	0.982	0.026

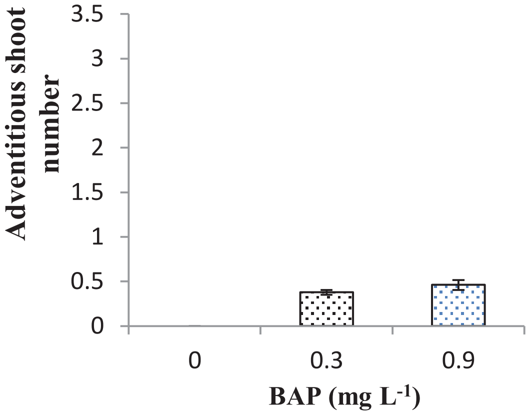


Figure 5.10 Number of adventitious shoots per plant of *Zantedeschia* plants grown *in vitro* as affected by main effect of BAP observed four weeks after treatment. The vertical bars indicate SE of the mean (n=4).

By week 8, a triple interaction effect amongst the three factors was evident on the number of adventitious shoots ($P < 0.05$; Table 5.3). Likewise, for the number of adventitious shoots at week 8, the interaction effect between cultivars and hormones was also significant with BAP ($P < 0.001$) and with GR24 ($P < 0.05$; Table 5.3). In the low branched cultivar (Best Gold), the number of adventitious shoots increased to be as twice as many with the increase in BAP concentration (Figure 5.11A), whereas this effect was not evident in highly branched cultivars (Figure 5.11B & C), resulting in a greater number of adventitious shoots in 'Best Gold' (about 80% more)compared to the two normally highly branched cultivars (Figure 5.11). Importantly ‘GR24’ significantly reduced almost by half the formation of BAP-induced adventitious shoots in ‘Best Gold’ (Figure 5.11A).

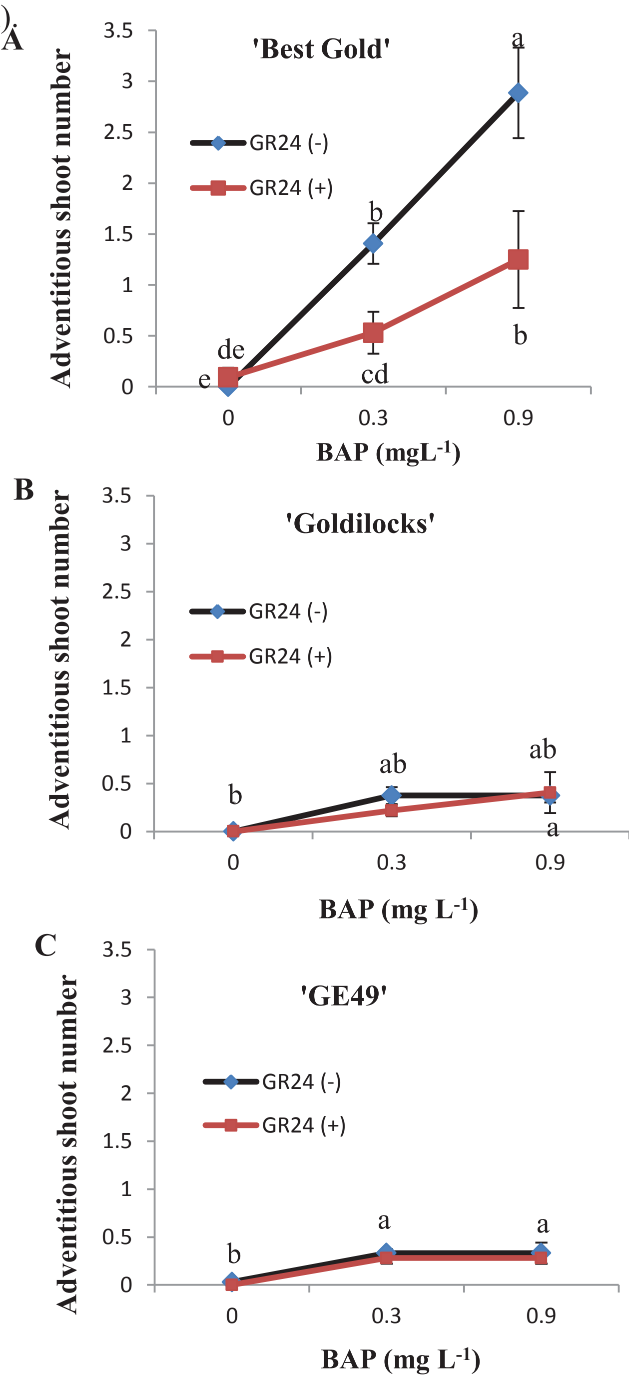


Figure 5.11 Number of adventitious shoots per plant in three cultivars (A) Best Gold, (B) Goldilocks and (C) GE49 of *Zantedeschia* grown *in vitro* supplied with GR24 (±) and/or BAP (±) (in mgL^{-1}) observed eight weeks after treatment. The vertical bars indicate SE of the mean (n=4). For each cultivar, means with same letters do not differ significantly at $P \leq 0.05$, based on DMRT.

5.3.3.3 Number of nodes

The effect of BAP on the average number of nodes visible on the primary shoot varied between cultivars at week 4 ($P < 0.05$; Table 5.4). For cultivar GE49, BAP at the lower concentration significantly increased the number of nodes but no further increase occured at the higher concentration of BAP (Table 5.5); however, the difference between maximum and minimum node number was only about 0.4. In other cultivars, BAP apeared to have no influence on the number of nodes at week 4. After eight weeks, the effect of BAP was not different from that in the control in all cultivars (Table 5.5), except in 'GE49'. In this cultivar, BAP (0.9 mgL^{-1}) significantly reduced the number of nodes, however GR24 reversed this effect of BAP by enhancing node number.

Table 5.4 Statistical main and interaction effects between cultivars and hormones as represented by *P* values for the number of nodes on the primary shoot in *Zantedeschia* grown *in vitro* observed in week 4 and week 8.

Variables	***P value***	
	Week 4	**Week 8**
<u>**Main Effects**</u>		
Cultivars	<0.0001	<0.0001
BAP	<0.0001	0.757
GR24	0.213	0.695
<u>**Interaction Effects**</u>		
Cultivars X BAP	0.043	0.020
Cultivars X GR24	0.180	0.170
BAP X GR24	0.094	0.451
Cultivars X BAP X GR24	0.745	0.113

Table 5.5 Hormonal influence on the number of nodes on the primary shoot (±SE) per plant of three *Zantedeschia* cultivars grown *in vitro* observed in week 4 and week 8.

Cultivars	BAP (mgL^{-1})	GR24	Week 4	Week 8
Best Gold	0.0	-	1.74 ±0.05	2.46 ±0.16
	0.0	+	1.60 ±0.08	2.38 ±0.12
	0.3	-	1.77 ±0.04	2.36 ±0.04
	0.3	+	1.75 ±0.09	2.28 ±0.13
	0.9	-	1.72 ±0.08	2.68 ±0.11
	0.9	+	1.74 ±0.09	2.43 ±0.07
P value			0.544	0.2153
Goldilocks	0.0	-	1.28 ±0.11	2.51 ±0.08
	0.0	+	1.28 ±0.10	2.54 ±0.11
	0.3	-	1.38 ±0.13	2.58 ±0.11
	0.3	+	1.43 ±0.06	2.75 ±0.09
	0.9	-	1.09 ±0.05	2.59 ±0.07
	0.9	+	1.41 ±0.06	2.39 ±0.16
P value			0.159	0.342
GE49	0.0	-	1.23 ±0.03 c	2.25 ±0.09 a
	0.0	+	1.25 ±0.04 c	2.04 ±0.13 ab
	0.3	-	1.63 ±0.07 a	2.15±0.12 a
	0.3	+	1.66 ±0.08 a	2.17 ±0.10 a
	0.9	-	1.39 ±0.07 bc	1.77 ±0.07 b
	0.9	+	1.50±0.05 ab	2.10 ±0.03 a
P value			0.0003	0.0616

For each cultivar and time of assessment, means with same letters within a column do not differ significantly at $P \leq 0.05$ based on DMRT. Presence and absence of GR24 (0.1 mgL^{-1}) is indicated by symbols.

5.4 Discussion

5.4.1 Influence of BAP on axillary and adventitious shoot formation

The effect of CKs in promoting development of axillary shoots of *Zantedeschia* in the current experiments has also been reported many times for plant species *in vitro* (George et al., 2008) including *Zantedeschia* (Naor et al., 2005). At the highest concentration of BAP used in these experiments, the number of axillary shoots reduced in all cultivars studied from that achieved at lower concentrations, which is in line with other findings (Aasim et al., 2009; Venkatachalam et al., 2012). However, the supra-optimal concentration of BAP varied between the highly branched and a low branched cultivars. For highly branched cultivars, 0.9 mg L^{-1} BAP was supra-optimal whereas for the low branched cultivar, the supra-optimal concentration occurred between 0.9 and 3.0 mg L^{-1} (Figure 5.5, 5.8) suggesting differences in sensitivity of these cultivars to respond to externally applied CK. Despite ‘Best Gold’ being a naturally low branched cultivar, this cultivar produced a higher number of axillary shoots as compared to ‘Goldilocks’ and ‘GE49’ in response to CK *in vitro* (Figure 5.8). The higher responsiveness of ‘Best Gold’ to applied CK might be due to this cultivar being deficient in endogenous CK; a hypothesis for future examination by others perhaps.

In the absence of BAP axillary shoot number in all three cultivars grown *in vitro*, was either nil or negligible (Figure 5.8). In highly branched cultivars grown *in vitro*, the absence of roots on stem cuttings in the current experiments may have contributed to branching deficiency, as roots are a major site of CK synthesis (Letham, 1994). When roots are present, CKs would be produced to stimulate branching but in the low branching cultivars like ‘Best Gold’ both CKs production may be low and production of SLs high, as described in Chapters 2 and 4. The antagonistic interaction between CKs and SL is further discussed in Section 5.4.2.

Interestingly, some axillary shoots developed within the primary shoot of ‘Best Gold’ even without BAP (Figure 5.5) in Experiment Two. It must be acknowledged that this result might be due to a carryover effect of CK, as the stem cuttings used for this experiment were derived from plants previously grown in the media that contained low levels of CKs (see Section 5.2.3.1). For verification of this hypothesis, shoots of 'Best Gold' were sub-cultured using media that had no CK and stem cuttings from these shoots were further grown with and without CK. Within this additional experiment, no axillary shoots were produced when BAP was absent from the basal medium (Appendix VI). Hence, for stimulating the growth of axillary shoots within *Zantedeschia* grown *in vitro,* external application of CK was essential.

In all cultivars studied, CK also induced more adventitious shoots, in addition to stimulating release of axillary shoots. This is supported by previous studies on plant hormones in which CK either alone (Mendi et al., 2010), or when combined with auxins (Naz & Anis, 2012), increased the number of adventitious shoots. Similar to the response with the number of axillary shoots, 'Best Gold' also produced more adventitious shoots compared to both 'Goldilocks' and 'GE49' (Figure 5.11), indicating that 'Best Gold' has a higher potential for producing shoots if CKs are supplied. Consistent with the varied response of genotypes, in *Populus deltoids* the number of shoots (including adventitious shoots) in response to CK varied from four to zero depending upon the genotype (Coleman & Ernst, 1989).

Figure 5.12 Adventitious shoots (within red circles) of *Zantedeschia* grown *in vitro* arising from plant parts other than being subtended by leaf axils

An increase in the number of nodes with applied CK was not evident in all cultivars, suggesting that the apex of the primary shoot had not been affected in terms of its capacity for active growth and differentiation (Table 5.5). For some plants grown *in vivo,* increased node numbers on the primary shoot in response to CKs might have been expected to contribute to an increased number of axillary shoots, but for *Zantedeschia* cultivars used in this experiment node number was essentially independent of CK treatment. Moreover, release of axillary buds to develop into shoots occurred at the lowest node (first node) (Figure 5.1) in all cultivars studied. It therefore follows that axillary shoot number was dependant on bud release in response to the applied CK, rather than more axillary buds being present due to more nodes.

In ‘Best Gold’, the presence of CK reduced root growth (Figure 5.6), supporting other observations that CK has an inhibitory effect on root growth (Cary et al., 1995; D'Arth et al., 2002; Ioio et al., 2008). Hence, CK-induced effects on root and shoot growth are opposite. In addition, the optimal concentration of BAP on root and shoot growth was also different. For shoot growth, the optimal concentration of BAP was less as compared to optimal concentration for root growth (Figure 5.5, Figure 5.6). This result not only illustrates the difference in hormonal sensitivity for different tissue (Firn, 1986) but, more importantly, it suggests that in the absence of BAP, even though roots (a major site of CK synthesis) were formed, ‘Best Gold’ was not able to release axillary buds to develop into shoots. When BAP was applied, axillary shoots developed although root growth was reduced, indicating a lack of endogenous CK both within the root and shoot as the presence of CK had a large influence in stimulating axillary buds in *Zantedeschia* in the current experiments. However, it is also acknowledged that the reduced CK in ‘Best Gold’ might also have been associated with a higher level of SL, as discussed in Section 5.4.3.

5.4.2 Influence of GR24 on axillary and adventitious shoot formation

The synthetic strigolactone, ‘GR24’, was able to reduce the number of axillary shoots when applied to the highly branched *Zantedeschia* cultivars grown *in vitro* (Figure 5.2, 5.8), in a concentration dependent manner, mainly during the early weeks after treatment commenced (Figure 5.2A). Reduction in the number of axillary shoots occurred with increases in concentration of GR24 up to 0.1 mgL^{-1} (Figure 5.2A). The dose dependent response to GR24 on the development of axillary shoots follows a typical near sigmoidal pattern, with the optimal zone at higher concentration followed by supra-optimal concentration as defined by Wigchert et al. (1999). Thus the optimal concentration was determined to be 0.1 mg L^{-1}, which could act as a reasonable concentration to test in interaction studies between GR24 and BAP; a fact used in Experiment Three (refer Section 5.3.1).

The reduction in axillary shoot number in response to externally applied SL, in highly branched *Zantedeschia* cultivars (Experiment One and Experiment Three), supports previous reports on the inhibitory influence of GR24 on axillary shoot number in highly branched SL deficient mutants of *Arabidopsis* and rice (Gomez-Roldan et al., 2008; Umehara et al., 2008). Likewise, Gomez-Roldan et al. (2008) and Dun et al. (2012) also observed inhibition of axillary bud growth by GR24 in pea plants, but only in terms of shoot length rather than bud release. However, the current results in this chapter clearly showed that GR24 affected bud release *per se*, demonstrating the specific important effect it has on shoot branching, since inhibition of shoot branching begins with an inhibition of bud release. A subsequent effect on length of the shoot is secondary, and probably via a different mechanism (Chapter 1, refer Section 1.2).

Despite reduction in the total number of axillary shoots after 4 weeks and 8 weeks in highly branched *Zantedeschia* plantlets treated with GR24 (Figure 5.2), the rate of increase in shoot number of axillary shoots released was not similar in early and later weeks of assessment (Figure 5.3). The effect on rate of increase in the number of axillary shoots declined between week 4 to 8 (Figure 5.3, 5.4), suggesting that the effect of GR24 in the basal medium weakens, possibly due to degradation of the GR24. This hypothesis of degradation is supported within the paper by Crawford et al. (2010) in which the effect of SL on the number of axillary shoots reduced after five weeks when applied in the agar medium. However, in Experiment Three, all cultivars appeared to respond to GR24 in week 8 (Figure 5.8), suggesting variability in the duration of time over which GR24 is effective. To further confirm whether the reduced response of axillary buds to GR24 after week 4 observed in Experiment One was due to loss in effectiveness of GR24 applied in the culture medium, an experiment that includes at least two applications of GR24 at an interval of four weeks is recommended. In other studies in which either the length or number of axillary shoots in response to GR24 was observed, either repeated supply of GR24 was adopted throughout the experiments, depending on the method of application (Gomez-Roldan et al., 2008; Dun et al., 2012;

Umehera et al., 2008), or just once for short duration studies of about a week (Liang et al., 2010).

Interestingly, in the low branched cultivar Best Gold, the effect of GR24 on release of axillary buds was not evident in the first four weeks (Figure 5.8), though GR24 significantly reduced the outgrowth of axillary buds after eight weeks (Figure 5.8). Hence, it appears that the response to GR24 by axillary buds of 'Best Gold' was slow compared to that evident in the highly branched cultivars and that the rate of degradation of GR24 may differ between genotypes.

To further demonstrate that inhibition of axillary and/or adventitious shoot number caused by GR24 was not due to hormonal toxicity, node number on the primary was also taken into consideration in Experiment Three. In all three cultivars, the number of nodes was not reduced in SL-treated shoots compared to the control (Table 5.5) so, as for CKs, the reduced number of axillary shoots in GR24-treated *Zantedeschia* plantlets was independent of the number of nodes, but dependent on the ability of this hormone to inhibit the release of axillary buds (Chapter 6). Thus, this result strongly supports the hypothesis that the effect of GR24 is directly on release of axillary buds, without involving any toxic effect on plant growth *per se*.

BAP increased adventitious bud number, but only in the low branched cultivar Best Gold (Figure 5.11). Interestingly, GR24 significantly reduced the number of adventitious shoots induced by BAP. Thus, in future, it would be worthwhile to investigate whether any highly branched cultivars produce adventitious buds (with or without exogenous CKs) and, if so, whether SL inhibits this formation. This is the first time the inhibitory effect of SL on adventitious shoot number has been reported, hence adding a new role for SL in plant canopy architecture.

Reduced adventitious shoot number with SL suggests that SL may have inhibited the formation of adventitious buds. Hence, with SL, it is initiation rather than release that is affected. However, if it were inhibition at an early stage of development, that means if SL has inhibited the growth at an early microscopic state of development of the adventitious bud, the mechanism involved could not be canalization. The canalization theory suggests SL inhibits the early stage of bud development or the release via reducing auxin transport out of bud into the main stem in the presence of apically derived auxin (according to auxin-SL canalization model, refer Section 1.4.3.2, Theory B). Unlike axillary buds (Figure 5.7), adventitious buds (also see Figure 5.12) are not directly connected to the vascular tissue of the main stem (Samarakoon, 2012) as they may form at any part of the tissue such as root, stem, or leaf (Evert, 2006; Figure 5.12). The ability of SL to reduce the BAP stimulated adventitious bud number (Figure 5.11) in fact supports the direct localized action of SL on the buds (see Section 1.4.3.1, Theory C).

5.4.3 Interaction between strigolactone and cytokinin in shoot branching

CKs are well known for enhancing bud release (Shimizu-Sato et al., 2009), whereas SL inhibits the release (Figure 5.8, also see Section 3.3.1, Chapter 3) although it is difficult to distinguish between release and subsequent growth. In the current experiments presented in this chapter, SL reduced CK-induced axillary shoot number (Figure 5.8), supporting the model of an interaction between SL and CK at the early stage of shoot branching i.e., during initiation of bud release. Dun et al. (2012) also found an interaction in pea seedlings but, in their study, the parameter measured was shoot length rather than bud release, as SL reduced CK-induced shoot length suggesting that the interaction between these two hormones was on the subsequent growth. Hence, it is possible that SL interacts with CK at a different stage of shoot branching in *Zantedeschia* but, due to lack of data on SL and/or CK on axillary shoot length of *Zantedeschia* when grown *in vitro*, the interaction between these two hormones on axillary shoot length of *Zantedeschia* is unknown.

On the other hand, in contrast to the inhibition effect of GR24, Crawford et al. (2011), Ward et al. (2013) and Liang et al. (2010) observed no inhibition of axillary shoot length in node/s of stem segments with basally-supplied GR24. In those studies, adding auxin to the cut top internode, in the presence and/or absence of GR24, inhibited axillary shoot length. Hence, polar auxin transport (PAT) appears as the main cause of axillary bud inhibition via preventing auxin transport out of the buds to the main stem, which is crucial for bud release (Domagalska & Leyser, 2011; Prusinkiewicz et al., 2009). Contrary to this hypothesis, in the current experiment, despite the presence of auxin from the apical meristem (Figure 5.1), axillary buds (stimulated by BAP) were not inhibited. Wickson & Thimann (1958) and Turnbull et al. (1996) showed that CK could release the axillary bud, even in the presence of the apex. Thus, the lack of release was probably due to deficiency of CK rather than lack of auxin transport out of the buds. Since GR24 inhibited the BAP stimulated axillary bud release, it is concluded that an antagonist interaction between CK and SL has occurred on release of buds.

Efforts by Liang et al. (2010) and Ward et al. (2014) to inhibit axillary shoot length with GR24 failed, possibly because application of GR24 was insufficient to antagonize the effect of a higher level of CK contributed by *de novo* synthesized CK (Shimizu-Sato et al.,2009) and/or storage CK (Woolley & Wareing, 1972) near buds after removal of apex, since effectiveness of GR24 might depend on relative amounts of CK available in axillary buds (Dun et al., 2012). On applying auxin to the decapitated stem, CK might get reduced due to the inhibitory effect of auxin on CK biosynthesis (Nordström et al., 2004), thus allowing GR24 to begin to have an effect due to an increased SL/CK ratio (Dun et al., 2012). Gomez-Roldan et al. (2008) noted that applying GR24 directly to the buds, as compared to via the vascular stream, might differ in their response to bud inhibition, with more effect via direct bud supply. In the experiments presented in this chapter, although GR24 was applied only in the basal medium, it is possible that GR24 was received directly by the axillary buds as nodes present at the base of the stem cutting were completely immersed in the media (Figure 5.1A). In addition, possibly, the level of SL was higher due to the presence of apically-derived auxin, and less availability to CKs. Supporting this hypothesis, at higher concentrations (0.9 mgL^{-1}) of BAP, GR24 failed to inhibit axillary shoot number in all cultivars studied (Figure 5.8).

Stimulation of axillary shoot number by CKs in plants grown *in vitro* suggests that high frequency of branching of some cultivars in the field might be due to a higher level of endogenous CKs. Highly branched 'Goldilocks' contained high levels of CKs as compared to the low branched 'Best Gold' (Appendix IV) supporting that CK enhances shoot branching. However, D'Arth et al. (2007) found no correlation between CK concentration and the incidence of "bushy syndrome" (i.e. excessive multiple branching) in *Zantadeschia* 'Florex Gold'. The possible explanation for lack of correlation between CK level and branching in 'Florex Gold' could be due to the fact that D'Arth et al. (2007) analysed only zeatin type CKs (non-aromatic) , whereas the aromatic CKs such as meta-topolins are predominantly present in 'Best Gold' (Subbaraj, 2011). It is also worthy of noting that Subbaraj (2011) also showed that aromatic CKs increased in plants of 'Best Gold' grown *in vivo* after external application of BAP, which successfully stimulated branching.

The alternative explanation for the lack of correlation between CK and branching is that may be 'Florex Gold' is a SL deficient mutant. Like other SL mutants such as max of *Arabidopsis,* without having a high level of CK (Foo et al., 2007), 'Florex Gold' is highly branching. However, in the highly branched 'Goldilocks' which is not a SL mutant (refer Chapter 2), enhanced branching due to CK is highly likely. In the low branched 'Best Gold', production of higher levels of endogenous SLs in roots of 'Best Gold' (Chapter 2 and 4) might have suppressed the level of endogenous CKs, resulting in inhibition of release of axillary buds. It is possible that SL may have reduced CK via increasing CK metabolism. Overall, it is possible that the differences in level of these two hormones might alter branching of *Zantedeschia* plants grown *in vivo*.

5.5 Conclusion

Supply of SL in the media of *Zantedeschia* cuttings grown *in vitro* can inhibit both CK-stimulated axillary buds and the formation of CK-stimulated adventitious buds in *Zantedeschia* grown *in vitro*, which may explain the phenotypic differences between high branched and low branched cultivars. It would be interesting to explore further the generality of the observed effects of SLs on adventitious bud formation. Similarly, analysis of endogenous CKs (including aromatics) in high and low branched cultivars of *Zantedeschia* grown either *in vitro* or *in vivo*, could be useful to understand shoot branching in plants like *Zantedeschia*.

6 Hormonal interaction influencing shoot branching of pea plants

6.1 Introduction

Different hormonal interactions occur during shoot branching, a process that involves different stages from initiation of axillary meristems through to the development of lateral branches (Vanstraelen and Benkova, 2012) (also refer Section 1.2, Chapter 1). Auxin and cytokinins (CKs) are the well-known hormones that are involved during bud release and/or subsequent growth (Shimizu-Sato et al., 2009). Recently, strigolactones (SLs) are also known to be a putative hormone that is involved in controlling bud outgrowth (Gomez-roldan et al., 2008). Studies in pea (*Pisum sativum*) plants reported inhibition effects of SLs on the growth of the axillary shoot (Boyers et al., 2012; Dun et al., 2012) suggesting SL reduced subsequent growth of the released bud. In Chapter 5, externally applied SL reduced the number of axillary shoots in *Zantedeschia* grown *in vitro* suggesting a role for SL on inhibition of bud release. Hence, it was hypothesized that SL inhibits bud release as well as subsequent growth. However, the mechanism of how SL inhibits bud release and/or subsequent growth as well as interact with auxin and CK in this regard, is still not clear.

Different theories have been hypothesized over many years to explain mechanisms of shoot branching (refer Section 1.4, Chapter 1), with many not stating the stage of shoot branching being considered however. The classical theory of shoot branching suggests that auxin, originating from the shoot apex, inhibits axillary bud outgrowth (Thimann & Skoog, 1933) and thereby reducing shoot branching. Removal of the shoot apex induces bud release, and application of auxin to the decapitated stump restores inhibition, at least for a short period (Thimann & Skoog, 1934). However, auxin from the apical meristem does not act directly on the buds to inhibit them (Morris, 1977), but indirectly influences the outgrowth of the buds. Based on indirect auxin action, several theories were hypothesized as described in Chapter 1 (refer Section 1.4.3) among which the most recent two theories that incorporate the role of SLs have become a current subject of debate. One theory is based on direct localized action of SLs and CK in buds (see Theory C under the section 1.4.3.1, Chapter 1). In this theory, auxin indirectly inhibits bud outgrowth via stimulating biosynthesis of SLs (Foo et al., 2005; Johnson et al., 2006; Sorefan et al., 2003) and reducing CK level (Nordström et al., 2004). The other theory is 'Auxin-Strigolactone canalization' (see Theory B under the section 1.4.3.2, Chapter 1) in which apically derived auxin, and basally applied SLs, restrict auxin transport out of axillary buds, crucial for bud outgrowth (Crawford et al., 2010). However, these two theories are not mutually exclusive, as no single model can fully explain the exact mechanism in different situations (Waldie et al., 2014). In fact, it is important to note that many previous studies related with shoot branching are based on hormonal effects on axillary shoot length, rather than release of axillary buds. Hence a careful study is needed to distinguish between the mechanism involved in bud release compared to subsequent shoot growth (Cline, 1991). It is possible that the two mechanisms may integrate with each in the overall shoot branching phenomenon.

In Chapter 3, a synthetic SL, GR24, applied at basal node/s of decapitated wild-type pea seedlings, reduced axillary shoot length as well as number of shoots growing, supporting the hypothesis that SL inhibits both release and the subsequent growth. However, the hormonal pathway of axillary shoot branching in decapitated plants might not be the same as in the plants that have a shoot apex (Ferguson & Beveridge, 2009; Morris et al., 2005) as a rapid signal other than hormone can exist (Mason et al., 2014) (also refer Section 1.4.4, Chapter 1). Hence un-decapitated pea stems were used in the current experiments, which were also part of an effort to develop a quick and easy assay based on shoot branching (preferably bud release) in order to track purification of the branch inhibiting hormone, as described in Chapter 3 (see Section 3.1). The current chapter describes studies associated with hormonal interactions primarily between auxin, SLs and CKs. A highly apically dominant, un-decapitated wild-type pea stem, variety 'Ashton', was used. This variety did not produce axillary shoot growth at any nodes when grown in a controlled environment. Application of a synthetic CK 'BAP'

(6-benzylaminopurine) was adopted to stimulate axillary shoot growth of un-decapitated pea stems.

In the experiments presented in this chapter, the application method of hormones was vascular and/or direct to the bud. One experiment involved studying the hormonal interaction using pea stems laid horizontally, resulting in the hormonal supply being both vascular and direct to the bud (Figure 6.3A, Appendix VIII). In contrast, vertically oriented pea seedlings were used in the second and third experiment, resulting in uptake of hormonal solution only via the vascular system, as the bud was not in contact with the hormonal solution (Figure 6.3B). The first two experiments also allowed comparison of the interaction between SLs and CK at high and low levels of apically derived auxin in lower nodes, since shoots orientated horizontally exhibit considerably less IAA transport from the apex when compared to vertical shoots (Sanyal & Bangerth, 1998).

In addition to the roles of auxin, CK and SLs, there is also some evidence that gibberellins (GAs) may be involved in shoot branching. In *Zantedeschia* K. Spreng, application of GAs along with CK promotes more (number or length or both) axillary shoot development compared to CK alone (Subbaraj et al., 2010) (also refer Section 2.3.2.1). In *Actinida chinensis and deliciosa* (kiwifruit) application of GAs alone stimulates more axillary shoot formation (Vattiprolu, 2012), suggesting that the effect of GAs at the initial stage of axillary shoot development i.e., bud release. Therefore, within this chapter, the role of GA for both bud release as well as subsequent growth has been explored using pea plants.

Specific objectives of this study were:

- to compare the difference in response on axillary shoot development when hormones (CK or SL) were transported through the vascular stream or with all tissue lying in the hormonal solution including buds;
- to determine the stage of branching at which strigolactones or cytokinins act, i.e. bud release or subsequent bud growth;

- to examine the interaction between strigolactone and cytokinin in bud release and/or subsequent shoot growth of pea plants;
- to examine the role of gibberellin in bud release and subsequent growth.

6.2 Materials and methods

6.2.1 General methods

Germination and transplantation of the pea variety (var) 'Ashton' followed the method described in Section 3.2.1.1, Chapter 3. Transplanted seedlings (3 days old) were grown for 11 days in a growth cabinet (Contherm Biosyn Series 6000, CAT No. 630, Contherm Scientific Ltd., Hutt City, New Zealand) (Figure 6.1) at 23°C/18°C (day/night regime) supplied with 100 µmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) through cool white tubes for a 16 hour photoperiod.

Figure 6.1 Pea seedlings growing in plastic containers half filled with moist sand in a growth cabinet.

Before hormonal treatment, cotyledons and node 1 of each pea seedling were removed by cutting the main stem above node 1 (Figure 6.2). Cotyledons were removed in order to avoid loss of pea seedlings due to microbial contamination of the cotyledon, and node 1 was removed to eliminate any potential interaction between buds (Dun et al., 2012) at nodal positions 1 and 2. Hence at the commencement of the experiment the pea seedlings comprised a shoot apex, three nodes including basal node 2 and, at least three pairs of fully expanded leaves (Figure 6.2).

In preliminary experiments, axillary buds of intact pea seedlings which had attained two to three visible nodes (10 days old), when grown without cotyledons, did not develop into axillary shoots even using externally applied CKs. In contrast, however, supplying some nutrients (half strength macro & full strength micro salts of Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) to the excised stems, allowed axillary buds to develop into shoots when supplied with BAP, as also found by Ferguson and Beveridge (2009). When seedlings had attained a stage of growth of 4 visible nodes or more, CK was effective at inducing axillary buds to develop into shoots without cotyledons or supplementary nutrients, presumably because sufficient nutrients from the cotyledons were now stored in the leaves, which also produced photosynthate.

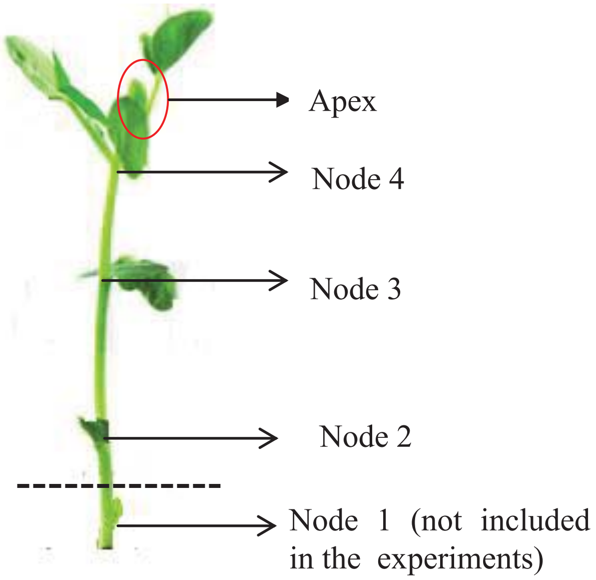


Figure 6.2 An excised stem of a 14 day old pea seedling var Ashton that was used for the experiments. Basal nodes (1 & 2) beared a scale leaf, while the remaining nodes beared true leaves. Plant parts above the dash line were used for the experiments.

6.2.2 Preparation of hormonal solutions

The concentration of GR24 used in this experiment, 5 μM (1.5 mg L^{-1}) or 1 μM (0.3 mg L^{-1}) were selected because they had previously been shown to be effective with the pea variety Ashton for achieving a branching inhibition response (Chapter 3). For BAP, the same range of concentrations i.e., 5 µM (1.1 mg L^{-1}) and 1 µM (0.23 mg L^{-1}) was used, based on a preliminary dose-response trial (refer Appendix VII).

The stock solution of 333 µM (100 mg L^{-1}) GR24 was prepared as described previously (refer Section 2.2.2.1, Chapter 2), followed by dilution to obtain treatment solutions. For BAP (molecular weight = 225.25 g/M) 100 mls of stock solution (100 µM) was prepared by dissolving 22.5 mg BAP in 10% ethanol. Similarly, for GA_3 (molecular weight = 345.38 g/M), 100 mls of stock solution (100 µM) was made by dissolving 34.53 mg GA_3 in 10 % ethanol. The final concentrations of each hormone were made up with distilled water through dilution, so that the final concentration of ethanol was less than 0.5%.

6.2.3 Experiment One - Horizontal orientation

6.2.3.1 Treatment application

Excised stems of pea seedlings, with an intact apex, were placed horizontally in 9 cm diameter glass Petri dishes, each filled with 20 ml of treatment solution (Figure 6.3A). Another glass Petri dish of the same size was used as a lid, and partially sealed with insulating tape (ensuring some level of aeration to the growing stems through the portion between the two dishes with no tape), so as to minimize loss of solution due to evaporation. The cut end of the stem base and node 2 were directly in contact with the solution, allowing for both vascular and direct-bud supply of hormones. However, the bud received major amount of hormone through the vascular stream than directly (refer Appendix VIII). Subsequently during the course of the experiment, both before and 5 days after placing the Petri dish in a growth cabinet, the Petri dishes were shaken at a rate of 50 rpm (rotations per minute) for about a minute using a gyrotary shaker (Model –G2, New Bunswick Scientific Co. Inc, USA), in order to provide aeration of the solution.

6.2.3.2 Treatments and experimental design

The treatments comprised three concentrations (0, 1 and 5 µM) of GR24 and three concentrations of BAP (0, 1 and 5 µM) arranged in a 3 × 3 factorial design. There were a total of nine treatments each replicated three times, represented by pairs of Petri dishes, each containing four stems. Within the growth chamber in which the experiment was conducted, treatments were arranged in a completely randomised design.

6.2.4 Experiment Two- Vertical orientation

6.2.4.1 Treatment application

When conducted with excised stems positioned in a vertical orientation, stems were upright in a glass jar containing 4 ml of treatment solution (Figure 6.3B). The open end of the glass jar was covered with a 5 cm glass Petri dish, so as to reduce water loss due to evaporation (Figure 6.3B). With stems held in this orientation, only the cut end of the stem base was in contact with the treatment solution. Stems were aerated as noted for horizontally oriented pea seedlings (refer Section 6.2.3.1)..

6.2.4.2 Treatments and experimental design

Hormonal treatments and experimental design were the same as that used in Experiment One (horizontal orientation), except that only three excised stems were placed within each replicate glass jar.

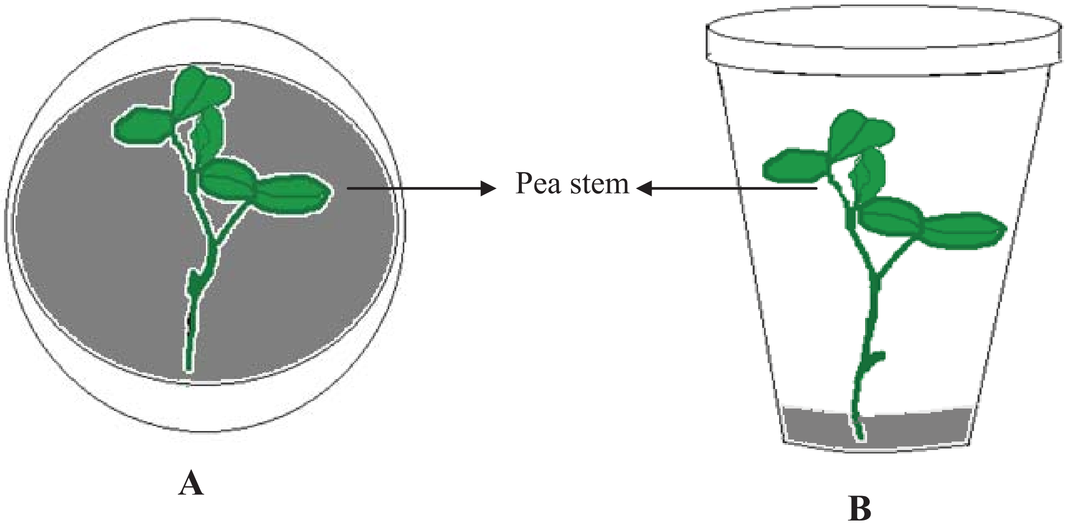


Figure 6.3 Diagrammatic illustration of excised stem with an intact apex, of pea seedlings var 'Ashton', orientated either; (A) horizontally in a Petri dish filled with 20 ml of treatment solution (Plan view) or, (B) vertically upright in a glass vessel filled with 4 ml of treatment solution (Side view). Grey colour in each figure represents treatment solution.

6.2.5 Experiment Three- Application of GA_3

This experiment involved pea stems held vertically upright as described in Section 6.2.4.1, except the stems were devoid of both node 1 and node 2, in order to observe the hormonal influence in the bud at node 3. Four treatments that included BAP, GA_3, BAP+GA_3 and control (water) were arranged in a completely randomized design. The control and concentration of 1.5 μM for each hormone were tested on two replicated glass jars, each containing three stems.

6.2.6 Variables recorded and data analysis

For Experiments One and Two, total length of all shoots at node 2 and node 3 was measured. Also the length of the longest shoot at node 2 was recorded. In addition, the number of axillary shoots arising from node 2 and node 3, nodes per stem and roots per stem were counted. Total length of all roots in each stem was also considered. For Experiment Three, the length of the axillary shoot at node 3 (also see Section 6.2.5) was measured, and the number of axillary shoots was noted. In all experiments, data were recorded 12 days after treatment.

Data were analysed by ANOVA utilising the GLM procedure of Minitab 16 (Minitab Inc., State College PA, USA). Mean separation were made at $P \leq 0.05$ using Fisher's mean separation method, and Excel (Microsoft Corp., USA) was used for data entry, graphs generation and associated standard error values. Comparison between the means was made at $P \leq 0.05$ unless otherwise stated. Residual plots were checked for normality and constant variance through Minitab. If data were not normally distributed, square root transformations were carried out.

6.3 Results

6.3.1 Experiment One- Horizontal orientation

6.3.1.1 Axillary shoot length and number at node 2

The hormone BAP significantly increased total axillary shoot length and the longest shoot length equally at both concentrations, whereas GR24 significantly reduced (by about more than half) BAP-stimulated growth of such shoots (Figure 6.4A & B). GR24 completely inhibited the small amount of growth that occurred in the absence of BAP.

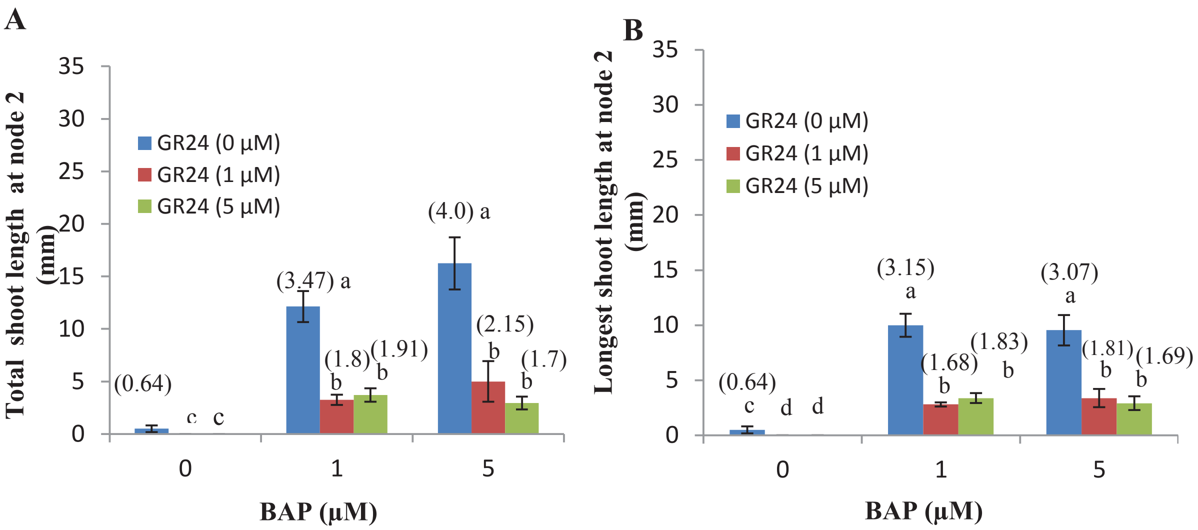


Figure 6.4 Effect of different combinations of GR24 on; (A) average total axillary shoot (maximum 3 shoots) length and, (B) average length of the longest shoot length at node 2 of pea stems var Ashton that were laid horizontally. Hormones were applied on excised stems (devoid of cotyledons and node 1 of 14 day-old pea seedlings. Data was collected 12 days after treatment. Nodes were numbered acropetally with basal node named as node 1. In each graph, means with different letters indicate significant difference at *P* < 0.05, through Fisher's mean separation test. Numbers within brackets in each graph are the transformed square root values of the mean. Vertical bars indicate standard errors (n=3).

With increase in BAP concentration, the number of axillary shoots increased. GR24 reduced the number of such shoots at each level of BAP, but the difference in effect between 1 and 5 µM of GR24 was not statistically evident (Figure 6.5).

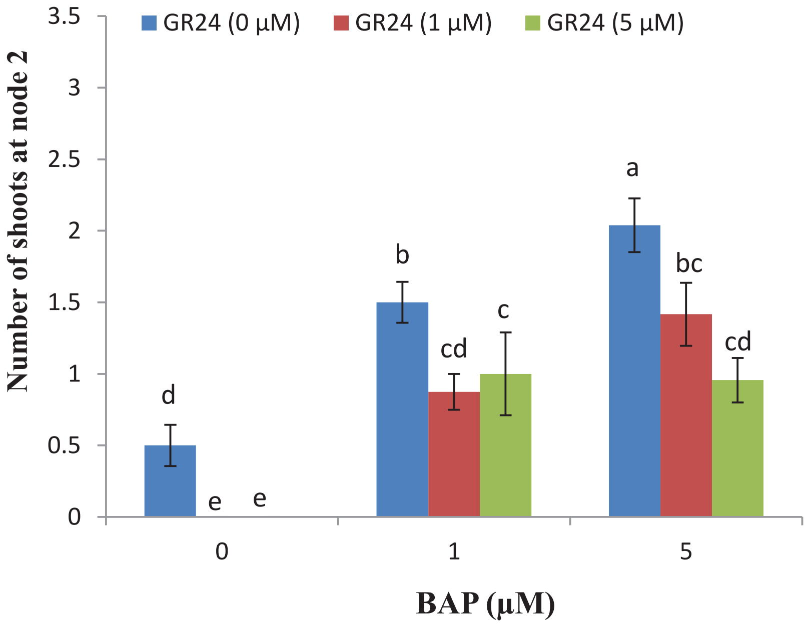


Figure 6.5 Effect of different combinations of GR24 and BAP concentration on the average number of axillary shoots at node 2 of pea stems of var Ashton that were laid horizontally. Hormones were applied on excised stems (devoid of cotyledons and node 1) of 14 day-old pea seedlings. Data was collected 12 days after treatment. Nodes were numbered acropetally with basal node named as node 1. Means with different letters indicate significant difference at $P < 0.05$, through Fisher's mean separation test. Vertical bars indicate standard errors (n=3).

6.3.1.2 Axillary shoot length and number at node 3

BAP stimulated the axillary shoot length at node 3 but the highest concentration of BAP produced five times greater length of the shoot than that produced by 1 µM BAP, while GR24 significantly reduced the shoot length by about half that produced by 5 µM BAP ($P < 0.05$; Figure 6.6).

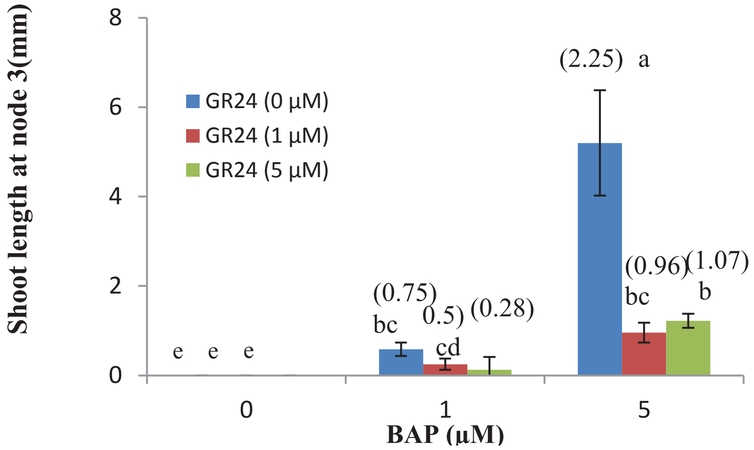


Figure 6.6 Effect of different combinations of GR24 and BAP concentration on average total axillary shoot (maximum two shoots) length at node 3 of pea stems of var Ashton that were orientated horizontally. Hormones were applied on excised stems (devoid of cotyledons and node 1) of 14 day-old pea seedlings. Data was collected 12 days after treatment. Means with different letters indicate significant difference at *P* < 0.05 through Fisher's mean separation test. Numbers within brackets are the transformed square root values of the mean. Vertical bars indicate standard errors (n=3).

At node 3, BAP (5 µM) significantly increased the number of axillary shoots compared to the control ($P \leq 0.05$). Like at node 2, GR24 reduced the number of such shoots but without the difference in effect between 1 and 5 µM of GR24 (Figure 6.7).

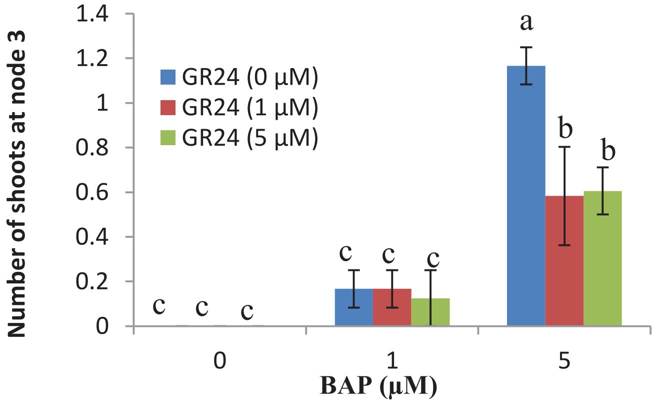


Figure 6.7 Effect of different combinations of GR24 and BAP concentration on average number of axillary shoots at node 3 of pea stems of var Ashton that were orientated horizontally. Hormones were applied on excised stems (devoid of cotyledons and node 1) of 14 day-old seedlings. Data was collected 12 days after treatment. Nodes were numbered acropetally with basal node named as node 1. Means with different letters indicate significant difference at *P* < 0.05 through Fisher's mean separation test. Vertical bars indicate standard errors (n=3).

6.3.1.3 Number of nodes on main stem

In horizontal pea stems, the hormonal effect on node number was insignificant, with a *P* value of 0.101 for BAP and 0.287 for GR24. Interaction between these two hormones was also insignificant (*P* = 0.311).

6.3.1.4 Root growth

Roots did not grow in BAP-treated pea stems (Table 6.1). Although the effect of GR24 was statistically not significant (*P* = 0.703) for root length, the lower concentration (1 µM) of GR24 produced the greatest average total root length (about 20 cm greater than control) (Table 6.1).

Table 6.1 Effect of different combinations of BAP and GR24 concentrations on average number of roots and total length of the roots (±SE) in horizontally orientated pea stems of var Ashton.

BAP	GR24	Number of roots	Total root length (mm)
0 µM	**0 µM**	6.00 ± 2.18 a[z]	(6.20) 41.50 ±18.10 a[z]
1 µM	**0 µM**	0.00 ± 0.00 b	(0.00) 0.00 ± 0.00 b
5 µM	**0 µM**	0.00 ± 0.00 b	(0.00) 0.00 ± 0.00 b
0 µM	**1 µM**	6.92 ± 2.25 a	(7.80) 64.10 ± 23.60 a
1 µM	**1 µM**	0.00 ± 0.00 b	(0.00) 0.00 ± 0.00 b
5 µM	**1 µM**	0.00 ± 0.00 b	(0.00) 0.00 ± 0.00 b
0 µM	**5 µM**	5.73 ± 1.75 a	(7.10) 53.90 ± 19.10 a
1 µM	**5 µM**	0.00 ± 0.00 b	(0.00) 0.00 ± 0.00 b
5 µM	**5 µM**	0.00 ± 0.00 b	(0.00) 0.00 ± 0.00 b

[z] Within the same column, different letters indicate significant difference at *P* < 0.05 between treatments (n=3). Mean separation by *Fisher's* method. Data within parenthesis are the transformed square root values of the mean.

6.3.2 Experiment Two-Vertical orientation

6.3.2.1 Axillary shoot length and number at node 2

Total axillary shoot length increased by about 15 times with increase in concentration of BAP (Figure 6.8A) but the length of the longest shoot remained similar at 1 and 5 µM BAP (Figure 6.8B). Without BAP, the growth of the axillary shoot was close to zero (Figure 6.8). GR24 at 5 µM appeared to reduce (by about half) the total axillary shoot length and the longest axillary shoot stimulated by BAP (5 µM) (Figure 6.8).

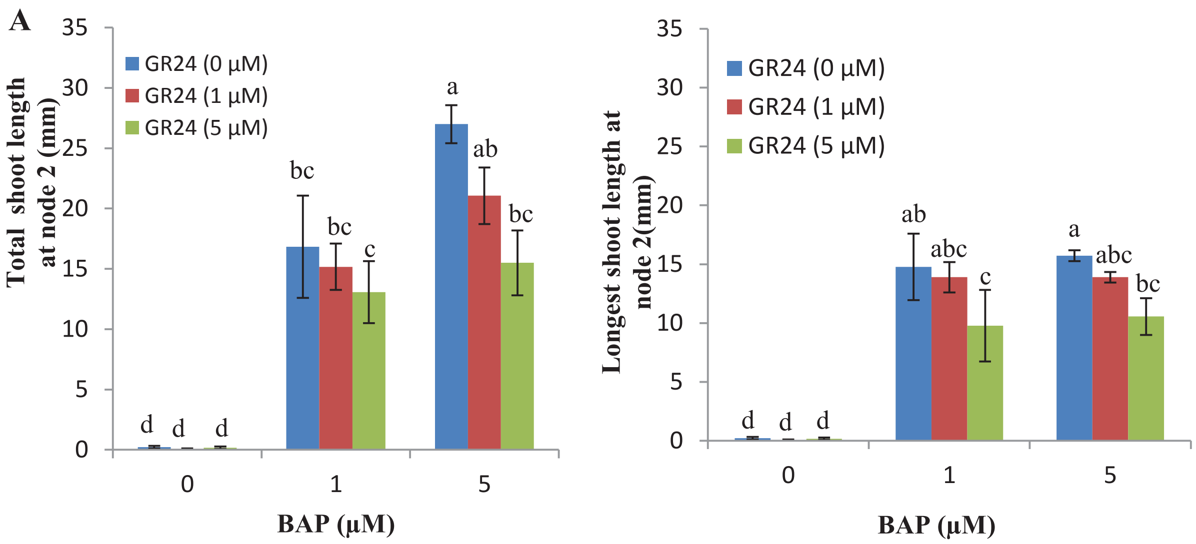


Figure 6.8 Effect of different combinations of GR24 and BAP concentration on (A) average total axillary shoot (maximum 3 shoots) length and, (B) average length of the longest shoot length at node 2 of vertically oriented pea stems of var Ashton. Hormones were applied to excised stems (devoid of cotyledons and node 1) of 14 day-old pea seedlings. Data was collected 12 days after treatment. Nodes were numbered acropetally with basal node named as node 1. In each graph, means with different letters indicate significant difference at *P* < 0.05, through Fisher's mean separation test. Vertical bars indicate standard errors (n=3).

BAP increased number of axillary shoots at node 2 with increase in concentration (Figure 6.9). GR24 (1 µM) reduced the axillary shoot number stimulated by BAP 5µM but with no further reduction at higher concentration of GR24 (Figure 6.9).

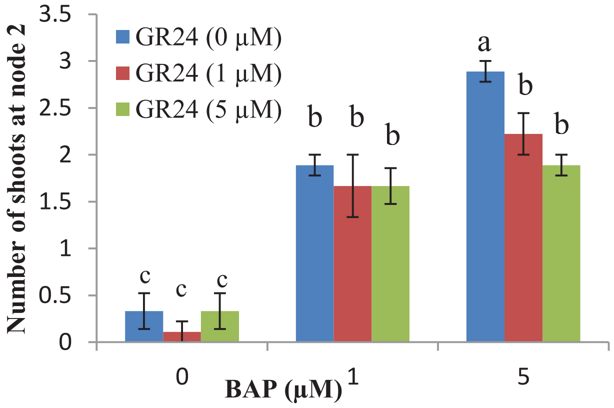


Figure 6.9 Effect of different combinations of GR24 and BAP concentration on the average number of axillary shoots at node 2 of vertically orientated pea stems of var Ashton. Hormones were applied on excised stems (devoid of cotyledons and node 1) of 14 day-old pea seedlings. Data was collected 12 days after treatment. Nodes were numbered acropetally with basal node named as node 1. Means with different letters indicate significant difference at *P* < 0.05, through Fisher's mean separation test. Vertical bars indicate standard errors (n=3).

6.3.2.2 Axillary shoot length and number at node 3

BAP (5 µM) stimulated slight (about one mm) axillary shoot growth at node 3. The lower concentration of GR24 (1 µM) appeared to reduce the length of BAP-stimulated shoots, but the highest concentration of GR24 did not (Figure 6.10). However, both 1 and 5 µM GR24 significantly reduced the axillary shoot number stimulated by 5 µM BAP (Figure 6.11).

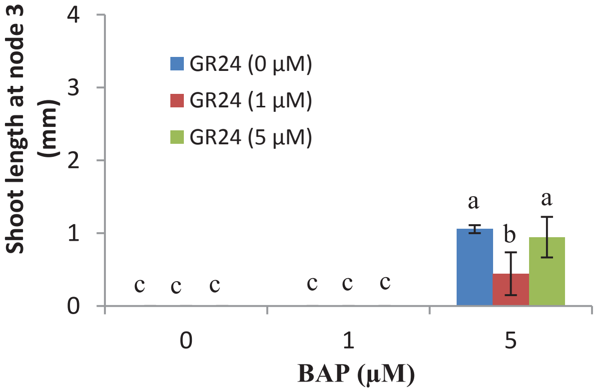


Figure 6.10 Effect of different combinations of GR24 and BAP concentration on average axillary shoot length at node 3 of vertically orientated pea stems var Ashton. Hormones were applied on excised stems (devoid of cotyledones and node 1) of 14 day-old pea seedlings. Data were collected 12 days after treatment. Nodes were named acropetally with basal node named as node 1. For each variable, means with different small letters indicate significant difference at *P* < 0.05 through Fisher's mean separation test. Vertical bars indicate standard errors (n=3).

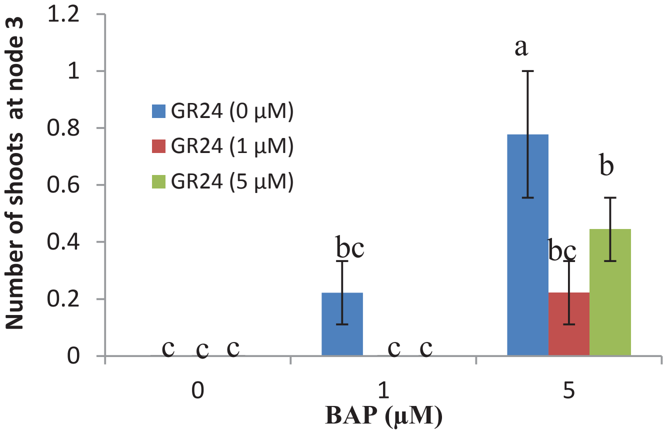


Figure 6.11 Effect of different combinations of GR24 and BAP concentration on average number of axillary shoots at node 3 of vertically orientated pea stems of var Ashton. Hormones were applied on excised stems (devoid of cotyledones and node 1) of 14 day-old pea seedlings. Data were collected 12 days after treatment. Nodes were named acropetally with basal node named as node 1. For each variable, means with different small letters indicate significant difference at $P < 0.05$ through Fisher's mean separation test. Vertical bars indicate standard errors (n=3).

6.3.2.3 Number of nodes on main stem

Application of BAP alone produced a small (about 0.6) but significantly ($P \leq 0.05$),greater average number of nodes per stem as compared to the control but the average number of nodes in the rest of the treatments were fairly similar (ranged from 4.5 to 5) (Figure 6.12).

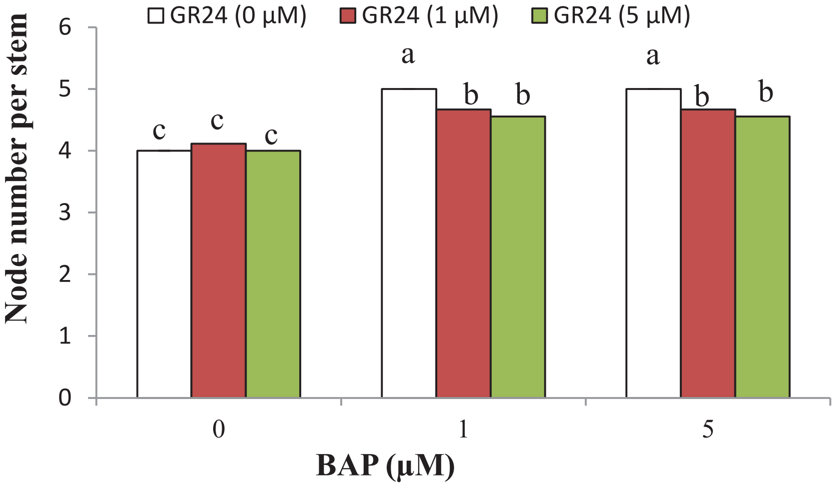


Figure 6.12 Effect of different combinations of GR24 and BAP concentration on the average number of nodes of vertical primary shoots of pea stems var Ashton. Means with different letters indicate significant difference at $P < 0.05$, through Fisher's mean separation test.

6.3.2.4 Root growth

Similar to horizontally laid pea stems, roots did not grow in BAP-treated vertically orientated pea stems (Table 6.2). Pea stems treated with GR24 (1 µM) appeared to produce roots that were about 20 cm longer than within the control, but the effect of GR24 was not statistically significant.

Table 6.2 Effect of different combinations of BAP and GR24 concentration on average number of roots and total length of the roots (±SE) in vertically orientated pea stems of var Ashton

BAP	GR24	Root number	Total root length (mm)
0 µM	**0 µM**	6.44 ±1.44 a[z]	(6.60) 46.20 ±15.20 a[z]
1 µM	**0 µM**	0.00 ±0.00 b	(0.00) 0.00 ±0.00 b
5 µM	**0 µM**	0.00 ±0.00 b	(0.00) 0.00 ±0.00 b
0 µM	**1µM**	6.78 ±0.62 a	(8.00) 65.33 ±9.97 a
1 µM	**1µM**	0.00 ±0.00 b	(0.00) 0.00 ±0.00 b
5 µM	**1µM**	0.00 ±0.00 b	(0.00) 0.00 ±0.00 b
0 µM	**5µM**	6.67 ±0.51 a	(7.40) 55.70 ±11.70 a
1 µM	**5µM**	0.00 ±0.00 b	(0.00) 0.00 ±0.00 b
5 µM	**5µM**	0.00 ±0.00 b	(0.00) 0.00 ±0.00 b

[z] Within the same column, different letters indicate significant difference at $P < 0.05$ between treatments (n=3). Mean separation by *Fisher's* method. Data within parenthesis are the transformed square root values of the mean.

6.3.3 Experiment Three- Application of GA_3

With application of GA_3 along with BAP to vertically orientated pea stems, about 15 times more growth of axillary shoot length occurred at node 3 than for BAP alone (Figure 6.13A). Pea stems that were supplied with only GA_3 or water did not show any bud outgrowth at node 3 (Figure 6.13). At this node only one axillary shoot grew when stimulated.

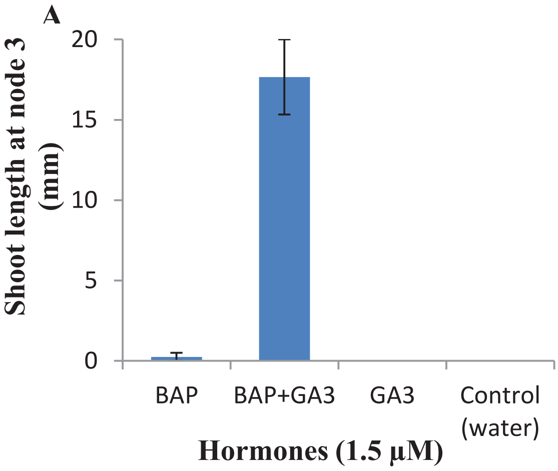


Figure 6.13 Effects of different combination of hormones including control (water) on average axillary shoot (maximum 1 shoot) length at node 3 of vertically orientated pea stems of var Ashton. Hormones were applied on excised stems (devoid of cotyledons, node 1 & 2) of 14 day-old pea seedlings. Data were collected 12 days after treatment. Nodes were named acropetally with basal node named as node 1. Vertical bars indicate standard errors (n=3).

6.4 Discussion

6.4.1 Hormonal influences and their interaction on bud release and subsequent growth

It is well established that CK stimulates bud release, however, in past decades, there has been a controversy about the exact role of CK in bud release (Chapter 1, refer Section 1.4.3.1, Theory A). Studies using branching mutants revealed that the release of buds is associated with reduced SLs (Beveridge, Symons et al., 1997; Umehara et al., 2008) or SL-auxin canalization (Prusinkiewicz et al., 2009). The concept related to SL-auxin canalization was not studied in detail, but the experiments within this chapter have investigated the role of CK and SL as well as the interaction of different hormones in the development of axillary shoots, particularly release and the subsequent growth.

At node 2, BAP increased the number of axillary shoots with an increase in BAP concentration both in horizontal (Figure 6.4) and vertical (Figure 6.8) orientated pea stems. Thus CK supplied through the vascular stream alone or through the bud, vascular stream, and all other parts in contact with the hormone, was able to release axillary buds. There have been several reports, using intact plants of different species (Panigrahi & Audus, 1966; Sachs & Thimann, 1967; Turnbull et al., 1997), in support of BAP-induced bud release. In the current experiments, with both orientations of stems, BAP induced considerable growth (about 10 mm in horizontal and 15 mm in vertical orientation) of the longest shoot, but increased concentration of BAP failed to further increase the length of such shoots (Figure 6.4B & 6.8B). Hence, while an increase in BAP concentration did increase bud release, the subsequent growth of shoots was limited by some other factor, possibly GAs (refer Section 1.2). It is likely that none of the shoots grew longer as sufficient GA was not available in the nodal stem or the buds to enhance the length of all the released buds. Supporting this, GA along with CK was able to enhance the length of all the shoots that grew from or were released in node 2 (data not shown), suggesting GA had a role in subsequent growth of the released buds.

A higher concentration of BAP (5 µM) induced axillary shoot length as well as shoot number at node 3 of horizontally orientated pea stems (Figure 6.5), but the length was about half of that produced at node 2 (Figure 6.4). Failure to increase axillary shoot length at node 3, compared to node 2, might be due to correlative inhibition (Ferguson & Beveridge, 2009; White & Mansfield, 1977). The buds at node 2 might have been released earlier with the presence of BAP, and therefore grew longer; hence the axillary shoot at node 3 was inhibited. In several experiments in pea plants, the buds at the more basal nodes (i.e. node 1 and 2) were removed in order to promote axillary shoot growth at node 3 or above (Beveridge, Murfet et al., 1997; Dun et al., 2012; Ferguson & Beveridge, 2009). The varying responsiveness to applied CK between the buds of node 2 and node 3 is also supported by King & van Staden (1988). They found that the axillary bud at node 2 grew longer than node 3 in response to CK applied directly to the bud, also suggesting decreased sensitivity of the bud at node 3 in response to CK compared to node 2. As suggested by Ferguson & Beveridge (2009), the increased response of buds at node 2 compared to node 3 might be due to a better vascular connection or larger buds than those at node 3. In fact, it is highly likely that the auxin from the apex may have inhibited the access of CK to the bud at node 3 but not at node 2. This may be because node 3 was closer to the apex (Figure 6.2).

With application of 5 µM BAP, the shoot at node 3 grew five times longer in horizontal pea stems (Figure 6.6) than in pea stems with vertical orientation (Figure 6.10). The difference in response to BAP between vertical and horizontal pea stems might also be due to the mode of hormone supply; the buds of horizontally orientated pea stems received BAP from the tissues (e.g. leaves) that were in contact with the hormonal solution in addition to the vascular stream (see Figure 6.3A & B). In contrast, the access to BAP for the buds of vertically orientated stems was only via the vascular stream. The fact that there was negligible or a much less (about 1 mm) mean length of shoot at node 3 in the presence (Figure 6.10) or absence of node 2 (Figure 6.13) through BAP in the vascular stream, is consistent with Dun et al. (2012). In their study, an increased BAP concentration of up to 50 µM did not stimulate axillary shoot length at node 3 in wild-type pea plants, suggesting that a deficiency of CK may not be the only cause of inhibition of subsequent growth. In contrast, there was considerable growth of the shoot at node 3 in SL mutants in response to BAP applied vascularly (Dun et al., 2012; Dun, Hanan, et al., 2009), suggesting the influence of genotypes over bud response to CK. It is possible that in SL mutants, due to high auxin transport (Crawford et al., 2010), the buds grew longer than that in the wild-type. So, it appears that, apart from CK, for subsequent growth, auxin transport out of the bud is important. Auxin transport out of the buds for increased shoot growth may also explain the reason why axillary buds at node 3 of the horizontal stems had elongated shoots compared to the vertical stems. Although the buds received BAP mainly through the vascular stream in horizontally orientated stems (Appendix VIII), the elongated shoots at node 3 might be due to the increased transport of auxin from the released bud. When pea stems were horizontally orientated, possibly due to less auxin transport from the apex (Sanyal & Bangerth, 1998) compared to that in vertical stems, the buds might have easy access to transport its auxin after released in response to BAP.

In the current experiments, interestingly, in vertically orientated pea stems, GA_3 and BAP produced a substantial increase in growth (about 18 mm) of the axillary shoot (Figure 6.13) at node 3, despite GA_3 alone failing to stimulate the bud release. Also, with BAP alone, the mean shoot length was negligible (Figure 6.13) but, because the enlargement of the bud was clearly more visible than those treated with GA_3 alone, the buds might have been released. Hence, it seems clear that gibberellins are involved in growth of the buds that have been released by CK. Supporting the role of CK on bud release and GA for subsequent growth, in *Glycine max* (soyabean) buds at an early stage (7 days old) required only GA as they have already been released by endogenous CK. However, both CK and GA were required for release and subsequent growth at the later stage of 14 days (Ali & Fletcher, 1970). Likewise, in *Zantedeschia* (Subbaraj et al., 2010) and *Jatropha curcas* (Ni et al., 2015), a combined application of CK and GA promoted bud release rather than when the hormones were applied alone, suggesting a synerstic interaction between these two hormones. However, in the paper by Ni et al. (2015), combined CK and GA was effective in young plants (3 weeks) but these two hormones, even though applied alone, significantly increased the bud release in older plants (2 yrs old) of *Jatropa curcas,* suggesting there is a difference in response to hormones with plant age. It is also likely that in perennials, apart from CK, GA may also be important not only in increasing the subsequent growth but also in releasing buds. Supporting increased axillary bud release by GA, in *Actinidia* spp. (kiwifruit), GA was able to produce a greater number of sylleptic shoots (axillary shoots) (Vattiprolu, 2012). Hence, for annual plants like pea and young perennials (e.g. few weeks old), it can be interpreted that CK is essential for bud release, and dependent upon GA for the bud to grow out longer. Since GAs, which are known to increase shoot length through increasing cell division and cell elongation (de Saint Germain et al., 2013; Lee et al., 2012), it is likely that this hormone was involved in increasing the subsequent growth of the released bud at node 3, but not the initial release. Hence, the reduced growth at node 3, even in the presence of applied CK, might be due to a deficiency of GA.

An ability to stimulate axillary shoot growth by CK suggests the importance of CK for bud release whether or not this hormone enhances the subsequent growth. In contrast however, Dun, Hanan et al. (2009) proposed vasculary supplied CK increases the subsequent growth but not the release. The bud at node 3 of rms4 (SL response mutant) responded to the applied CK but not in the wild-type. It was suggested that in the rms4 mutant, the buds were previously released and vascularly applied CK stimulated the further shoot extension. It remains controversial that although the tiny growth (about 4 mm) in the wild-type of pea in response to CK was considered inhibited in the study by

Dun, Hanan et al. et al. (2009), the buds might have actually been released. Later, Dun et al. (2012) suggested that the difference in response of buds to BAP at the same node between the wild-type and SL mutant may be due to the larger buds in the SL mutant compared to that in wild-type. However, more importantly, the varied response to CK might be due to the buds within the SL mutant being more responsive to applied CK as compared to the wild-type buds. Thus, it seems possible that in SL mutants the large buds are in fact released, or partly released. Partial release in SL mutants could be envisaged as a down-regulation of BRC1 and increased sensitivity to CK (refer Section 1.4.3.1., Theory C). However, to confirm that the buds were released or not in the mutants, the buds may be supplied with GA_3 as this hormone strongly stimulates growth of the released but not the inhibited buds (Figure 6.13).

In horizontally orientated pea stems, at node 2, GR24 reduced BAP stimulated axillary shoot length (Figure 6.4) and number (Figure 6.5). GR24 inhibition of CK-stimulated axillary shoot number and length supports the hypothesis that these hormones antagonistically interact with each other on both bud release as well as the subsequent growth. Antagonistic interaction between SL and CK on axillary shoot length is in line with the results of Dun et al. (2012). Since the mean length was measured in their experiment, it was not clear whether or not GR24 has reduced the CK-induced bud release. In fact, GR24 inhibition over the CK-stimulated bud at node 3 of the SL deficient mutant (rms1) suggests that SL inhibits the subsequent growth rather than bud release because buds in SL mutants were actually fully or partially released. In their experiment, with the mutant, seedlings with no hormone treatment (i.e., within control) produced an axillary shoot length of about 1 mm or more whereas in the wild-type, the axillary shoot was not evident. The reduced shoot number by GR24 is consistent with the finding in SL mutants of different plant species such as *Arabidopsis* and rice (Umehara et al., 2008). In SL deficient mutants, max3/max4 of *Arabidopsis* and d17/d10 of *Oryza sativa* (rice), SL reduced the number of branches/tillers suggesting the role of SL on inhibition of bud release. However, as noted for SL mutants of pea (Dun et al., 2012), and perhaps SL mutants of *Arabidopsis* and rice, it is likely that instead of inhibition of bud release, in mutants, SL has actually inhibited the subsequent growth. For example, before SL was applied to two-week-old rice seedlings, it is possible that the buds were previously released as it is difficult to know if a bud is released. Thus, within the SL mutants, the effect of SL might actually have been noted as affecting the subsequent growth of the released buds. However, in the current experiment, using the pea var Ashton, the wild-type seedlings had completely inhibited axillary buds unless stimulated by application of CK (see Section 6.1) or decapitated (Chapter 3). Even if the buds were released in some plants without BAP, for example in horizontal orientated stems, GR24 completely inhibited them (Figure 6.5). Hence, the ability of GR24 to reduce the axillary shoot number in pea plants supports that SL inhibits the release of axillary buds. However, as suggested previously in the preceding paragraph, the release of buds can be determined by assuring that GA is not limited. That means application of SL along with GA_3 may help to know whether or not buds are inhibited.

The interaction between SL and CK is dependent on the regulation of PsBRC1 (Braun et al., 2011), the gene known for reducing bud outgrowth (also refer Chapter 1, Section 1.4.3.1 Theory C). However, it is likely that the expression of PsBRC1 may differ between buds located at different nodal positions in pea plants. For example, buds at node 2 might be expected to have reduced expression of PsBRC1 compared to node 3, leading to an increased response of buds at node 2 to CK application. Consistent with this hypothesis, an increased response to CK application in the SL mutant compared to wild-type may be associated to the down regulation of PsBRC1 in the buds of SL mutants (Braun et al., 2011). However, for the apical bud, since the node number was not affected by SL (Section 6.3.1.3, Figure 6.12), interaction between SL and CK on growth of the primary shoot is less likely. It would seem that BRC1 does not operate in the main stem.

Recently, SL was also found to interact with GA via regulation of BRC1 on bud outgrowth in the woody perennial *Jatropha curcas* (Ni et al., 2015). As with CK, BRC1 was down regulated with GA. Although GA alone stimulated the release of an axillary bud in *J. curcas*, it may be possible that such release is associated with increased sensitivity of buds to endogenous CK due to down-regulation of BRC1. As GA_3 was found to have a strong effect on the subsequent growth but not on the release in pea plants (Figure 6.13), the interaction between SL and GA on subsequent growth is highly likely.

Regarding the effectiveness of GR24 in reversing the effect of direct or indirect supply of CK to the bud at node 2, there was a reduced response to GR24 in vertically orientated pea stems compared to horizontal stems, both in terms of number of buds released (Figure 6.5, 6.8) as well as shoot length (Figure 6.4, 6.7). A higher concentration of GR24 was required to reduce the length and number of axillary shoots stimulated by high concentrations of BAP in vertically orientated pea stems (Figure 6.7, 6.8) in which supply of hormones was entirely vascular (Figure 6.3B). Likewise, the magnitude of reduction by GR24 was less compared to horizontal pea stems. GR24 reduced the shoot length five fold in horizontal stems, but only by half in vertically oriented stems. As for CK, the reduced response to GR24 between vertical and horizontal pea stems might be due to the differences in the mode of hormonal supply. In horizontally oriented pea stems, in addition to the vascular supply of the hormones, GR24 was directly received by the buds, as the buds (at node 2), nodes and internodal tissue were in direct contact with the hormone (Figure 6.3A). The reduced effect on bud growth at node 2 with vascularly supplied SL in both the wild-type and rms mutants is also supported by the results of Boyers et al. (2012) and de Saint Germain et al. (2013). However, GR24 was able to reduce the axillary shoot length at node 3 of SL deficient mutants pea seedling with either the hormone supplied directly to the bud or through the vascular stream (Boyer et al., 2012; Dun et al., 2012; Gomez-Roldan et al., 2008). So, the vascular supply of GR24 was effective at node 3 or above in SL mutants, again suggesting the varying responsiveness to hormones depends on the position of the buds on the main stem.

Buds at node 2 appear to be highly responsive in relation to bud release and/or subsequent growth compared to the buds at upper nodes in pea plants. In some wild-type pea plants, basal buds grow out even when CK is not applied (de Saint Germain et al., 2013). Apart from the responsiveness of the buds in different locations, the alternative explanation relating to the reduced response of buds to GR24 at node 2 might be that the buds at node 2 of pea plants (wild-type or mutants) may contain higher levels of GA than in node 3 or above. Certainly, the growth rate of pea axillary shoots is highly responsive to GA (Figure 6.13). Variation in hormonal balances at different nodal positions within a main stem was also demonstrated in *Lupinus angustifolius*

(Emery et al., 1998). In their study the upper and basal nodes, where bud outgrowth was vigorous, contained a higher ratio of CK/IAA compared to the middle nodes. It was suggested that the lower ratio of CK/IAA was related to reduced bud outgrowth, further emphasising the importance of an increased CK level for bud release. However, within pea plants, apart from the increased CK level, it is possible that the increased shoot growth/bud outgrowth is also related to an increased GA level in the buds; an aspect not specifically investigated in the current series of experiments.

6.4.2 Impact on proposed model of shoot branching

As described in Chapter 1, there has been several arguments about models of shoot branching. From the current experiments, some conclusions can be drawn about the involvement of hormones particularly CK and SL on bud release and the subsequent growth separately. However, further experiments are required to verify the ideas. The ability of SL to reduce CK-induced bud release supports the model based on the direct localized action between SL and CK (see Theory C under the section 1.4.3.1, Chapter 1). Further supporting the direct localized action model, in horizontally orientated stems, although apically derived auxin may have been depleted, SL was able to reduce the CK-stimulated axillary bud release and/or subsequent growth (Figure 6.5). This is consistent with the finding that GR24, particularly at a higher concentration (1 mgL^{-1}), inhibited bud release in decapitated plants (Chapter 3) in which apically derived auxin is greatly reduced (Thimann & Skoog, 1933, Morris et al., 2005). Hence, apically derived auxin is not required for SL to act, which is against the SL-Auxin canalization model that suggests the necessity of apically derived auxin for SL to inhibit bud outgrowth (Crawford et al., 2010, Liang et al., 2010) (also refer Chapter1, Section 1.4.3.2, Theory B).

For subsequent growth, in addition to CK, auxin transport out of the bud may also be important. Buds at node 3 grew longer in the presence of BAP in horizontally oriented pea stems (Figure 6.6), but not when vertically orientated (Figure 6.10), suggesting that the elongated shoots may have been due to increased auxin transport from the released buds as the apically derived auxin became depleted (Sanyal & Bangerth, 1998). However, since in the current experiments the auxin transport from the released bud/shoot was not measured, the increased shoot length at node 3 in horizontal compared to vertical stems being due to increased auxin transport, has yet to be confirmed. In fact, there has been much evidence that auxin transport out of the bud stimulates growth of the shoot (Li & Bangerth, 1999; Morris, 1977). In SL deficient pea seedlings, when NPA was applied near to the buds, bud release was not prevented but the subsequent growth was reduced (Brewer et al. 2009). Hence, auxin transport is required for the subsequent growth but not necessarily, for bud release. However, it is not clear whether auxin transport from the bud after it is released is a consequence or a cause. That is, does more auxin production lead to more growth, or does more growth lead to more auxin production? Hence, the proposed two models may work in an integrated way, depending upon the stage of shoot branching being considered, either bud release or subsequent growth. Since GA was also effective in stimulating the subsequent growth of the released bud (Figure 6.11), it is probable that in addition to the auxin transport hypothesis, interaction of GAs with auxin, CK and SL can be important, at least for the subsequent growth.

6.5 Conclusion

In pea plants, there appears to be an antagonist interaction between SLs and CK for bud release as well as subsequent growth. At least in an annual plant like pea, CK was found to be crucial for bud release and SL can reduce this CK stimulated bud release. For subsequent growth, apart from CK, the role of GAs and possibly auxin transport out of the released buds are also important.

7 General discussion

7.1 Introduction

Discovery of strigolactones (SLs) has aided a fuller understanding of the mechanism controlling shoot branching. Shoot branching involves several stages (Chapter 1, Section 1.2) but within this thesis, the role of SL was explored for two stages, i.e. the release and subsequent growth of the axillary buds. The fact that many previous studies failed to indicate the particular stage of shoot branching while formulating hormonal-based models/mechanisms of shoot branching has made understanding of these mechanisms more complicated. Hence, Chapters 5 and 6 of this thesis have attempted to clarify the hormonal role at these two stages separately. Another aspect of the current study (Chapter 3) was to identify bioactive compound/s that directly inhibit/s axillary bud outgrowth at either the bud release stage or subsequent growth. Such bioactive compound/s was hypothesized as being a SL, or a strigolactone derivative (Boyer et al., 2012). The approach included biological assays in combination with liquid chromatography and mass spectrometry, in order to identify a bioactive inhibitor(s). Within the thesis, an assay based on the germination of a parasitic weed seed was used for detection (Chapter 2, 3, 4) and/or quantification (Chapter 2) of endogenous SLs in a range of genotypes with different degrees of branching.

7.2 Detection of strigolactones and its correlation with branching inhibition

In Chapter 2, different commercially important horticultural species were chosen for SLs identification and quantification using a biological assay based on germination of a parasitic weed seed *Orobanche minor*. Unlike other parasitic weed seeds (*Striga* and some other *Orobanche* species) there is no evidence to suggest that the germination of *O. minor* seeds is stimulated by any compound other than SLs (refer Section 2.1). Hence, the germination assay of *O. minor* seeds, for detection of SLs is highly specific.

Before the experiments within Chapter 2 were started (early 2010), SLs had only been identified in some grass and legume species but not in horticulturally important species, except *Pisum sativum*. So, one of the objectives was to detect SLs in different horticultural species. However, during the course of this thesis, several other studies on different horticultural species like *Solanum lycopersicum* (Vogel et al., 2011), kiwifruit (*Actinidia* Lindl.) (Ledger et al., 2010), and *Chrysanthemum* (Liang et al., 2010) showed SL biosynthesis gene expression, suggesting the existence of SLs in a range of horticultural crops. In line with the hypothesis that SLs are present in horticultural species, SLs were detected in all the species studied (Table 2.1). In fact, the existence of SL in a wide community of the plant kingdom is highly likely. The arbuscular mycorrhizal fungus which requires SLs for association with the host plant is present in 80% of the land plant kingdom (Wang & Qiu, 2006). However, in relation to shoot branching, rather than simply detecting the presence of SLs, the relative concentration of SLs between highly and low branching cultivars within the species was further explored (Chapter 2), so as to identify the importance of SL in shoot branching across a range of horticultural species.

Most studies have used branching mutants while correlating SL and branching inhibition. However, this thesis has studied naturally high or low branching cultivars, plus mutants of *Petunia* (Table 2.1, Chapter 2). Interestingly, in all the species studied, highly branched cultivars contained less SLs compared to the low branched ones (Table 2.2, Figure 2.6, 2.7), thus supporting the hypothesis that SLs inhibit shoot branching in terms of bud release or subsequent growth (see Section 7.4). In *Zantedeschia* and *Acer*, however, the correlation between SL and bud outgrowth inhibition was noted during an early stage of the growth cycle when branches were not visibly evident, but not after the branches were fully developed (Table 2.2, 2.3, also refer Section 2.3.2.3). Hence, as the highly branched cultivars Goldilocks of *Zantedeschia* and Sango Kaku of *Acer* studied within this thesis were not SL mutants; the result is interpreted to suggest that the concentration of SL varies at different stages of the growth cycle (Ledger et al., 2010). The possible explanations related to the correlation between SLs concentration and growth stages have been described in Chapter 2 (refer Section 2.3.2.1). More importantly, the reduced concentration of SLs in highly branched cultivars at the early stage of the annual growth cycle when the process of bud outgrowth begins, suggests that SLs inhibit axillary bud outgrowth (a hypothesis explored more fully in Chapters 5 and 6). Since, cytokinin (CK) was able to increase shoot branching at the early stage of the annual growth cycle of *Zantedeschia* (Subbaraj et al., 2010), it was hypothesized that SL may interact with CK in controlling bud outgrowth, but in an antagonistic way. Supporting an antagonistic interaction between these two hormones (also refer Section 7.5.1.2), SL was able to reduce the CK-stimulated axillary shoot number in *Zantedeschia* grown *in vitro* (Chapter 5, refer Figure 5.9) and pea stems (Chapter 6). In fact, GA_3 also stimulated shoot branching in *Zantedeschia*, but had to be applied along with CK (Subbaraj et al, 2010). It was suggested that CK-induced cell division for bud release while, due to shoot elongation stimulated by gibberellins (GAs), axillary shoots become more visible. Interaction of these hormones, including SLs, on shoot branching is further elaborated in Section 7.4.2.

Correlation between SLs and branching, based on germination assays, supports SL inhibition of branching. However, the magnitude of *O. minor* seed germination depends on the forms of SLs (Kim et al., 2010) with three monohydroxy SLs (e.g. Orobanchol) being the most active form of SL, whereas strigol and solanacol are less active (Xie et al., 2007). For branching inhibition as well, the inhibition varies with different forms of SLs. For example, fabacyl acetate at 100 nmoles reduced the shoot length six times more than by solanacol at the same concentration (Boyer et al., 2012). Hence it is acknowledged that there may not necessarily be a strong association between percentage germination of *O. minor* seed and amount of shoot branching. That is, different forms of SLs may differentially affect germination and branching thus, the lower concentration of SL observed in a highly branched cultivar may be due to the presence of a form of SL that has low stimulatory effect on seed germination. Although more than one form of SL may be present in a single species (Matsuura et al., 2008), there is no evidence for different forms of SLs in different cultivars of a single species. Existence of different forms of SL in different cultivars within a single species or a genus is less likely. In fact, using the technique of LC/MS, both ‘Goldilocks’ and ‘Best Gold’ contained 5-deoxy-strigol but not strigol or orobanchol (Appendix IV), suggesting that the form of SL present in these two cultivars was the same. Hence, the fact that germination of *O. minor* seeds was activated by the same form of SL within a single species, at least in *Zantedeschia*, supports the view that germination percentage is well correlated to branching inhibition.

To verify that the correlation between concentration of SLs and branching inhibition was causal, SL analysis was further carried out using guttation fluid of *Zantedeschia.* It was found that SLs concentration was independent of the volume of the guttation fluid (Figure 4.8, Section 4.3.2.1). The varied volume of guttation arising from different plants under natural conditions (i.e., without imposition of any external pressure) was, in fact, due to the difference in the lag period between transpiration stopping and guttation starting (refer Section 4.3.1). In contrast however, hormonal concentration decreased with the increase in the amount of xylem sap extracted from detached shoots using external pressure (Beck & Wagner, 1994). It is likely that an increase in external pressure may increase the volume of xylem sap, but not the synthesis of hormones within roots. It is also possible that when plants are injured during detachment of shoots for sap extraction, the stress may create an alteration in hormonal balance such as increasing the level of ABA or jasmonic acid (Titarenko et al., 1997). This leads to a hypothesis that the hormonal quantification within samples collected via destructive methods such as shoots being cut off, may not be accurate. Since guttation fluid is as a result of a normal sap flow rate (Section 4.3.1), the process of hormone synthesis in roots and transport to the shoot may not be affected. Hence, the concentration of SLs measured within high branched and low branched cultivars of *Zantedeschia* (Table 2.2, 2.3) are considered to truly reflect the normal concentration within the shoot, at least during the night (dark period when transpiration is suppressed, refer Figure 4.2). Consequently, the difference between the SLs in high and low branched cultivars of *Zantedeschia* was due to a difference in the potential of SLs production between the genotypes rather than the difference in volume of guttation fluid.

7.3 Is strigolactone a branching inhibitor?

Although the bioassay based on germination of *O. minor* is highly sensitive (see Section 2.3.1) in detecting almost all forms of SLs (Yoneyama et al., 2010; Boyer et al., 2012), it is not necessarily specific to branching. It may be possible that a branching inhibitor may be only one form of SL or its derivative. Hence, an assay specific to branching was developed (Chapter 3). Unlike in the paper of Boyers et al. (2012), rather than testing different forms of SL for bud outgrowth, within the thesis it was intended to identify a bioactive compound which may or may not be a SL. Although grafting and genetic approaches have shown a strong association between SLs and branching (Foo et al., 2011, see Section 1.3.1), such studies do not prove SLs are the active branching inhibitor, although it seems likely that a specific SL or closely related compound may be the elusive branch-inhibiting hormone. Interestingly, the branching assay using decapitated pea seedlings was able to detect the LC-fraction of a *Petunia* sample that had inhibition activity. The LC-fraction from a low branched 'V26' *Petunia*, collected between 11-16 minutes, reduced axillary shoot length of pea seedlings (Table 3.1), suggesting the presence of a bioactive inhibitor within this fraction. Moreover, LC/MS analysis showed four unknown compounds in V26 but not in dad3 (a high branched mutant) fraction collected within the same range of time, indicating that the compounds may be the branching inhibitors. Although the chemical identification of these compounds was not successful, each compound contained 'N' as a common element (Figure 3.13), suggesting that the compounds are not SLs (refer structural configuration of different forms of SLs within Figure 1.11), but may be downstream products or precursors. A further attempt was made using the guttation fluid of 'Goldilocks' (high branched) and 'Best Gold' (low branched) *Zantedeschia* cultivars, following the procedure used for *Petunia*. However, no conclusive results were obtained from the samples of *Zantedeschia* in identifying the bioactive compound/s. Due to limitation of time, no further attempt was made, as the entire method (Figure 7.1) probably requires improvements such as the use of concentrated samples, LC/MS based on MRM mode in addition to HRMS, easy and highly sensitive branching assay that requires less volume of samples etc. It is possible that the inhibitor may be highly unstable, so, apart from highly sensitive equipment, careful handling of the samples during analysis is likely to become of upmost importance for future research.

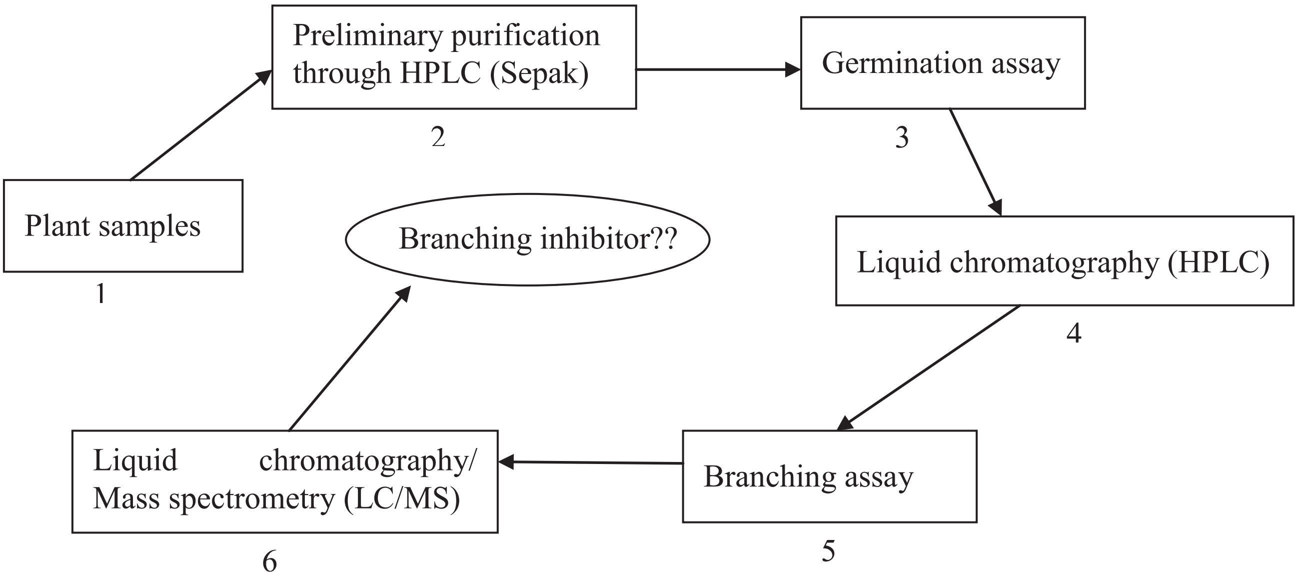


Figure 7.1 Sequential steps utilized while identifying a bioactive compound/s that inhibit/s shoot branching

Despite only partially achieving the main objective of identification of bioactive compound(s), the experiments within Chapter 3 demonstrate a rapid, easy, sensitive branching assay. This assay utilized decapitated pea seedlings that were laid horizontally in a Petri dish filled with treatment solution (Section 3.2.1.2), with axillary bud growth found to respond to GR24 (Figure 3.3; also refer Section 3.3.1). However, the limitation of this branching assay was the requirement of a high volume of solution (Section 3.2.2.4), thus it may not be always practically feasible for some samples. Considering further improvement of the branching assay, a method using vertically orientated decapitated pea seedlings within a test-tube filled with one ml of GR24 solution was attempted. Unfortunately, in contrast to horizontally orientated pea seedlings, GR24 applied basally within a test-tube did not reduce the axillary shoot length of vertically orientated pea seedlings (data not shown). Hence, the method was not investigated further for testing the influence of different HPLC fractions of plant root exudates on branching inhibition. In fact, a reduced response to GR24 between pea seedlings of vertical versus horizontal orientations is consistent with the response of pea stems investigated in experiments within Chapter 6. This might be due to the spatial and temporal changes in the sensitivity of the tissue to respond to hormones (Klee et al., 2004). Further explanation of the reason behind the disparity in effect of GR24 between these two orientations is mentioned in Section 7.5.1.2.

7.4 Hormonal mechanism of axillary bud outgrowth

In Chapter 1, different theories of the shoot branching (bud outgrowth) mechanism based on hormonal influence have been described (Section 1.4), particularly for bud release and/or subsequent growth (refer Section 1.2). There is still a debate between the current two theories 'direct localized action' between CK and SL (refer Theory B under Section 1.4.3.1) and 'SL auxin canalization' (refer Theory B under Section 1.4.3.2). In both theories, the role of SL was mainly based on evidence for the length of axillary shoot (Dun et al., 2012, Crawford et al., 2010); so the role of SL and its interaction with other hormones on actual bud release remains unclear.

7.4.1 Bud release versus subsequent growth

Stimulation of axillary shoot number in response to externally applied CK in *Zantedeschia* grown *in vitro* (Figure 5.8) and un-decapitated pea stems (Figure 6.5, 6.7, 6.9, 6.11), supports the hypothesis that CK directly releases axillary buds. Bud release or bud activation is related to the up-regulation of the cell cycle in the axillary meristems. A role for CK in maintaining meristematic cells of the shoot apical meristem (SAM) via increased cell division (Riou-Khamlichi et al., 1999) is clear, as CK deficiency reduced shoot meristems size and activity (Werner et al., 2003). Hence, it seems likely that CK is involved in bud release. However, a recent model of SL-auxin canalization (Crawford et al., 2010; see Theory B under Section 1.4.3.2) suggests that bud outgrowth is associated with buds being able to transport their own auxin. Since shoot length, as opposed to shoot number, was considered in their experiment that developed the model, evidence for the release of a bud being associated with the canalization is not clear. In decapitated plants, although the level of CK increases within buds or nodes after decapitation (Li et al., 1995), the role of CK on bud release is questioned as the increase in CK level in buds and/or nodes was too slow to cause the initial release after decapitation (Turnbull et al., 1997). Hence a rapid signal that induces the initial trigger of bud release was proposed (Turnbull et al., 1997). Recently, sucrose was considered as a strong candidate to be this rapid signal (Mason et al., 2014). Hence the mechanism of shoot branching involved in decapitated plants may not be the same as in un-decapitated plants. It is possible that SL may interact with sucrose to inhibit the decapitation-induced bud release when applied directly to the bud (Chapter 3, Brewer et al., 2009). To this end, it is proposed that in the future experiments that include blocking the translocation of endogenous sugar level into the bud and measurement of the endogenous SL within two hours after decapitation be performed. This will help in developing our understanding of the interaction between sucrose and SL on decapitation-induced bud release. However, as suggested within Chapter 1 (Section 1.4.4), rather than sucrose, the role of CK inducing the initial trigger of bud release after decapitation is also possible. Since sucrose down-regulates BRC1 within two hours (Mason et al., 2014), buds become sensitive to respond to the CK that is already present in the buds/or nodes (Chen et al., 1985; Foo et al., 2007). In this thesis the role of sucrose was not investigated in decapitated plants, but bud release did not occur in *Zantedeschia* grown *in vitro* with an apex present, despite the presence of 3% sucrose in the medium (refer Section 5.2.1), unless CK was applied, both in highly and low branched cultivars (Figure 5.9). Hence, at least in plants having a shoot apex, it seems more likely that CK rather than sucrose *per se* is vital for bud release.

Although the release induced by CK applied directly to the bud is usually accepted as indicating a primary role for CK in bud release, the release of buds by CK transported from root to shoot is questioned (Beveridge et al., 1997; Dun, Hanan et al., 2009; Section 1.4.3.1, Theory A). Dun, Hanan et al. (2009) suggested that CK supplied through the vascular stream does not release axillary buds, but promotes the subsequent growth (also refer Section 6.4.1). In contrast however, CK released axillary buds both in horizontal and vertically orientated pea stems. In vertical pea stems, in which the CK flow is entirely vascular (Figure 6.3B), buds were released at all the nodes (node 2 and node 3, refer Figure 6.9, 6.13) studied. In horizontal pea stems, although the buds received some CK through buds, the major amount of CK was received through the vascular stream (Appendix VIII). Hence, the release of buds by CK transported from root to shoot is highly supported. The fact that buds are not released in wild-type plants may be due to CK deficiency caused by apically derived auxin (Section 1.4.3.1, Theory A, Chapter 1). In SL-deficient plants, although apically derived auxin is present and also reduced xylem CK (X-CK) (Beveridge, Symons et al., 1997), bud release may be due to the BRC1 gene (a gene regulator that inhibits bud outgrowth, refer Section 1.4.3.1, Theory C) being highly down-regulated (Braun et al., 2011), since reduced expression of BRC1 may increase the sensitivity of the buds to respond to the CK already present in the buds and/or nodes.

The presence of SLs reduced the number of axillary shoots stimulated by CK in pea stems (Figure 6.5, 6.9) and *Zantedeschia* grown *in vitro* (Figure 5.8), supporting the idea that SLs interact antagonistically to inhibit bud release. Likewise, in decapitated pea seedlings (laid horizontally in a Petri dish), GR24 at 1.0 mgL^{-1} reduced the number of axillary shoots observed 10 days after treatment (Figure 3.5), supporting the hypothesis that SL reduced decapitation-induced bud release. Brewer et al. (2009) and Dun et al., (2013) also reported SL-inhibition of axillary bud growth in decapitated pea seedlings. They claimed that SL completely inhibits bud outgrowth, including the release. Since in their experiments the observation was based on the mean length of the bud, the effect of SL on bud release was not clear. Hence, rather than the mean length, a number of bud released should be considered in order to clearly indicate the effect of hormones on bud release. However, supplying GA_3 along with SL may help to assure whether buds are inhibited or not as GA_3 did not stimulate release of bud but strongly enhance the subsequent growth of the released bud in pea stems (Figure 6.13, also refer Section 6.4.1).

The reduced shoot number caused by GR24 application is consistent with the finding in SL mutants of different plant species, such as *Arabidopsis* and rice (Umehara et al., 2008). In SL deficient mutants, max3/max4 of *Arabidopsis* and d17/d10 of *Oryza sativa* (rice), SL reduced the number of branches/tillers. However, in such SL mutants, it is likely that instead of inhibition of bud release, SL has actually inhibited the subsequent growth. For example, before SL was applied to two-week old rice seedlings, it is possible that the buds were previously released. In SL mutants of another herbaceous annual plant such as pea, the mean length of the bud at node 3 of sixteen-day-old seedlings was about 2 mm, whereas the length of buds in wild-type plants was close to zero (Dun et al., 2013), suggesting that the buds of mutant seedlings were fully or partially released. Thus, in SL mutants, the effect of SL might actually have been an effect on subsequent growth of the released buds, assuming the authors had recorded SL effects on subsequent growth rather than bud release. However, in the experiments within the thesis, the pea var Ashton (wild-type) has completely inhibited axillary buds unless stimulated by application of CK (Chapter 6) or decapitation (Chapter 3). Hence, the ability of GR24 to reduce the axillary shoot number in pea plants suggests that SL inhibits the release of axillary buds.

7.4.2 Interaction between hormones on bud release and subsequent growth

SL-inhibition of CK-stimulated axillary shoot number, suggests an antagonistic interaction between SL and CK on bud release, as distinct from subsequent growth. Likewise, in *Zantedeschia* grown *in vivo* (Chapter 2), concentration of SLs was higher in low branched cultivars compared to a high branched cultivar (refer Section 2.3.2.1). In contrast, the highly branched cultivar contained more CK than the low branched cultivar (Appendix IV). Thus the correlation between these two endogenous hormones with shoot branching not only suggests that these two hormones are involved in bud release, but further supports the interaction between these hormones in bud release. High SLs but less CK in the low branched cultivar Best Gold suggests that SLs might have affected the synthesis of CK in *Zantedeschia*. However, unlike auxin, SL probably did not reduce the biosynthesis of CK, as GR24 did not reduce the expression of IPT (see Section 1.2.4.) genes (Dun et al., 2012) suggesting the interaction between these two hormones is less likely to be at the level of biosynthesis. Hence, it is possible that SLs may have affected the metabolism of CK, and like auxin, SL reduced the effect of CK via increasing the metabolism of CK (refer Section 1.4.3.1, Figure 1.7); but this has to be verified. So far, the most convincing explanation is that these two hormones interact via regulation of a 'PsBRC1', a gene analogue to the BRC1 known to inhibit bud outgrowth (Aguilar-Martizez et al., 2007; also refer Section 1.4.3.1 Theory C, Chapter 1). The expression of BRC1 is enhanced by SL but reduced by CK (Braun et al., 2011). Since the gene BRC1 is exclusively expressed in axillary buds, the interaction between SL and CK for bud release may have interacted within the axillary buds. As evident from the recent reports, sucrose is also found to be important in bud release, at least in decapitated buds (Barbier et al., 2015; Mason et al., 2014; Rabot et al., 2012), so it would be interesting in the future to study the interaction between SL and sucrose in bud release (also see Section 7.4.1).

Enhanced shoot length with GA_3 applied together with CK, led to a suggestion that GA_3 promotes the subsequent growth. Supporting the hypothesis that CK is required for the initial trigger of bud release and GA for subsequent growth; CK acts on the apical meristem, up-regulates the cell cycle and maintains the meristematic cells of shoot apical meristems (Müller & Leyser, 2011) (refer Section 7.5.1.1) whereas GA acts on the sub-apical meristem for internode elongation (Talon et al., 1991). The requirement of GA in order for CK to stimulate visible axillary shoot growth was also observed in three-week-old seedlings of *Jatropha curcas*, a woody perennial species (Ni et al., 2015). However, GA_3 alone, but not CK, produced elongated axillary shoots in two-year-old plants of the same species. Consistent with this finding, in kiwifruit, another perennial species, GA_3 promoted a greater number of sylleptic, axillary shoots, whereas BAP did not (Vattiprolu, 2012). It was suggested that possibly the buds may contain sufficient endogenous CK for release, but due to GA deficiency there was little or no subsequent growth resulting in reduced numbers of axillary shoots. However, it is also possible that SLs might have affected the response as other perennials such as kiwifruit and *Acer*, also contain SLs (Chapter 2). As a consequence, in the future it would be useful to carry out experiments investigating interactions between SLs, CKs and GA on bud release and shoot elongation, especially in horticultural species.

During the later stage (or fully matured) of perennial plants, the concentration of SL in the bud may have been less and, as a result, endogenous CK might be sufficient for bud release or activation. It is possible that despite the fact that the bud may have been released, the subsequent growth may have been retarded. Upon GA application, due to shoot extension, shoots were visibly evident. To know whether buds were released or not, examination at the cell level is required because bud activation or release refers to the increased cell cycle activity but may lack cell differentiation and/or growth.

Differences in response to hormones depending on the stage of growth and species also lead to an assumption that the mechanism of shoot branching of perennial species is not completely comparable to annual species such as *Pisum sativum*. The buds of perennial species may be affected by many other factors such as; the environment, endo-dormancy, and plant ontogeny among others, which may not be explained by the similar mechanism existing in annual plants. Apart from auxin, SL, CK and GA, hormones like abscisic acid which has an important role in endo-dormancy of bud (Hocking & Hillman, 1975) may also be involved in regulating shoot branching in perennial plants.

There has been much evidence to show that auxin transport from the buds is required for the growth of the shoot (Li & Bangerth, 1999; Morris, 1977). From the experiments in Chapter 6, it is evident that CK also stimulate the subsequent growth of the bud at node 2 (Figure 6.4, 6.7, also refer Section 6.4). As suggested by Li & Bangerth (2003), for the subsequent growth rather than the release (also refer Section 7.5.1.1.1), the role of CK is indirect. CK appears to enhance auxin transport out of the released buds (Li and Bangerth, 2003) via increased accumulation of PIN1 (auxin transporter or auxin efflux carrier) proteins (Kalousek et al., 2014); so as well as releasing buds via BRC1, CK may have stimulated auxin transport out of the bud resulting in further growth. However, in contrast to the suggestion of Li & Bangerth (2003), Dun et al. (2012) found that even though CK was applied at up to 50 μM to the bud at node 3 of wild-type pea seedlings, there was no substantial growth. Unlike wild-type buds, in SL mutants, CK was able to enhance the shoot length at node 3. A possible explanation for the failure to enhance shoot length in wild-types but not in SL-mutants through the application of CK, might be because of the reduced auxin transport caused by SL, as SL is known to reduce PIN1 accumulation (Crawford et al., 2010; Shinohara et al., 2013). However, as shown in Figure 6.11, the most effective way to stimulate subsequent growth is the application of GA_3 to the pea stems along with BAP, which resulted in a considerably longer shoot at node 3, suggesting that subsequent growth was halted due to GA deficiency. The synergistic interaction between auxin and GA in shoot elongation is well known (Ross et al., 2000) and, therefore, poses the question of interaction of different hormones including SLs on the subsequent growth.

With regard to the role of SL in subsequent growth, following the SL-auxin canalization model (Crawford et al., 2010; Prusinkiewicz et al., 2009), SL acts systematically to diminish polar auxin transport (PAT), ultimately enhancing competition between buds for transporting auxin to a common auxin sink i.e., the main stem. If that is the case for subsequent growth, as described earlier within this section, SL may have reduced the subsequent growth via inhibiting the auxin transport out of the buds. This also supports the fact that SL inhibits the CK-mediated auxin transport out of the bud, which leads to an assumption that SL has no direct interaction with CK for the subsequent growth.

As suggested earlier, since gibberellins appear important for subsequent growth (Figure 6.13A), it is possible that SL may have interacted with GA. However, for stem elongation of the primary shoot of pea plants, there was no interaction between SL and GA (de Saint Germain et al., 2013). In their experiment, rather than inhibition of primary shoot length, SL was found to have promoted internode length via increased cell division. This result was based on internode cell number which was less in SL mutants (rms1) than that in non mutants, whether they were endogenous GA_1 deficient ('le' mutant) or not. In their experiment, SL applied basally via hydroponics also increased the internode length of the primary shoot. In contrast however, externally applied SL inhibited the subsequent growth of the axillary shoot in a dose response manner (Chapter 3, Figure 3.7) suggesting that SL directly inhibits subsequent growth of the axillary buds, which is also supported by several past works (Gomez-roldan et al., 2008; Boyers et al., 2012; Dun et al., 2012). De Saint Germain et al. (2013) attempted to clarify this contradictory result by suggesting that the effect of SL on the cell division varies with the type of the tissue. For example, SLs repress cell division in axillary meristem but promotes cell division in the primary shoot apical meristem. However, the alternative explanation would be that SL represses the axillary shoot length via upregulating PsBRC1 which inhibits bud outgrowth and the gene is mainly expressed in axillary buds (Braun et al., 2011) but within apical bud, SL may act directly to promote the internode length. However, it is surprising that in rms1 (a SL deficient mutant), SL applied to the shoot tip did not increase the internode length whereas GA_1 applied through the same method substantially increased the stem elongation (de Germain et al., 2013). Possibly SLs only prolong cell division in internode at some distance from the apex. For inhibition of subsequent growth of the axillary buds, SL may interact with GA via PsBRC1 as GA_3 was found to downregulate the expression of BRC1 (Ni et al., 2015). In another experiment studying the association between SL and GA on axillary shoot length, higher concentration of SL compared to a lower concentration had a greater inhibition influence on the axillary shoot length in climbing pea cultivar having higher GA_1 level (Luisi et al., (2011). In contrast, a lower concentration of SL was more effective in a dwarf cultivar having lower GA_1 level. It was suggested that the sensitivity of the buds to respond to SL depends on the amount of endogenous gibberellins. However, the alternative explanation would be that in pea having higher GA_1 level, once decapitated, GA_1 might have translocated to the released buds thus reducing the effect of SL. If this is the case, interaction between SL and GA on the subsequent growth is highly supported and requires further investigation. Such an experiment might comprise that involves basal supply of GA_1 and SL in decapitated plants but provided that the decapitated stump is applied with auxin. This would potentially clarify the relationship between SL and GA on the subsequent growth and also whether or not GA_1 is being translocated to the growing axillary buds after apex being removed.

7.5 Strigolactones and the formation of adventitious shoots

Like with axillary shoots, GR24 also reduced CK-stimulated adventitious shoot number in *Zantedeschia* grown *in vitro*, suggesting that these two hormones antagonistically interact with each other in the formation of adventitious buds. There is much evidence to support CK stimulating formation of adventitious buds in different species grown *in vitro* (Von Arnold & Tillberg, 1987; Vyas et al., 2005). However, the role of SL in inhibition of adventitious bud formation is reported here for the first time. In *Gentiana*, ethylene stimulates formation of adventitious buds in a highly branched cultivar but not in low branched cultivar (Keshavarzi et al., 2014), which is in contrast to the effect of CK with *Zantedeschia*. In *Zantedeschia*, adventitious buds were stimulated to emerge/develop in the low branched cultivar but not in highly branched cultivars (Figure 5.11). It is possible that for adventitious bud formation, CK antagonized the effect of endogenous SL in the low branched cultivar, but ethylene did not, suggesting that ethylene may not interact with SL in adventitious bud formation. However, interaction between SL and ethylene in adventitious bud formation would be worth testing within this system in any future research. It will also be interesting to know the interaction between SL and ethylene on release of adventitious buds, as ethylene also stimulates the development or the release of pre-formed adventitious bud in gentians

(Marzieh Keshavarzi, PhD thesis, Massey University, unpublished result). In the experiment within Chapter 5, the effect of SL on the release of adventitious buds is not clear (Figure 5.11) but, if SL inhibits adventitious bud growth at an early microscopic stage of development, as suggested in Chapter 5 (refer Section 5.4.2), the canalization theory of shoot branching would not have been possible.

7.6 Proposed model of axillary bud outgrowth

A proposed model (refer Figure 7.2) of shoot branching control by plant hormones has been developed by integrating the findings presented within this thesis and past works. In line with the thinking developed within this thesis, the hormonal interaction for bud release and subsequent growth are described separately.

7.6.1 Bud release

Apically derived auxin indirectly inhibits bud release by enhancing the level of SL and reducing CK level (Figure 7.2) within a plant. These two hormones interact with each other via regulation of BRC1 (a transcription factor), a gene that inhibits the bud release. SL up-regulates BRC1 whereas CK down-regulates the gene. The relative concentration of SL and CK in the axillary bud effects the expression of the gene. If the gene is down-regulated, the bud releases. However, if SL affects the CK level directly, the interaction between these two hormones will be independent of BRC1 as well.

7.6.2 Subsequent growth

Once released, if a bud becomes able to transport its auxin to the main stem, the bud grows longer (Figure 7.2). Increased auxin transport may support the further growth of the bud via translocation of nutrients towards the bud (hypothesis not tested within this thesis). In the situation where if a released bud has less access to transport of its own auxin, but possesses adequate GA, the bud grows longer because GA stimulates shoot extension. The effect of CK on subsequent growth via increasing the auxin transport out of the bud is more likely than the direct effect of CK on the subsequent growth. SL inhibits the subsequent growth by reducing the auxin transport out of the bud, which is the most likely explanation. In addition, SL inhibition on subsequent growth via interacting antagonistically with GA is possible.

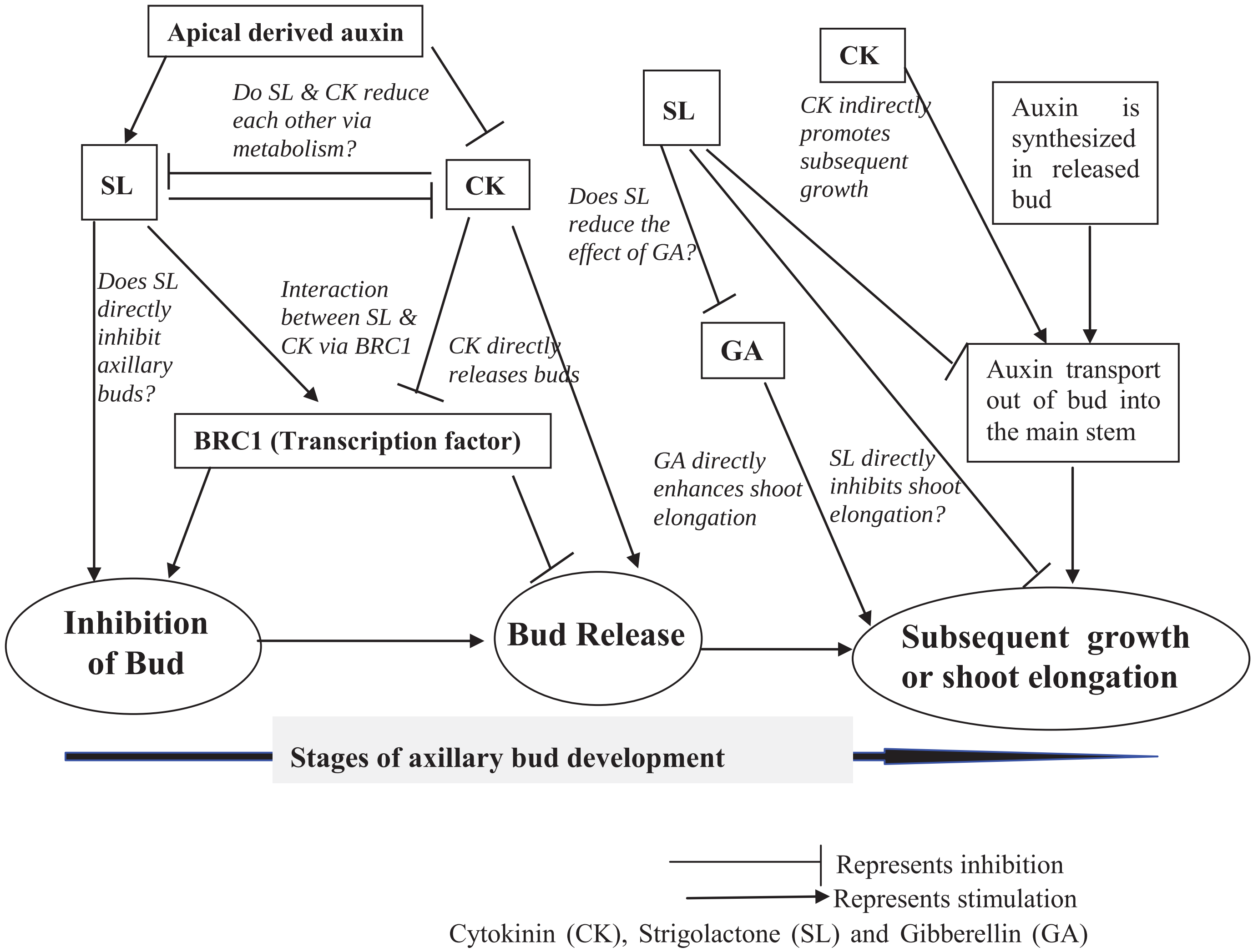


Figure 7.2 A proposed model of hormonal interaction in axillary bud release and subsequent growth of a shoot of an annual plant or a shoot produced from the bud of current season (or syllepic shoot). The model involves the hormonal interaction for two stages of shoot branching i.e., bud release and subsequent growth. For bud release, SL and CK interact with each other via BRC1 (transcription factor). Apically derived auxin indirectly inhibits bud release by increasing SL or reducing CK level. For subsequent growth, multiple pathways of hormonal interaction occur. SL inhibits the subsequent growth either directly or indirectly by reducing the transport of auxin from the released buds. SL may also inhibit the subsequent growth by reducing the effect of GA.

7.7 Implication of this research and recommendations for future work

The main horticultural implication of this study would be in the breeding of different horticultural crops such as apple and kiwifruit. The concentration of SLs could be used as a marker for early selection of plants that exhibit desirable branching phenotypes, as SLs are an important factor affecting shoot branching (Chapter 2, 5, 6). Also marker assisted selection can reduce some of the limitation of the traditional population breeding and selection method based on phenotypic traits (Collard & Mackill, 2008). Likewise, in different ornamental plants such as *Zantedeschia,* the crop value increases with increase number of flower bearing branches. In such cases, breeding would involve selection of high branched genotypes. As the germination bioassay using *O. minor* seeds enabled quantification of the relative concentration of SLs in different genotypes within a range of horticultural species (Chapter 2), this technique can be employed for the preliminary selection of genotypes based on the concentration of SLs. Methods such as liquid chromatography and mass spectrometry may not be practically feasible for screening on such a large scale as normally encountered within breeding programmes.

Although the application of SL in open field is not possible due to expense, SL could be used for generating plants with reduced branching within an *in vitro* system. In contrast, SL inhibitors, if developed, could be used for plants that require high branching.

- As the issue related to the identification of a bioactive compound was not fully resolved in this study, future investigations are required to identify which compound(s) have branching activity using an improved specific branching assay together with LC/MS. Since the bioactive compound(s) would probably be a SL or its derivatives, in addition to the branching assay, germination assays based on *O. minor* seed could be usefully employed during the identification process for separating the LC fractions that contains SLs.
- Within the thesis, particularly in *Zantedeschia* and *Acer*, SL content was correlated with shoot branching inhibition, at a stage before the branches were fully developed but not after the branches had appeared (Chapter 2). It is likely

that a particular form of SL produced at this stage is directly related to the branching, but not the SLs produced at other stages. Hence identification of endogenous SLs at different stages of the growth cycle of *Zantedeschia* is highly recommended.

- As GA_3 enhances the subsequent growth, it is possible that SL may interact with GA in subsequent growth. Using *Zantedeschia* or apple for example, knowing the interaction between SL and GA may add valuable insight to fully understand the mechanism on subsequent growth in horticulturally important plant species.
- In support of inhibition of bud release, GR24 appeared to reduce the number of axillary shoots in both decapitated and CK-induced plants. However, following the GR24 application, application of GA_3 may help to confirm whether buds are inhibited or not; as GA_3 did not release buds but stimulated the subsequent growth in pea stems.
- Although sucrose was not found to have a primary role in releasing buds of intact *Zantedeschia* plants grown *in vitro* (Chapter 5), it can be the cause of an initial trigger of bud release in decapitated plants (Mason et al., 2014). Since SL-inhibited bud release is evident in decapitated plants (Chapter 3), the interaction between sucrose and SL on bud release should be studied in the future by blocking the translocation of endogenous sugar level into the bud and measurement of the endogenous SL within two hours after decapitation.
- Knowing the effect of SL and CK on the metabolism of each other might be a useful prospect for future study since these two hormones antagonistically interact with each other on bud outgrowth.
- The effect of SL on adventitious bud formation and development (or release) needs to be further studied in naturally high branching cultivars of *Zantedeschia* or other species such as *Gentians* spp.
- It is true that quantification of SL based on the germination assay is less accurate than the LC/MS method, but the germination method is efficient for preliminary comparison of a large range of genotypes for the purpose of screening of relative content of SLs.

- Since guttation fluid is highly reliable in deriving a true representation of endogenous concentration of plant hormone(s) (Chapter 4, Figure 4.11), collection of guttation fluid (where possible) should be used for hormonal analysis.
- Study of SL within *in vitro* system is likely to be more cost effective as well as practically feasible, as compared to open field studies. Hence, research related to SL and its interaction with other hormones on comparative branching of commercially important cultivars, such as those of kiwifruit, within an *in vitro* system is highly recommended.
- In many previous reports on the hormonal role in shoot branching, the length of the shoot or the bud is taken as a measure of bud outgrowth, which does not clearly distinguish whether the effect of hormone(s) is on bud release or the subsequent growth. To address this issue, as considered within Chapter 3, the number of buds released or the % bud released may be useful as a measure of bud release rather than the mean shoot length.

Appendices

Appendix I. Effect of storage during preconditioning and age of seeds on germination in response to different concentration of GR24

Introduction

The optimum preconditioning period varies in parasitic weed populations of different or the same species (Logan & Stewart, 1992). Hence, germination assays using different populations of *Orobanche* seeds, either newly harvested or stored for some duration, were performed to obtain the optimum duration of preconditioning. Determination of the optimum preconditioning period for each population of *O. minor* seeds enabled the most sensitive seeds used for detecting minute levels of endogenous SLs throughout the thesis.

Materials and methods

New (stored for 15 days after harvest) and old (stored for 10 months) seeds of *Orobanche minor* were surfaced sterilized and preconditioned as described in Section 2.2.1. Both new and old seeds were stored in a dark for either 7 or 14 days at 21 °C before treatment with GR24. The preconditioned seeds were then treated with different concentrations of GR24 solutions as described in Section 2.2.2.1. Germinated seeds were noted as described in Section 2.2.2.2.

Results and discussion

There was a variation in dose response between the old seeds (10 months storage) and new seeds (½ month storage) for the same preconditioning duration (Figure 1A & C). A short preconditioning duration of seven days was effective for old seeds to induce sensitivity to GR24 at a low concentration (0.0001 mg L^{-1}) (Figure 1A), whereas new seeds had low sensitivity as they required a higher concentration of GR24 (0.1 mg L^{-1})

to initiate germination (Figure 1C). According to Chae et al. (2004), at the same concentration of germination stimulant, the maximum germination percentage was greater for seeds stored for 3 months as compared to seeds stored for 15 months using *O. minor* seeds preconditioned for 7 days, suggesting age of seeds when stored tend to reduce sensitivity for germination. In the current experiment, the fact that seeds stored for only 15 days did not produce more germinated seeds as compared to those stored for 10 months, might be due to the low amount of plant hormones like gibberellins in new seeds, since gibberellins are known to be effective in promoting germination of seeds of *Orobanche* by shortening the preconditioning duration (Chae et al., 2004). Supporting this hypothesis, with the extended period of preconditioning, after 14 days of preconditioning new seeds germinated at a lower concentration of GR24 (0.0001 mg L^{-1}) (Figure 1D).

With an extended period of preconditioning of 14 days, more than 50% of old seeds germinated without GR24, which was not observed in previous experiments (Chae et al., 2004; Matusova et al., 2004; Wigchert et al., 1999). This could have been due to a long duration of storage and/or the preconditioning period, and shows that the seeds lost their dependence on SLs and became insensitive to germination stimulants.

The new seeds which did not germinate at low SL concentration when preconditioned for seven days, showed some germination at the lowest concentration of GR24 (0.0001 mg L^{-1}) after preconditioning for 14 days, which is in line with that reported by Matusova et al. (2004). They also found a prolonged preconditioning period increased sensitivity of seeds to germinate at lower concentrations of SL; though no mention was made about the age of the seeds. Hence, it is evident from the results that new seeds require a longer preconditioning duration as compared to the older seeds, in order to induce sensitivity to germinate at lower concentration of applied SL.

Generally, the germination percentage of seeds was reduced by concentration of GR24 above 1.0 mg L^{-1} (Figure 1), though the supra-optimal concentration was different in each germination assay. The fact that a higher concentration inhibits parasitic seed germination has been found by several other authors (Wigchert et al., 1999; Malik et al., 2010; Matusova et al., 2004; Joel et al., 1995). The supra-optimal concentration differed in different experiments, which is consistent with the current results, and could be due to different storage periods, and to different species being used.

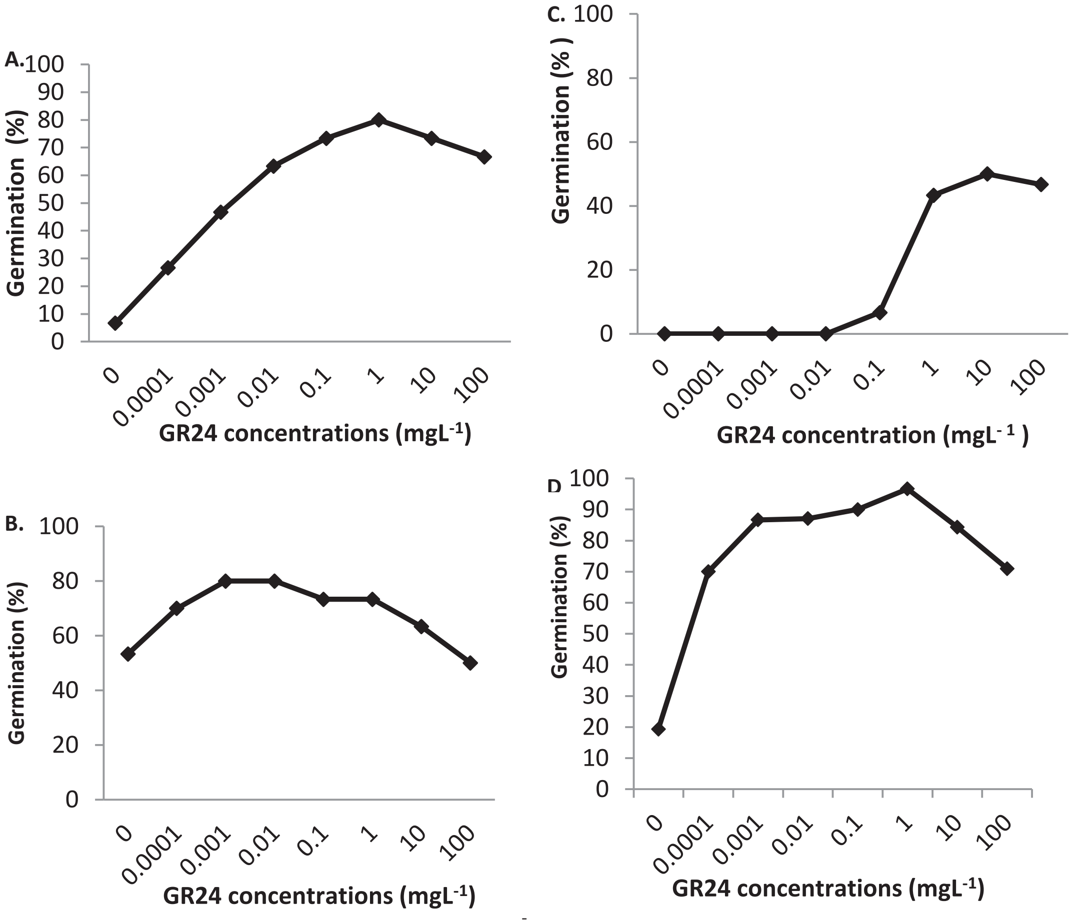


Figure 1. Germination percentage of seeds of *Orobanche minor* treated with different concentrations of GR24. "Old" *Orobanche minor* seeds (December, 2009 harvest, stored for 10 months) preconditioned at: (A) 21 °C for 7 days, (B) 21 °C for 14 days; and "New" seeds (December, 2010 harvest, stored for 15 days) preconditioned at: (C) 21 °C for 7 days and, (D) 21 °C for 14 days.

Appendix II: Composition within medium and fertilizer used for growing *Zantedeschia*

Composition of structural components (in % volume) of the bark-based medium used for growing *Zantedeschia*.

1. C.A.N-Fines A grade (50%)
2. Fibre (30%)
3. Pacific pumice-7mm (20%)

A. Composition of mineral elements (%) within each fertilizer ingredient added to growing medium. Actual weights per unit volume of medium added noted within relevant materials and methods section.

1. Dolomite: Mg (11.5%), Ca (24%)
2. 8-9 month Osmocote: N (16%), P (3.5%), K (10%), S (2.4%), Mg (1.2 %), B (0.02%), Cu (0.05%), Fe (0.4%), Mn (0.06%), Mo (0.02%)
3. 3-4 month Osmocote: N (15%), P (4.8%), K (10.8%), S (3%), Mg (1.2%), B (0.02%), Cu (0.05%), Fe (0.4%), Mn (0.06%), Mo (0.02%), Zn (0.015%)

Appendix III: A dose response curve of GR24 on germination of seeds of *O. minor* for use while quantifying endogenous SLs in different cultivars of *Zantedeschia*

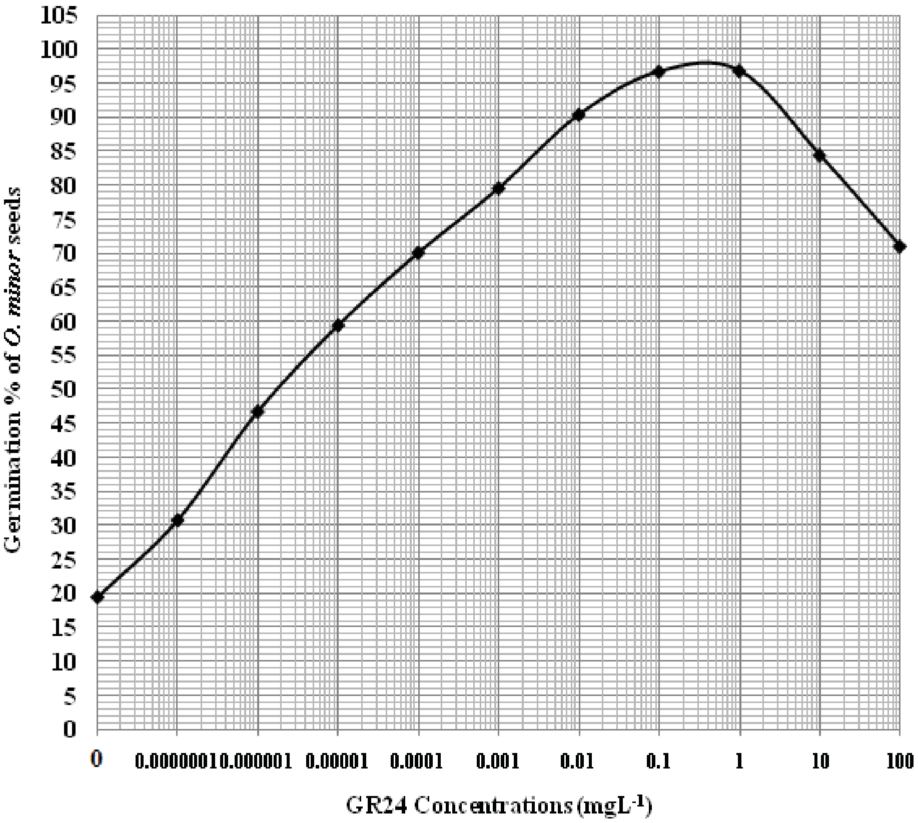


Figure 2. A dose response curve for germination of *O. minor* seeds at different concentrations of GR24 (a synthetic strigolactone)

Appendix IV. LC/MS analysis of cytokinins and strigolactones within the samples of high and low branched cultivars of *Zantedeschia* and *Acer*.

Janine Cooneyand her team at Plant & Food Research Ltd., Ruakura are acknowledged for conducting the analysis of the samples using LC/MS**.**

Sample collection

Samples from *Zantedeschia* and *Acer* were collected as described in Chapter 2 (Section 2.2.3.2). Shoot xylem sap of *Acer* was filtered using Phenex filters (0.2 µm) as described in Section 2.2.3.2.3. The samples of cultivars from both genera were collected when the branches had not visibly developed during the Year 2013. Unfortunately, with these samples, analysis of the strigolactone was not successful. So a second attempt was made for SL analysis, but using the samples collected within the plants that had already developed branches.

Sample Clean-up

Guttation fluid of *Zantedeschia* cultivars Goldilocks and Best Gold (stored at -80 ^{o}C) and shoot xylem sap of *Acer* cultivars Sango Kaku and Red Emperor were thawed and then spiked with 10 ng of a labelled internal standard mix ($[^2H_5]$ t-Z, $[^2H_5]$ t-Z9G, $[^2H_6]$ 2iP, $[^2H_6]$ iPR, $[^2H_5]$ t-ZR; OlchemIm Ltd, Olomouc, Czech Republic). Samples were acidified with formic acid to be equivalent to 1 M formic acid and vortexed for 1 min prior to column clean-up on a mixed mode, reverse-phase, cation-exchange cartridge (Oasis MCX 60mg/3mL; Waters, ON, Canada). Cartridges were activated using 3 mL acetonitrile and equilibrated using 3 mL 1 M formic acid. After equilibration, the sample was loaded and washed with 3 mL of 1 M formic acid followed by 3 mL of water. The cartridge was then washed with 2 mL of water then 4 mL of 0.35 M ammonium hydroxide. Cytokinins were eluted with 3 mL of 0.35 M ammonium hydroxide in 60 % acetonitrile. Cartridges were activated with 3 mL acetonitrile and equilibrated using 3 mL of 25 mM ammonium bicarbonate (pH 6.4 adjusted with formic acid). The elution was evaporated to dryness using a CentriVap concentrator (Labcon) and stored at -20°C until analysis. Prior to mass spectrometric analysis, the eluted cytokinins were reconstituted with 200 µL of 10 % methanol/water plus 1 % acetic acid.

LC-MS Analysis

LC-MS/MS experiments were performed on a 5500 QTrap triple quadrupole/linear ion trap (QqLIT) mass spectrometer equipped with a TurboIon-SprayTM interface (AB Sciex, ON, Canada) coupled to an Ultimate 3000 UHPLC (Dionex, CA, USA).

Cytokinins were separated on a Poroshell 120 SB-C18 2.7 µm 2.1 x 150 mm ID column (Agilent Technologies, CA, USA) maintained at 80°C. Solvents were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid and the flow rate was 600 μL^{-1}. The initial mobile phase, 0% B than ramped linearly to 3% B at 9 min, then to 10% B at 15 min and 100% B at 22 min. The column was flushed at 100% B for 1 min before resetting to the original conditions. Injection size was 10 µL. MS data was acquired in the positive mode using a scheduled multiple reaction monitoring (MRM) method. The operating parameters were as follows: ionspray voltage, 4500 V; temperature, 600 °C; curtain gas, 45 psi; ion source gas 1, 60 psi; ion source gas 2, 60 psi.

Strigolactones were separated on a Poroshell 120 SB-C18 2.7 µm 2.1 x 150 mm ID column (Agilent Technologies, CA, USA) maintained at 60°C. Solvents were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid and the flow rate was 400 μL^{-1}. The initial mobile phase, 25% B which was held isocratic for 5 min before being ramped linearly to 35% B at 8 min, then to 50% B at 11.0 min and 100% B at 12 min. The column was flushed at 100% B for 2 min before resetting to the original conditions. Injection size was 5 µL. MS data was acquired in the positive mode using a MRM method. Other operating parameters were as follows: dwell time, 20 ms; ionspray voltage, 3500 V; temperature, 700 °C; curtain gas, 25 psi; ion source gas 1, 50 psi; ion source gas 2, 60 psi.

Results

Cytokinins

Among the cytokinins analysed, t-Z and t-ZR were abundant in *Zantedeschia* particularly in the highly branched cultivar. In addition to these two cytokinins, *Acer* contained considerably high concentration of iPR, DHZR and c-ZR. Interestingly, in both *Zantedeschia* and *Acer*, highly branched cultivars contained a higher concentration of cytokinin compared to the low branched cultivars (Table 1).

Table 1. Concentration of cytokinin (ng/ml) in high and low branched cultivars of *Zantedeschia and Acer.*

Genus	**Cultivars**	**Different forms of cytokinins ***							
		2iP	**t-Z**	**c-Z**	**iPR**	**t-ZR**	**DHZR**	**iP9G**	**c-ZR**
Zantedeschia**	Goldilocks (highly branched	1	164	3	6	568	nd	nd	7
	Best Gold (low branched)	nd	2	nd	0.1	9	nd	nd	nd
Acer* **	Sango Kako (highly branched)	7	122	2	353	3774	96	1	73
	Red Emperor (low branched)	2	55	1	48	583	58	2	20

*DHZ, T-Z9G, t-ZOG, iPRMP, t-ZRMP, t-ZROG and DHZROG were not detected
**guttation fluid of *Zantedeschia* collected during the leaf emergence stage.
***shoot xylem sap of *Acer* collected at the stage of bud release in early spring i.e., August.

Abbreviations

t-Z, trans zeatin; c-Z, cis zeatin; t-ZR, trans zeatin riboside; DHZR, dihydrozeatin riboside; 2iP, N^6-isopentenyladenine; iPR, N^6-isopentenyladenosine; iP9G, N^6-isopentenyladenine-9-glucoside; DHZ, dihydrozeatin; DHZROG, dihydrozeatin-O-glucoside riboside; t-ZOG, trans zeatin-O-glucoside; nd, non-detected.

Strigolactones

Out of three strigolactones tested, only one form of strigolactone i.e., 5-deoxy-strigol was detected in both cultivars of *Zantedeschia*. Similar level of strigolactones was found in both highly branched and low branched cultivars at the stage when the branches had already appeared which is consistent to the results obtained from the germination assay of Year 2 samples (Chapter 2, refer Table 2.3).

Table 2. Concentration of strigolactones (pg/ml) and germination % of *O. minor* seeds within the guttation fluid of high and low branched cultivars of *Zantedeschia*

Cultivar	5-deoxy-strigol	Strigol	Orobanchol	**Germination %
Best Gold	31	nd*	nd	91.29 ±4.18
Goldilocks	42	nd	nd	81.65 ±5.36

*'nd' means non-detected

** Germination % of *O. minor* seeds within controls, GR24 (89.05±0.8) and water (0 ±0)

Appendix V. Correlation between volume of guttation fluid and number of days stressed

In Chapter 4, it was intended to vary the volume of guttation fluid of *Zantedeschia* plants to investigate whether or not the concentration of strigolactones altered with the volume of guttation exudation. Before the main experiments were commenced, a trial experiment was conducted to identify the number of days to withhold water in order to create an alteration in the volume of guttation fluid. As expected, with the increase in days that water was withheld before guttation fluid was collected, the volume of guttation fluid decreased. The volume of the guttation fluid was the least at four days (the maximum days of water withheld tested) of water withheld and was about 3 times less compared to that with zero days of water withheld (Figure 3).

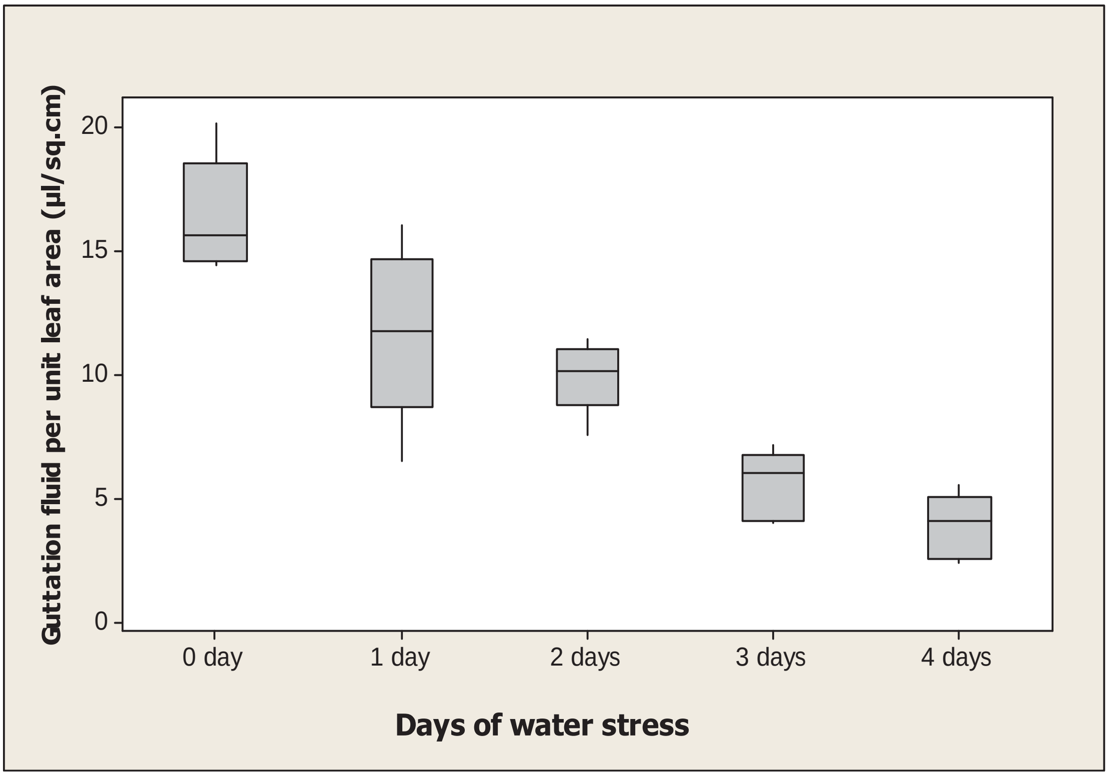


Figure 3. Volume of guttation fluid per unit leaf area (µl/sq.cm) collected from plants of *Zantedeschia* cultivar Goldilocks subjected to different durations of water withheld (withholding irrigation water) before collection of guttation fluid commenced.

Appendix VI. Effect of externally applied cytokinin on axillary or adventitious shoots number of low branched *Zantedeschia* cultivar Best Gold grown *in vitro*

Within Experiment Two of Chapter 5, *Zantedeschia* 'Best Gold' grown *in vitro* produced axillary or adventitious shoots even with no BAP present (Figure 5.5, 5.6). For this experiment, the stem cuttings were derived from the plants grown in the media that contained 0.3 mgL^{-1} of BAP (refer Section 5.2.3.1). It was suggested that such axillary or adventitious shoots were produced due to the carryover effect of previously applied BAP. Hence, to test this hypothesis and avoid the carryover effect of BAP, some stem cuttings derived from the plants grown in the media without BAP were further tested with or without BAP. Interestingly, no axillary or adventitious shoots developed in the absence of externally applied cytokinin. Thus, within an *in vitro* system, exogenous application of cytokinin is necessary for multiple branching (either axillary or adventitious) of the low branched cultivar Best Gold.

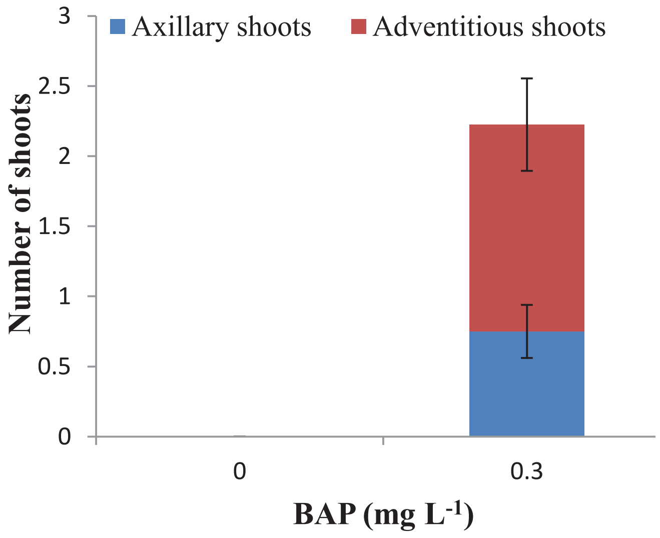


Figure 4. Effect of BAP on the number of axillary or adventitious shoots per plant of the low branched cultivar of *Zantedeschia,* Best Gold, grown *in vitro*. Stem cuttings used for this experiment were derived from the plants that were previously grown in the media (*in vitro*) containing no BAP.

Appendix VII Dose response of un-decapitated pea stems to BAP

Before conducting experiments on the hormonal interaction in shoot branching of pea plants (Chapter 6), the optimum concentration of BAP to be tested was obtained by applying five concentrations (0, 1, 2, 5 and 10 µM) of BAP to un-decapitated pea stems orientated vertically (refer Figure 6.3B). Within this experiment, the response of axillary shoot growth at node 1 and node 2 was considered. Since BAP concentration at 10 µM was toxic,as indicated by death of pea stems about 3-5 days after treatment (before the completion of final observation i.e., 10 days after hormone treatment), a response to 10 µM BAP on axillary shoot growth was not obtained.

Lower concentrations, i.e. 1 and 2 µM BAP enhanced shoot length and shoot number at node 1 only, whereas the highest concentration 5 µM stimulated the length and the number at both node 1 and node 2 (Figure 5). With 5 µM BAP, although shoot number was increased, total shoot length decreased.

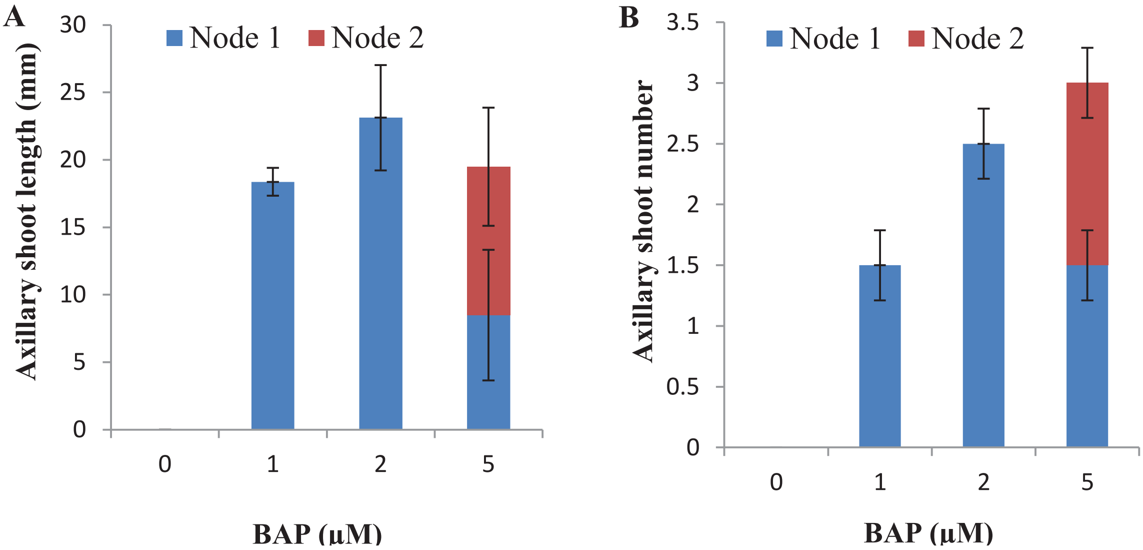


Figure 5. Response of 14 day-old un-decapitated pea stems (devoid of cotyledons) to different concentration of BAP on; average axillary shoot length (A) and number (B), at node 1 and node 2.

Appendix VIII Axillary shoot growth of intact pea stems laid horizontally in a Petri dish in response to BAP with or without lanolin paste at stem base.

Within Experiment One of Chapter 6, it was intended that the axillary bud of horizontally orientated (refer Figure 6.2A) un-decapitated pea stems received hormones directly. To test the magnitude of hormone delivery to the bud directly, the supply of BAP through the vascular stream was either blocked via sealing the cut end with lanolin paste or remained unblocked (i.e. untreated control). Interestingly, in response to BAP, the axillary shoot grew about 5 times less in the stems having no vascular supply (cut end sealed with lanolin) of BAP compared to the stems having vascular supply (cut end unsealed) of BAP (Figure 6). Hence, the ability of axillary shoots to grow longer in the stems with no blockage of BAP at vascular stream suggests that rather than directly, buds within the horizontally laid pea stems received the major amount of the hormone through the vascular stream.

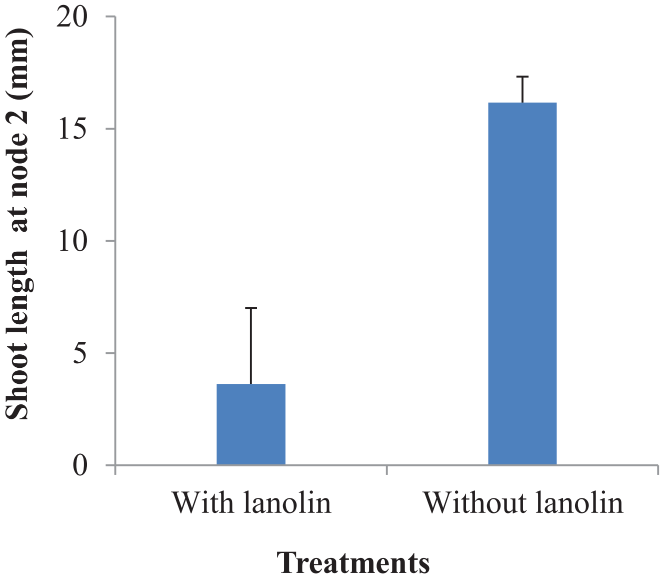


Figure 6. Axillary shoot length in response to BAP (1µM) at node 2 of horizontally orientated pea stems with the cut base either sealed with lanolin or left unsealed. Stems sealed with lanolin at the cut base blocked the vascular supply of hormone to the bud at node 2, whereas the unsealed stems did not. Pea stems were laid horizontally in a Petri dish filled with 20 ml of hormonal solution.

References

Aasim, M., Khawar, K. M., & Özcan, S. (2009). In vitro micropropagation from plumular apices of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Scientia Horticulturae, 122*(3), 468-471.

Aceña, J., Stampachiacchiere, S., Pérez, S., & Barceló, D. (2015). Advances in liquid chromatography–high-resolution mass spectrometry for quantitative and qualitative environmental analysis. *Analytical and bioanalytical chemistry, 407*(21), 6289-6299.

Aguilar-Martínez, J. A., Poza-Carrión, C., & Cubas, P. (2007). *Arabidopsis* BRANCHED1 acts as an integrator of branching signals within axillary Buds. *The Plant Cell, 19*(2), 458-472. doi: 10.1105/tpc.106.048934

Akiyama, K., & Hayasi, H. (2006). Strigolactones: chemical signals for fungal symbionts and parasitic weeds in plant roots. *Annals of Botany, 97*(6), 925-931. doi: 10.1093/aob/mcl063

Alder, A., Jamil, M., Marzorati, M., Bruno, M., Vermathen, M., Bigler, P., . . . Al-Babili, S. (2012). The path from β-carotene to carlactone, a strigolactone-like plant hormone. *Science, 335*(6074), 1348-1351. doi: 10.1126/science.1218094

Ali, A., & Fletcher, R. A. (1970). Hormonal regulation of apical dominance in soybeans. *Canadian Journal of Botany, 48*(11), 1989-1994. doi: 10.1139/b70-290

Aloni, R., Schwalm, K., Langhans, M., & Ullrich, C. I. (2003). Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in Arabidopsis. *Planta, 216*(5), 841-853.

Arite, T., Umehara, M., Ishikawa, S., Hanada, A., Maekawa, M., Yamaguchi, S., & Kyozuka, J. (2009). d14, a strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. *Plant and Cell Physiology, 50*(8), 1416-1424.

Awad, A., Sato, D., Kusumoto, D., Kamioka, H., Takeuchi, Y., & Yoneyama, K. (2006). Characterization of strigolactones, germination stimulants for the root parasitic plants *Striga* and *Orobanche,* produced by maize, millet and sorghum. *Plant Growth Regulation, 48*(3), 221-227. doi: 10.1007/s10725-006-0009-3.

Balla, J., Kalousek, P., Reinöhl, V., Friml, J., & Procházka, S. (2011). Competitive canalization of PIN-dependent auxin flow from axillary buds controls pea bud outgrowth. *The Plant Journal, 65*(4), 571-577.

Bangerth, F. (1989). Dominance among fruits/sinks and the search for a correlative signal. *Physiologia Plantarum, 76*(4), 608-614. doi: 10.1111/j.1399-3054.1989.tb05487.x

Bangerth, F. (1994). Response of cytokinin concentration in the xylem exudate of bean (*Phaseolus vulgaris L.*) plants to decapitation and auxin treatment, and relationship to apical dominance. *Planta, 194*(3), 439-442. doi: 10.1007/bf00197546

Barbier, F., Péron, T., Lecerf, M., Perez-Garcia, M.-D., Barrière, Q., Rolčík, J., . . . Sakr, S. (2015). Sucrose is an early modulator of the key hormonal mechanisms controlling bud outgrowth in *Rosa hybrida*. *Journal of Experimental Botany*. doi: 10.1093/jxb/erv047

Barker, E. R., Press, M. C., Scholes, J. D., & Quick, W. P. (1996). Interactions between the parasitic angiosperm *Orobanche aegyptiaca* and its tomato host: growth and biomass allocation. *New Phytologist, 133*(4), 637-642. doi: 10.1111/j.1469-8137.1996.tb01932.x

Barrs, H. D., & Weatherley, P. E. (1962). A re-examination of the relative turgidity technique for estimating water deficits in leaves *Australian Journal of Biological Sciences, 15*, 413-428.

Beck, E. H. (1996). Regulation of shoot/root ratio by cytokinins from roots in *Urtica dioica*: Opinion. [journal article]. *Plant and Soil, 185*(1), 1-12. doi: 10.1007/bf02257560

Beck, E. H., & Wagner, B. M. (1994). Quantification of the daily cytokinin transport from the root to the shoot of *Urtica dioica* L.*. *Botanica Acta, 107*(5), 342-348.

Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C., & Leyser, O. (2006). The *Arabidopsis* MAX pathway controls shoot branching by regulating auxin transport. *Current Biology, 16*(6), 553-563. doi: DOI: 10.1016/j.cub.2006.01.058

Berger, A., Oren, R., & Schulze, E.-D. (1994). Element concentrations in the xylem sap of *Picea abies* (L.) Karst. seedlings extracted by various methods under different environmental conditions. *Tree physiology, 14*(2), 111-128.

Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., . . . Séjalon-Delmas, N. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PLoS Biol, 4*(7), e226.

Beveridge, C. A. (2000). Long-distance signalling and a mutational analysis of branching in pea. *Plant Growth Regulation, 32*(2), 193-203. doi: 10.1023/a:1010718020095

Beveridge, C. A., & Kyozuka, J. (2010). New genes in the strigolactone-related shoot branching pathway. *Current Opinion in Plant Biology, 13*(1), 34-39. doi: DOI: 10.1016/j.pbi.2009.10.003

Beveridge, C. A., Murfet, I. C., Kerhoas, L., Sotta, B., Miginiac, E., & Rameau, C. (1997). The shoot controls zeatin riboside export from pea roots. Evidence from the branching mutant rms4. *The Plant Journal, 11*(2), 339-345. doi: 10.1046/j.1365-313X.1997.11020339.x

Beveridge, C. A., Ross, J. J., & Ian, C. M. (1996). Branching in pea' action of genes Rms3 and Rms4 *Plant Physiol 110*, 859-865.

Beveridge, C. A., Ross, J. J., & Murfet, I. C. (1994). Branching mutant rms-2 in *Pisum sativum* (grafting studies and endogenous indole-3-acetic acid levels). *Plant Physiology, 104*(3), 953-959. doi: 10.1104/pp.104.3.953

Beveridge, C. A., Symons, G. M., Murfet, I. C., Ross, J. J., & Rameau, C. (1997). The rms1 mutant of pea has elevated indole-3-acetic acid levels and reduced root-sap zeatin riboside content but increased branching controlled by graft-transmissible signal (s). . *Plant Physiology, 115*(3), 1251-1258.

Beveridge, C. A., Symons, G. M., Murfet, I. C., Ross, J. J., & Rameau, C. (1997). The rms1 mutant of pea has elevated indole-3-acetic acid levels and reduced root-sap zeatin riboside content but increased branching controlled by graft-transmissible signal(s). *Plant Physiology, 115*(3), 1251-1258.

Beveridge, C. A., Symons, G. M., & Turnbull, C. G. N. (2000). Auxin Inhibition of Decapitation-Induced Branching Is Dependent on Graft-Transmissible Signals Regulated by Genes Rms1 andRms2. *Plant Physiology, 123*(2), 689-698. doi: 10.1104/pp.123.2.689

Bhattacharya, A., Kourmpetli, S., & Davey, M. (2010). Practical applications of manipulating plant architecture by regulating gibberellin metabolism. *Journal of Plant Growth Regulation, 29*(2), 249-256. doi: 10.1007/s00344-009-9126-3

Bollard, E. G. (1953). The use of tracheal sap in the study of apple tree nutrition. *Journal of Experimental Botany, 4*, 363-368.

Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., . . . Leyser, O. (2005). MAX1 encodes a cytochrome P450 family member that acts downstream of MAX3/4 to produce a carotenoid-derived branch-inhibiting hormone. *Developmental cell, 8*(3), 443-449.

Bouwmeester, H. J., Matusova, R., Zhongkui, S., & Beale, M. H. (2003). Secondary metabolite signalling in host-parasitic plant interactions. *Current Opinion in Plant Biology, 6*(4), 358-364. doi: Doi: 10.1016/s1369-5266(03)00065-7

Boyer, F. D., Germain, A. S., Pillot, J. P., Pouvreau, J. B., Chen, V. X., Ramos, S., . . . and Rameau, C. (2012). Structure-activity relationship studies of strigoalctone-related molecules for branching inhibition in garden pea: molecule design for shoot branching. *Plant Physiology, 159*, 1524-1544.

Bradford, K. J., & Trewavas, A. J. (1994). Sensitivity thresholds and variable time scales in plant hormone action. *Plant Physiology, 105*(4), 1029-1036.

Braun, N., de Saint Germain, A., Pillot, J.-P., Boutet-Mercey, S., Li, X., Antoniadi, I., . . . Rameau, C. (2011). The pea TCP transcription factor PsBRC1 acts downstream of strigolactones to control shoot branching. *Plant Physiology*. doi: 10.1104/pp.111.182725

Brewer, P. B., Dun, E. A., Ferguson, B. J., Rameau, C., & Beveridge, C. A. (2009). Strigolactone acts downstream of auxin to regulate bud outgrowth in pea and *Arabidopsis*. *Plant Physiology, 150*(1), 482-493. doi: 10.1104/pp.108.134783

Brown, C. L., McAlpine, R. G., & Kormanik, P. P. (1967). Apical dominance and form in woody plants: A reappraisal. *American Journal of Botany, 54*(2), 153-162.

Brzobohatý, B., Moore, I., & Palme, K. (1994). Cytokinin metabolism: implications for regulation of plant growth and development. *Plant Molecular Biology, 26*(5), 1483-1497. doi: 10.1007/bf00016486

Cardoso, C., Zhang, Y., Jamil, M., Hepworth, J., Charnikhova, T., Dimkpa, S. O., . . . Meng, X. (2014). Natural variation of rice strigolactone biosynthesis is associated with the deletion of two MAX1 orthologs. *Proceedings of the National Academy of Sciences, 111*(6), 2379-2384.

Cary, A. J., Liu, W., & Howell, S. H. (1995). Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. *Plant Physiology, 107*(4), 1075-1082. doi: 10.1104/pp.107.4.1075

Catalano, M., & Hill, T. A. (1969). Interaction between gibberellic acid and kinetin in overcoming apical dominance, natural and induced by IAA, in Tomato (*Lycopersicum esculenturn* Mill. Cultivar Potentate). [10.1038/222985a0]. *Nature, 222*(5197), 985-986.

Ćavar, S., Zwanenburg, B., & Tarkowski, P. (2014). Strigolactones: occurrence, structure, and biological activity in the rhizosphere. *Phytochemistry Reviews*, 1-21.

Chae, S. H., Yoneyama, K., Takeuchi, Y., & Joel, D. M. (2004). Fluridone and norflurazon, carotenoid-biosynthesis inhibitors, promote seed conditioning and germination of the holoparasite *Orobanche minor*. *Physiologia Plantarum, 120*(2), 328-337. doi: 10.1111/j.0031-9317.2004.0243.x

Charrière, F., Sotta, B., Miginiac, É., & Hahne, G. (1999). Induction of adventitious shoots or somatic embryos on in vitro cultured zygotic embryos of *Helianthus annuus*: variation of endogenous hormone levels. *Plant Physiology and Biochemistry, 37*(10), 751-757.

Chen, C.-M., Ertl, J. R., Leisner, S. M., & Chang, C.-C. (1985). Localization of cytokinin biosynthetic sites in pea plants and carrot roots. *Plant Physiology, 78*(3), 510-513.

Chen, C., Zou, J., Zhang, S., Zaitlin, D., & Zhu, L. (2009). Strigolactones are a new-defined class of plant hormones which inhibit shoot branching and mediate the interaction of plant-AM fungi and plant-parasitic weeds. *Science in China Series C: Life Sciences, 52*(8), 693-700. doi: 10.1007/s11427-009-0104-6

Chen, C. C., & Chen, Y. R. (2005). Study on laminar hydathodes of *Ficus formosana* (Moraceae) I. Morphology and ultrastructure. *Botanical Bulletin of Academia Sinica, 46*.

Child, A. (Ed.). (1979). *A review of branching patterns in the Solanaceae.* : London , Academic Press.

Clearwater, M. J., Lowe, R. G., Hofstee, B. J., Barclay, C., Mandemaker, A. J., & Blattmann, P. (2004). Hydraulic conductance and rootstock effects in grafted vines of kiwifruit. *Journal of Experimental Botany, 55*(401), 1371-1382.

Clearwater, M. J., Seleznyova, A. N., Thorp, T. G., Blattmann, P., Barnett, A. M., Lowe, R. G., & Austin, P. T. (2006). Vigor-controlling rootstocks affect early shoot growth and leaf area development of kiwifruit. *Tree Physiology, 26*(4), 505-515. doi: 10.1093/treephys/26.4.505

Cline, M. G. (1991). Apical dominance. *The Botanical Review, 57*(4), 318-358. doi: 10.1007/bf02858771

Cline, M. G. (1997). Concepts and terminology of apical dominance. *American Journal of Botany, 84*(8), 1064-1069. doi: 10.2307/2446149

Cline, M. G. (2000). Execution of the auxin replacement apical dominance experiment in temperate woody species. *American Journal of Botany, 87*(2), 182-190.

Cody, C. A., Larsen, F. E., & Fritts, R. J. (1985). Stimulation of lateral branch development in tree fruit nursery stock with GA4+7 + BA. *HortScience 20* (4), 758-759.

Coenen, C., & Lomax, T. L. (1997). Auxin--cytokinin interactions in higher plants: old problems and new tools. *Trends in Plant Science, 2*(9), 351-356. doi: Doi: 10.1016/s1360-1385(97)84623-7

Coleman, G., & Ernst, S. (1989). *In vitro* shoot regeneration of *Populus deltoides*: effect of cytokinin and genotype. *Plant Cell Reports, 8*(8), 459-462. doi: 10.1007/bf00269048

Collard, B. C. Y., & Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences, 363*(1491), 557-572. doi: 10.1098/rstb.2007.2170

Cook, C. E., Whichard, L. P., Wall, M., Egley, G. H., Coggon, P., Luhan, P. A., & McPhail, A. T. (1972). Germination stimulants. II. Structure of strigol, a potent seed germination stimulant for witchweed (*Striga lutea*). *Journal of the American Chemical Society, 94*(17), 6198-6199. doi: 10.1021/ja00772a048

Crawford, S., Shinohara, N., Sieberer, T., Williamson, L., George, G., Hepworth, J., . . . Leyser, O. (2010). Strigolactones enhance competition between shoot branches by dampening auxin transport. *Development, 137*(17), 2905-2913. doi: 10.1242/dev.051987

Cutter, E. G. (1972). Regulation of branching in decussate species with unequal lateral buds. *Annals of Botany, 36*(1), 207-220.

D'Arth, S. M., Simpson, S., Seelye, J. F., & Jameson, P. E. (2002). Bushiness and cytokinin sensitivity in micropropagated *Zantedeschia*. *Plant Cell, Tissue and Organ Culture, 70*(1), 113-118. doi: 10.1023/a:1016034030557

D’Arth, S. M., Simpson, S. I., Seelye, J. F., & Jameson, P. E. (2007). Bushiness and cytokinin profile in dormant and sprouting tubers of *Zantedeschia*. *Plant Cell, Tissue and Organ Culture, 89*(2), 185-191. doi: 10.1007/s11240-007-9234-3 de Luque, A. P., Galindo, J. C. G., Macías, F. A., & Jorrín, J. (2000). Sunflower sesquiterpene lactone models induce *Orobanche cumana* seed germination. *Phytochemistry, 53*(1), 45-50. doi: http://dx.doi.org/10.1016/S0031-9422(99)00485-9

de Saint Germain, A., Ligerot, Y., Dun, E. A., Pillot, J.-P., Ross, J. J., Beveridge, C. A., & Rameau, C. (2013). Strigolactones stimulate internode elongation independently of gibberellins. *Plant Physiology, 163*(2), 1012-1025. doi: 10.1104/pp.113.220541

dePamphilis, C. W., & Palmer, J. D. (1990). Loss of photosynthetic and chlororespiratory genes from the plastid genome of a parasitic flowering plant. *Nature, 348*, 337-339.

Dieffenbach, H., Kramer, D., & Lüttge, U. (1980). Release of guttation fluid from passive hydathodes of intact barley plants. I. Structural and cytological aspects. *Annals of Botany, 45*(4), 397-401.

Domagalska, M. A., & Leyser, O. (2011). Signal integration in the control of shoot branching. [10.1038/nrm3088]. *Nat Rev Mol Cell Biol, 12*(4), 211-221.

Drummond, R. S., Sheehan, H., Simons, J. L., Martinez-Sanchez, N. M., Turner, R. M., Putterill, J., & Snowden, K. C. (2011). The expression of petunia strigolactone pathway genes is altered as part of the endogenous developmental program. *Front Plant Sci, 2*, 115. doi: 10.3389/fpls.2011.00115

Drummond, R. S. M., Ledger, S.E., Simons, J.L., Janssen, B.J., Snowden, K.C. . (2009). Vegetative branching in petunia In S. Gerats T., J. (Ed.), *Petunia: Evolutionary, Developmental and Physiological Genetics* (Second ed., pp. 157-178). New York: Springer.

Dun, E. A., Brewer, P. B., & Beveridge, C. A. (2009). Strigolactones: discovery of the elusive shoot branching hormone. *Trends in plant science, 14*(7), 364-372.

Dun, E. A., de Saint Germain, A., Rameau, C., & Beveridge, C. A. (2012). Antagonistic action of strigolactone and cytokinin in bud outgrowth control. *Plant Physiology, 158*(1), 487-498. doi: 10.1104/pp.111.186783

Dun, E. A., de Saint Germain, A., Rameau, C., & Beveridge, C. A. (2013). Dynamics of strigolactone function and shoot branching responses in *Pisum sativum*. *Molecular Plant, 6*(1), 128-140. doi: 10.1093/mp/sss131

Dun, E. A., Ferguson, B. J., & Beveridge, C. A. (2006). Apical dominance and shoot branching. Divergent opinions or divergent mechanisms? *Plant Physiology, 142*(3), 812-819. doi: 10.1104/pp.106.086868

Dun, E. A., Hanan, J., & Beveridge, C. A. (2009). Computational modeling and molecular physiology experiments reveal new insights into shoot branching in pea. *The Plant Cell Online, 21*(11), 3459-3472. doi: 10.1105/tpc.109.069013

Eliasson, L. (1975). Effect of indoleacetic acid on the abscisic acid level in stem tissue. *Physiologia Plantarum, 34*(2), 117-120. doi: 10.1111/j.1399-3054.1975.tb03803.x

Emery, R. J. N., Longnecker, N. E., & Atkins, C. A. (1998). Branch development in *Lupinus angustifolius L.* II. Relationship with endogenous ABA, IAA and cytokinins in axillary and main stem buds. *Journal of Experimental Botany, 49*(320), 555-562. doi: 10.1093/jxb/49.320.555

Evert, R. F. (2006). *Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function, and development*: John Wiley & Sons.

Ferguson, A. R. (1999). Kiwifruit cultivars: Breeding and selection. *Acta Hort. (ISHS), 498*, 43-52.

Ferguson, B. J., & Beveridge, C. A. (2009). Roles for auxin, cytokinin, and strigolactone in regulating shoot branching. *Plant Physiology, 149*(4), 1929-1944. doi: 10.1104/pp.109.135475

Fernández-Aparicio, M., Flores, F., & Rubiales, D. (2009). Recognition of root exudates by seeds of broomrape (Orobanche and Phelipanche) species. *Annals of Botany, 103*(3), 423-431. doi: 10.1093/aob/mcn236

Ferrante, A., Vernieri, P., Tognoni, F., Serra, G. (2006). Changes in abscisic acid and flower pigments during floral senescence of petunia. *Biologia Plantarum, 50*(4), 581-585. doi: 10.1007/s10535-006-0091-4

Firn, R. D. (1986). Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. *Physiologia Plantarum, 67*(2), 267-272. doi: 10.1111/j.1399-3054.1986.tb02454.x

Foo, E., Bullier, E., Goussot, M., Foucher, F., Rameau, C., & Beveridge, C. A. (2005). The branching gene RAMOSUS1 mediates interactions among two novel signals and auxin in pea. *The Plant Cell Online, 17*(2), 464-474. doi: 10.1105/tpc.104.026716

Foo, E., Morris, S. E., Parmenter, K., Young, N., Wang, H., Jones, A., . . . Beveridge, C. A. (2007). Feedback regulation of xylem cytokinin content is conserved in Pea and *Arabidopsis*. *Plant Physiology, 143*(3), 1418-1428. doi: 10.1104/pp.106.093708

Foo, E., & Reid, J. (2013). Strigolactones: New Physiological Roles for an Ancient Signal. *Journal of Plant Growth Regulation, 32*(2), 429-442. doi: 10.1007/s00344-012-9304-6

Forshey, C. G., & Elfving, D. C. (1989). The relationship between vegetative growth and fruiting in apple trees. *Horticultrual Reviews, 11*, 229-287.

FreshFacts. (2013). New Zealand Horticulture. *New Zealand Institute for Plant & Food Research*. Auckland.

Funnell, K., Seleznyova, A., Kaji, R., Chen, J., Manandhar, S., & Woolley, D. (2014). *Diverse growth and sylleptic branching patterns in Japanese maple cultivars.* Paper presented at the XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014): 1104.

Funnell, K. A. (2014). Interpreting Plant Architectural Design©. *Proceedings of the International Plant Propagator's Society-2013 1055*, 49-51.

Funnell, K. A., & Go, A. R. (1993). Tuber storage, floral induction, and gibberellin in *Zantedeschia*. *Acta Hort. (ISHS) 337*, 167-176.

Funnell, K. A., MacKay, B. R., & Lawoko, C. R. O. (1992). Comparative effects of promalin and GA3 on flowering and development of *Zantedeschia* 'Galaxy'. *Acta Hort. (ISHS), 292*, 173-180.

Garcia-Martinez, J. L., Sponsel, V. M., & Gaskin, P. (1987). Gibberellins in developing fruits of Pisum sativum cv. Alaska: Studies on their role in pod growth and seed development. *Planta, 170*(1), 130-137. doi: 10.1007/bf00392389

Gardner, W. H. (1986). Water content. In A. Klute (Ed.), *Methods of soil analysis- Part 1* (second ed., pp. 493-544): American Society of Agronomy, Inc., Soil Science Society of America, Inc., Madison, Wisconsin USA.

Gimeno-Gilles, C., Lelièvre, E., Viau, L., Malik-Ghulam, M., Ricoult, C., Niebel, A., . . . Limami, A. M. (2009). ABA-mediated inhibition of germination is related to the inhibition of genes encoding cell-wall biosynthetic and architecture: Modifying enzymes and structural proteins in *Medicago truncatula* embryo axis. *Molecular Plant, 2*(1), 108-119. doi: 10.1093/mp/ssn092

Gocal, G. F. W., Pharis, R. P., Yeung, E. C., & Pearce, D. (1991). Changes after decapitation in concentrations of indole-3-acetic acid and abscisic acid in the larger axillary bud of *Phaseolus vulgaris L*. cv. Tender Green. *Plant Physiology, 95*(2), 344-350. doi: 10.1104/pp.95.2.344.

George, E. F., Hall, M. A., & De Klerk, G.-J. (2008). Plant tissue culture procedure-background *Plant propagation by tissue culture* (pp. 1-28): Springer.

Goldwasser, Y., Yoneyama, K., Xie, X., & Yoneyama, K. (2008). Production of strigolactones by *Arabidopsis thaliana* responsible for *Orobanche aegyptiaca* seed germination. *Plant Growth Regulation, 55*(1), 21-28. doi: 10.1007/s10725-008-9253-z

Gomez-Roldan, V., Fermas, S., Brewer, P.B., Puech-Pages, V., Dun, E.A., Pillot, J.P., Letisse, F.,Matusova, R., Danoun, S., Portais, J.C., Bouwmeester, H., Becard, G., Beveridge, C.A., Rameau, C., Rochange, S.F. , Fermas, S., Brewer,, P.B., P.-P., V., , Dun, E. A., Pillot, J.P., , Letisse, F., Matusova, R., , Danoun, S., Portais, J.C., , . . . Rochange, S. F. (2008). Strigolactone inhibition of shoot branching. [10.1038/nature07271]. *Nature, 455*(7210), 189-194. doi: http://www.nature.com/nature/journal/v455/n7210/suppinfo/nature07271_S1.html

Gong, L., Yang, Y. J., & Zhou, J. (2012). Genes involved in the synthesis and signaling pathway of strigolactone, a shoot branching inhibitor. *Biologia Plantarum*, 1-6. doi: 10.1007/s10535-012-0001-x

Goodger, J. Q. D., Sharp, R. E., Marsh, E. L., & Schachtman, D. P. (2005). Relationships between xylem sap constituents and leaf conductance of well-watered and water-stressed maize across three xylem sap sampling techniques. *Journal of Experimental Botany, 56*(419), 2389-2400. doi: 10.1093/jxb/eri231

Grunwald, I., Rupprecht, I., Schuster, G., & Kloppstech, K. (2003). Identification of guttation fluid proteins: the presence of pathogenesis-related proteins in noninfected barley plants. *Physiologia Plantarum, 119*(2), 192-202. doi: 10.1034/j.1399-3054.2003.00202.x

Hall, S. M., & Hillman, J. R. (1975). Correlative inhibition of lateral bud growth in *Phaseolus vulgaris* L. timing of bud growth following decapitation. *Planta, 123*(2), 137-143. doi: 10.1007/bf00383862

Halligan, E. A., Brooking, I. R., Funnell, K. A., & Catley, J. L. (2004). Vegetative and floral shoot development of *Zantedeschia* 'Black Magic'. *Scientia Horticulturae, 99*(1), 55-65.

Hamiaux, C., Drummond, Revel S. M., Janssen, Bart J., Ledger, Susan E., Cooney, Janine M., Newcomb, Richard D., & Snowden, Kimberley C. (2012). DAD2 is an α/β hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. *Current Biology, 22*(21), 2032-2036. doi: http://dx.doi.org/10.1016/j.cub.2012.08.007

Harrison, M. A., & Kaufman, P. B. (1982). Does ethylene play a role in the release of lateral buds (Tillers) from apical dominance in oats? *Plant Physiology, 70*(3), 811-814. doi: 10.1104/pp.70.3.811

Hauck, C., Muller, S., & Schildknecht, H. (1992). A germination stimulant for parasitic flowering plants from *Sorghum bicolor*, a genuine host plant. *Journal of plant physiology 139*, 474-478.

Hayward, A., Stirnberg, P., Beveridge, C., & Leyser, O. (2009). Interactions between auxin and strigolactone in shoot branching control. *Plant Physiology, 151*(1), 400-412. doi: 10.1104/pp.109.137646

Heuvelink, E., & Buiskool, R. P. M. (1995). Influence of Sink-Source Interaction on Dry Matter Production in Tomato. *Annals of Botany, 75*(4), 381-389. doi: 10.1006/anbo.1995.1036

Hocking, T. J., & Hillman, J. R. (1975). Studies on the role of abscisic acid in the initiation of bud dormancy in Alnus glutinosa and Betula pubescens. [journal article]. *Planta, 125*(3), 235-242. doi: 10.1007/bf00385600

Ioio, R. D., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., . . . Sabatini, S. (2008). A Genetic Framework for the Control of Cell Division and Differentiation in the Root Meristem. *Science, 322*(5906), 1380-1384. doi: 10.1126/science.1164147

Ivanoff, S. S. (1963). Guttation injuries of plants. *The Botanical Review, 29*(2), 202-229. doi: 10.1007/bf02860821

Joel, D. M. (2000). The long-term approach to parasitic weeds control: manipulation of specific developmental mechanisms of the parasite. *Crop Protection, 19*(8-10), 753-758. doi: Doi: 10.1016/s0261-2194(00)00100-9

Joel, D. M., Steffens, J. C., & Matthews, D. E. (1995). *Germination of weedy root parasites*. New York: Marcel Dekker, Inc.

Johnson, X., Brcich, T., Dun, E. A., Goussot, M., Haurogné, K., Beveridge, C. A., & Rameau, C. (2006). Branching genes are conserved across species. Genes controlling a novel signal in pea are coregulated by other long-distance signals. *Plant Physiology, 142*(3), 1014-1026. doi: 10.1104/pp.106.087676

Kalousek, P., Buchtová, D., Balla, J., Reinöhl, V., & Procházka, S. (2014). Cytokinins and polar transport of auxin in axillary pea buds. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 58*(4), 79-88.

Kebreab, E., & Murdoch, A. J. (2001). Simulation of integrated control strategies for *Orobanche spp*. based on a life cycle model. *Experimental Agriculture, 37*(01), 37-51. doi: doi:null

Keshavarzi, M., Funnell, K. A., Heyes, J. A., & Woolley, D. J. (2014). Ethephon and secondary shoot induction in Gentian (*Gentiana* spp.) hybrids in vitro. *Scientia Horticulturae, 179*, 170-173.

King, R. A., & van Staden, J. (1988). Diffferential responses of buds along the shoot of *Pisum sativum* to isopentenyladenine and zeatin application. *Plant Physiol. Biochem., 26*(3), 253-259.

Kohlen, W., Charnikhova, T., Liu, Q., Bours, R., Domagalska, M.A., Beguerie, S., Verstappen, F., Leyser, O., Bouwmeester, H., Ruyter-Spira, C. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host *Arabidopsis*. *Plant Physiology, 155*(2), 974-987. doi: 10.1104/pp.110.164640

Koltai, H., & Prandi, C. (Eds.). (2014). *Strigolactones: Biosynthesis, Synthesis and Functions in Plant Growth and Stress Responses*. New York: Springer Science+Business Media.

Komarnytsky, S., Borisjuk, N. V., Borisjuk, L. G., Alam, M. Z., & Raskin, I. (2000). Production of recombinant proteins in tobacco guttation fluid. *Plant Physiology, 124*(3), 927-934. doi: 10.1104/pp.124.3.927

Koning, R. E. (1994). Transpiration. Plant Physiology. from http://plantphys.info/plant_physiology/transpire.shtml.

Kozak, D., & Stelmaszczuk, M. (2009). The effect of benzyladenine on shoot regeneration in vitro of *Zantedeschia aethiopica* 'Green Goddess'. *Annales UMCS, Horticultura, 19*(1), 14-18.

Kuehny, J. (2000). Calla history and culture. *HortTechnology 10*, 326-330.

Kulaeva, O. (1962). The effect of roots on leaf metabolism in relation to the action of kinetin on leaves. *Fiziol. rast, 9*, 229-239.

Kusumoto, D., Goldwasser, Y., Xie, X., Yoneyama, K., Takeuchi, Y., & Yoneyama, K. (2007). Resistance of red clover (*Trifolium pratense*) to the root parasitic plant *Orobanche minor* is activated by salicylate but not by Jasmonate. *Annals of Botany, 100*(3), 537-544. doi: 10.1093/aob/mcm148

Lang, G. A. (1987). Dormancy: a new universal terminology. *HortScience (USA).*

Leakey, R. R. B., & Longman, K. A. (1986). Physiological, environmental and genetic variation in apical dominance as determined by decapitation in *Triplochiton scleroxylon*. [10.1093/treephys/1.2.193]. *Tree Physiology, 1*(2), 193-207.

Lechat, M.-M., Pouvreau, J.-B., Péron, T., Gauthier, M., Montiel, G., Véronési, C., . . . Delavault, P. (2012). PrCYP707A1, an ABA catabolic gene, is a key component of Phelipanche ramosa seed germination in response to the strigolactone analogue GR24. *Journal of Experimental Botany, 63*(14), 5311-5322. doi: 10.1093/jxb/ers189

Ledger, S. E., Janssen, B. J., Karunairetnam, S., Wang, T., & Snowden, K. C. (2010). Modified CAROTENOID CLEAVAGE DIOXYGENASE8 expression correlates with altered branching in kiwifruit (*Actinidia chinensis*). *New Phytologist, 188*(3), 803-813. doi: 10.1111/j.1469-8137.2010.03394.x

Lee, L. Y. C., Hou, X., Fang, L., Fan, S., Kumar, P. P., & Yu, H. (2012). STUNTED mediates the control of cell proliferation by GA in Arabidopsis. *Development, 139*(9), 1568-1576.

Lersten, N. R., & Curtis, J. D. (1982). Hydathodes in physocarpus (Rosaceae: Spiraeoideae). *Canadian Journal of Botany, 60*(6), 850-855.

Letham, D. (1994). Cytokinins as phytohormones: sites of biosynthesis, translocation, and function of translocated cytokinin. *Cytokinins: chemistry, activity, and function. CRC Press, Boca Raton, FL*, 57-80.

Li, C. J., & Bangerth, F. (1999). Autoinhibition of indoleacetic acid transport in the shoots of two-branched pea (*Pisum sativum*) plants and its relationship to correlative dominance. *Physiologia Plantarum, 106*(4), 415-420. doi: 10.1034/j.1399-3054.1999.106409.x

Li, C. J., & Bangerth, F. (2003). Stimulatory effect of cytokinins and interaction with IAA on the release of lateral buds of pea plants from apical dominance. *Journal of plant physiology, 160*(9), 1059-1063. doi: http://dx.doi.org/10.1078/0176-1617-01042

Li, C. J., Guevara, E., Herrera, J., & Bangerth, F. (1995). Effect of apex excision and replacement by 1-naphthylacetic acid on cytokinin concentration and apical dominance in pea plants. *Physiologia Plantarum, 94*(3), 465-469. doi: 10.1111/j.1399-3054.1995.tb00955.x

Liang, J., Zhao, L., Challis, R., & Leyser, O. (2010). Strigolactone regulation of shoot branching in chrysanthemum (*Dendranthema grandiflorum*). *Journal of Experimental Botany, 61*(11), 3069-3078. doi: 10.1093/jxb/erq133

Linsmaier, E. M., & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum, 18*(1), 100-127. doi: 10.1111/j.1399-3054.1965.tb06874.x

Loeb, J. (1918). Chemical basis of correlation. I. Production of equal masses of shoots by equal masses of sister leaves in *Bryophyllum calycinum*. *Botanical Gazette, 65*(2), 150-174.

Logan, D. C., & Stewart, G. R. (1992). Germination of the seeds of parasitic angiosperms. *Seed Science Research, 2*(04), 179-190. doi: doi:10.1017/S0960258500001367

Luisi, A., Lorenzi, R., & Sorce, C. (2011). Strigolactone may interact with gibberellin to control apical dominance in pea (Pisum sativum). *Plant Growth Regulation, 65*(2), 415-419. doi: 10.1007/s10725-011-9603-0

Malik, H., Rutjes, F. P. J. T., & Zwanenburg, B. (2010). A new efficient synthesis of GR24 and dimethyl A-ring analogues, germinating agents for seeds of the parasitic weeds *Striga* and *Orobanche spp*. *Tetrahedron, 66*(35), 7198-7203. doi: DOI: 10.1016/j.tet.2010.06.072

Mason, M. G., Ross, J. J., Babst, B. A., Wienclaw, B. N., & Beveridge, C. A. (2014). Sugar demand, not auxin, is the initial regulator of apical dominance. *Proceedings of the National Academy of Sciences, 111*(16), 6092-6097. doi: 10.1073/pnas.1322045111

Matsuura, H., Ohashi, K., Sasako, H., Tagawa, N., Takano, Y., Ioka, Y., . . . Yoshihara, T. (2008). Germination stimulant from root exudates of *Vigna unguiculata*. *Plant Growth Regulation, 54*(1), 31-36. doi: 10.1007/s10725-007-9224-9

Matusova, R., & Bouwmeester, H. J. (2006). The effect of host-root derived cehmical signals on the germination of parasitic plants. . In M. Dicke & W. Takken (Eds.), *Chemical ecology: from gene to ecosystem.*: Springer, Wageningen.

Matusova, R., Mourik, T. v., & Bouwmeester, H. J. (2004). Changes in the sensitivity of parasitic weed seeds to germination stimulants. *Seed Science Research, 14*(04), 335-344. doi: doi:10.1079/SSR2004187

Matusova, R., Rani, K., Verstappen, F. W. A., Franssen, M. C. R., Beale, M. H., & Bouwmeester, H. J. (2005). The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp.are derived from the carotenoid pathway. *Plant Physiol., 139*(2), 920-934. doi: 10.1104/pp.105.061382

McGhie, T. K., Hudault, S. b., Lunken, R. C., & Christeller, J. T. (2011). Apple peels, from seven cultivars, have lipase-inhibitory activity and contain numerous ursenoic acids as identified by LC-ESI-QTOF-HRMS. *Journal of Agricultural and Food Chemistry, 60*(1), 482-491.

McIntyre, G. I. (1973). Environmental control of apical dominance in Phaseolus vulgaris. *Canadian Journal of Botany, 51*(2), 293-299. doi: 10.1139/b73-036

Mendi, Y. Y., Comlekcioglu, N., Ipek, M., Kocaman, E., Izgu, T., Tekdal, D., & Curuk, P. (2010). The effect of different hormone concentrations and dark pretreatment on adventitious shoot regeneration in snake melon (*Cucumis melo* var. flexousus) *Romanian Biotechnological Letters, 15*(4), 5392-5395.

Milborrow, B. V. (1974). The Chemistry and Physiology of Abscisic Acid. *Annual Review of Plant Physiology, 25*(1), 259-307. doi: doi:10.1146/annurev.pp.25.060174.001355

Miller, S. A., Broom, F. D., Thorp, T. G., & Barnett, A. M. (2001). Effects of leader pruning on vine architecture, productivity and fruit quality in kiwifruit (*Actinidia deliciosa* cv. Hayward). *Scientia Horticulturae, 91*(3-4), 189-199. doi: Doi: 10.1016/s0304-4238(01)00259-x

Morris, D. A. (1977). Transport of exogenous auxin in two-branched dwarf pea seedlings (*Pisum sativum* L.). *Planta, 136*, 91-96.

Morris, S. E., Cox, M. C. H., Ross, J. J., Krisantini, S., & Beveridge, C. A. (2005). Auxin dynamics after decapitation are not correlated with the initial growth of axillary buds. *Plant Physiology, 138*(3), 1665-1672. doi: 10.1104/pp.104.058743

Morris, S. E., Turnbull, C. G. N., Murfet, I. C., & Beveridge, C. A. (2001). Mutational analysis of branching in pea. Evidence that Rms1 and Rms5 regulate the same novel signal. *Plant Physiology, 126*(3), 1205-1213. doi: 10.1104/pp.126.3.1205

Müller, D., & Leyser, O. (2011). Auxin, cytokinin and the control of shoot branching. *Annals of Botany, 107*(7), 1203-1212. doi: 10.1093/aob/mcr069

Müller, S., Hauck, C., & Schildknecht, H. (1992). Germination stimulants produced by *Vigna unguiculata Walp* cv. Saunders Upright. *Journal of Plant Growth Regulation, 11*(2), 77-84. doi: 10.1007/bf00198018

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497.

Naor, V., Kigel, J., Ben-Tal, Y., & Ziv, M. (2008). Variation in endogenous gibberellins, abscisic acid, and carbohydrate content during the growth cycle of colored *Zantedeschia spp*., a tuberous geophyte. *Journal of Plant Growth Regulation, 27*(3), 211-220.

Naor, V., Kigel, J., & Ziv, M. (2005). The effect of gibberellin and cytokinin on floral development in *Zantedeschia* spp. *in vivo* and *in vitro*. *Acta Hort. (ISHS) 673*, 255-263.

Naor, V., Kigel, J., Ziv, M., & Flaishman, M. (2005). A developmental pattern of flowering in colored *Zantedeschia* spp.: Effects of bud position and gibberellin. [journal article]. *Journal of Plant Growth Regulation, 23*(4), 269-279. doi: 10.1007/s00344-004-0019-1

Napoli, C. A. (1996). Highly branched phenotype of the petunia dad1-1 mutant is reversed by grafting. *Plant Physiology, 111*(1), 27-37. doi: 10.1104/pp.111.1.27

Napoli, C. A., Beveridge, C. A., & Snowden, K. C. (1998). 5 reevaluating concepts of apical dominance and the control of axillary bud outgrowth. In A. P. Roger & P. S. Gerald (Eds.), *Current Topics in Developmental Biology* (Vol. Volume 44, pp. 127-169): Academic Press.

Napoli, C. A., & Ruehle, J. (1996). New mutations affecting meristem growth and potential in *Petunia hybrida* Vilm. *Journal of heredity, 87*, 371-377.

Naz, R., & Anis, M. (2012). Acceleration of adventitious shoots by interaction between exogenous hormone and adenine sulphate in *Althaea officinalis* L. *Applied Biochemistry and Biotechnology, 168*(5), 1239-1255. doi: 10.1007/s12010-012-9853-y

Ngamau, K. (2001). Promoting side shoot development in *Zantedeschia aethiopica* 'Green Goddess'. *European Journal of Horticultural Science, 66* ((2), 85-92.

Ni, J., Gao, C., Chen, M.-S., Pan, B.-Z., Ye, K., & Xu, Z.-F. (2015). Gibberellin promotes shoot branching in the perennial woody plant *Jatropha curcas*. *Plant and Cell Physiology*. doi: 10.1093/pcp/pcv089

Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Åstot, C., Dolezal, K., & Sandberg, G. (2004). Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin–cytokinin-regulated development. *Proceedings of the National Academy of Sciences of the United States of America, 101*(21), 8039-8044. doi: 10.1073/pnas.0402504101

Ofir, M., & Kigel, J. (1998). Abscisic acid involvement in the induction of summer-dormancy in Poa bulbosa, a grass geophyte. *Physiologia Plantarum, 102*(2), 163-170. doi: 10.1034/j.1399-3054.1998.1020202.x

Ongaro, V., & Leyser, O. (2008). Hormonal control of shoot branching. *Journal of Experimental Botany, 59*(1), 67-74. doi: 10.1093/jxb/erm134

Palni, L. M. S., Burch, L., & Horgan, R. (1988). The effect of auxin concentration on cytokinin stability and metabolism. [journal article]. *Planta, 174*(2), 231-234. doi: 10.1007/bf00394775

Panigrahi, B. M., & Audus, L. J. (1966). Apical Dominance in Vicia faba. *Annals of Botany, 30*(3), 457-473.

Phillips, I. D. J. (1975). Apical Dominance. *Annual Review of Plant Physiology, 26*(1), 341-367. doi: doi:10.1146/annurev.pp.26.060175.002013

Popenoe, J., & Barritt, B. H. (1988). Branch induction by growth regulators and leaf removal in 'Delicious' apple nursery stock. *Hort Science, 23*(5), 859-862.

Prasad, T., & Cline, M. (1985). Shoot inversion-induced ethylene in Pharbitis nil induces the release of apical dominance by restricting shoot elongation. *Plant Science, 38*(3), 163-172.

Prusinkiewicz, P., Crawford, S., Smith, R. S., Ljung, K., Bennett, T., Ongaro, V., & Leyser, O. (2009). Control of bud activation by an auxin transport switch. *Proceedings of the National Academy of Sciences, 106*(41), 17431-17436. doi: 10.1073/pnas.0906696106

Rabot, A., Henry, C., Ben Baaziz, K., Mortreau, E., Azri, W., Lothier, J., . . . Sakr, S. (2012). Insight into the role of sugars in bud burst under light in the rose. *Plant and Cell Physiology, 53*(6), 1068-1082. doi: 10.1093/pcp/pcs051

Rameau, C. (2010). Strigolactones, a novel class of plant hormone controlling shoot branching. *Comptes Rendus Biologies, 333*(4), 344-349. doi: DOI: 10.1016/j.crvi.2010.01.012

Rameau, C., Bertheloot, J., LEDUC, N., Andrieu, B., SAKR, S., & Foucher, F. (2015). Multiple pathways regulate shoot branching. [Review]. *Frontiers in Plant Science, 5*. doi: 10.3389/fpls.2014.00741

Reinhardt, D., & Kuhlemeier, C. (2002). Plant architecture. *EMBO Reports, 3*(9), 846-851. doi: 10.1093/embo-reports/kvf177

Riou-Khamlichi, C., Huntley, R., Jacqmard, A., & Murray, J. A. H. (1999). Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science, 283*(5407), 1541-1544. doi: 10.1126/science.283.5407.1541

Ross, J. J. (1998). Effects of auxin transport inhibitors on gibberellins in pea. [Article]. *Journal of Plant Growth Regulation, 17*(3), 141-146. doi: 10.1007/pl00007027

Ross, J. J., O'Neill, D. P., Smith, J. J., Kerckhoffs, L. H. J., & Elliott, R. C. (2000). Evidence that auxin promotes gibberellin A1 biosynthesis in pea. *The Plant Journal, 21*(6), 547-552. doi: 10.1046/j.1365-313x.2000.00702.x

Rumsey, F. J., & Jury, S. L. (1991). An account of *Orobanche* L. In Britain and Ireland *Watsonia, 18*, 257-295

Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter, N., . . . Bouwmeester, H. (2011). Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in *Arabidopsis*: Another belowground role for strigolactones? *Plant Physiology, 155*(2), 721-734. doi: 10.1104/pp.110.166645

Sachs, T. (1968). The role of the root in the induction of xylem differentiation in peas. *Annals of Botany, 32*(2), 391-399.

Sachs, T. (1981). The control of the patterned differentiation of vascular tissues. In H. W. Woolhouse (Ed.), *Advances in Botanical Research* (Vol. Volume 9, pp. 151-262): Academic Press.

Sachs, T., & Thimann, K. V. (1967). The Role of Auxins and Cytokinins in the Release of Buds From Dominance. *American Journal of Botany, 54*(1), 136-144. doi: 10.2307/2440896

Sakai, S. (1990). Sympodial and monopodial branching in Acer: implications for tree architecture and adaptive significance. *Canadian Journal of Botany, 68*(7), 1549-1553. doi: 10.1139/b90-198

Sale, P. R., & Lyford, P. B. (1990). Cultural, management and harvesting practices for kiwifruit in New Zealand. In I. J. Warrington & G. C. Weston (Eds.), *Kiwifruit: science and management* (pp. 247-296): New Zealand Society for Horticultural Science, Auckland, New Zealand.

Sanyal, D., & Bangerth, F. (1998). Stress induced ethylene evolution and its possible relationship to auxin-transport, cytokinin levels, and flower bud induction in shoots of apple seedlings and bearing apple trees. *Plant Growth Regulation, 24*(2), 127-134. doi: 10.1023/a:1005948918382

Seleznyova, A. N., Thorp, T. G., Barnett, A. M., & Costes, E. (2002). Quantitative analysis of shoot development and branching patterns in *Actinidia*. *Annals of Botany, 89*(4), 471-482. doi: 10.1093/aob/mcf069

Shani, E., Yanai, O., & Ori, N. (2006). The role of hormones in shoot apical meristem function. *Current Opinion in Plant Biology, 9*(5), 484-489. doi: http://dx.doi.org/10.1016/j.pbi.2006.07.008

Sherriff, L. J., McKay, M. J., Ross, J. J., Reid, J. B., & Willis, C. L. (1994). Decapitation reduces the metabolism of gibberellin A20 to A1 in *Pisum sativum* L., decreasing the Le/le difference. *Plant Physiology, 104*(1), 277-280.

Shimizu-Sato, S., & Mori, H. (2001). Control of outgrowth and dormancy in axillary buds. *Plant Physiology, 127*(4), 1405-1413. doi: 10.1104/pp.010841

Shimizu-Sato, S., Tanaka, M., & Mori, H. (2009). Auxin-cytokinin interactions in the control of shoot branching. [Article]. *Plant Molecular Biology, 69*(4), 429-435. doi: 10.1007/s11103-008-9416-3

Shinohara, N., Taylor, C., & Leyser, O. (2013). Strigolactone can promote or inhibit shoot branching by triggering rapid depletion of the auxin efflux protein PIN1 from the plasma membrane. *PLoS Biol, 11*(1), e1001474. doi: 10.1371/journal.pbio.1001474

Siame, B. A., Weerasuriya, Y., Wood, K., Ejeta, G., & Butler, L. G. (1993). Isolation of strigol, a germination stimulant for *Striga asiatica*, from host plants. *Journal of Agricultural and Food Chemistry, 41*(9), 1486-1491. doi: 10.1021/jf00033a025

Simons, J. L., Napoli, C. A., Janssen, B. J., Plummer, K. M., & Snowden, K. C. (2007). Analysis of the DECREASED APICAL DOMINANCE genes of petunia in the control of axillary branching. *Plant Physiol., 143*(2), 697-706. doi: 10.1104/pp.106.087957

Singh, S., & Singh, T. N. (2013). Guttation 1: chemistry, crop husbandry and molecular farming. *Phytochemistry Reviews, 12*(1), 147-172. doi: 10.1007/s11101-012-9269-x

Singh, S., Singh, T. N., & Chauhan, J. S. (2009). Guttation in rice: Occurrence, regulation, and significance in varietal improvement. *Journal of Crop Improvement, 23*(4), 351-365. doi: 10.1080/15427520902970300

Smart, R. E., & Bingham, G. E. (1974). Rapid estimates of relative water content. *Plant Physiology, 53*(2), 258-260. doi: 10.1104/pp.53.2.258

Snow, R. (1937). On the nature of correlative inhibition. *New Phytologist, 36*(4), 283-300.

Snowden, K. C., & Napoli, C. A. (2003). A quantitative study of lateral branching in petunia *Functional Plant Biology, 30*, 987-994. .

Snowden, K. C., Simkin, A. J., Janssen, B. J., Templeton, K. R., Loucas, H. M., Simons, J. L., . . . Klee, H. J. (2005). The Decreased apical dominance1/Petunia hybrida CAROTENOID CLEAVAGE DIOXYGENASE8 Gene Affects Branch Production and Plays a Role in Leaf Senescence, Root Growth, and Flower Development. *The Plant Cell, 17*(3), 746-759. doi: 10.1105/tpc.104.027714

Sorefan, K., Booker, J., Haurogne, K., Goussot, M., Bainbridge, K., Foo, E., . . . Leyser, O. (2003). MAX4 and RMS1 are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. [Article]. *Genes & Development, 17*(12), 1469-1474. doi: 10.1101/gad.256603

Staswick, P. E., & Tiryaki, I. (2004). The Oxylipin Signal Jasmonic Acid Is Activated by an Enzyme That Conjugates It to Isoleucine in Arabidopsis. *The Plant Cell, 16*(8), 2117-2127. doi: 10.1105/tpc.104.023549

Stirnberg, P., van de Sande, K., & Leyser, H. M. O. (2002). MAX1 and MAX2 control shoot lateral branching in *Arabidopsis*. *Development, 129*(5), 1131-1141.

Subbaraj, A. K. (2011). *Hormonal control of branching and flowering in Zantedeschia species: A Thesis Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Plant Physiology at Massey University, Palmerston North, New Zealand*: Massey University, Palmerston North.

Subbaraj, A. K., Funnell, K. A., & Woolley, D. J. (2010). Dormancy and flowering are regulated by the reciprocal interaction between cytokinin and gibberellin in *Zantedeschia*. *Journal of Plant Growth Regulation, 29*(4), 487-499. doi: 10.1007/s00344-010-9160-1

Talon, M., Tadeo, F., & Zeevaart, J. D. (1991). Cellular changes induced by exogenous and endogenous gibberellins in shoot tips of the long-day plant Silene armeria. *Planta, 185*(4), 487-493. doi: 10.1007/bf00202957

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., & Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *The Plant Journal, 45*(6), 1028-1036. doi: 10.1111/j.1365-313X.2006.02656.x

Thimann, K. V., & Skoog, F. (1933). Studies on the Growth Hormone of Plants: III. The Inhibiting Action of the Growth Substance on Bud Development. *Proceedings of the National Academy of Sciences of the United States of America, 19*(7), 714-716.

Thimann, K. V., & Skoog, F. (1934). On the inhibition of bud development and other functions of growth substance in *Vicia faba*. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 114*(789), 317-339.

Thompson, A. J., Andrews, J., Mulholland, B. J., McKee, J. M. T., Hilton, H. W., Horridge, J. S., . . . Taylor, I. B. (2007). Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. *Plant Physiology, 143*(4), 1905-1917. doi: 10.1104/pp.106.093559

Thorogood, C. J., Rumsey, F. J., & Hiscock, S. J. (2009a). Host-specific races in the holoparasitic angiosperm *Orobanche minor*: implications for speciation in parasitic plants. *Annals of Botany, 103*(7), 1005-1014. doi: 10.1093/aob/mcp034

Thorogood, C. J., Rumsey, F. J., & Hiscock, S. J. (2009b). Seed viability determination in parasitic broomrapes (Orobanche and Phelipanche) using fluorescein diacetate staining. *Weed Research, 49*(5), 461-468. doi: 10.1111/j.1365-3180.2009.00716.x

Titarenko, E., Rojo, E., León, J., & Sánchez-Serrano, J. J. (1997). Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in Arabidopsis thaliana. *Plant Physiology, 115*(2), 817-826.

Tucker, D. J. (1976). Effects of Far-red Light on the Hormonal Control of Side Shoot Growth in the Tomato. *Annals of Botany, 40*(5), 1033-1042.

Tucker, D. J., & Mansfield, T. A. (1971). Effects of light quality on apical dominance in Xanthium strumarium and the associated changes in endogenous levels of abscisic acid and cytokinins. *Planta, 102*(2), 140-151. doi: 10.1007/bf00384868

Turnbull, C. G. N., Booker, J. P., & Leyser, H. M. O. (2002). Micrografting techniques for testing long-distance signalling in *Arabidopsis*. [Article]. *Plant Journal, 32*(2), 255-262.

Turnbull, C. G. N., Raymond, M. A. A., Dodd, I. C., & Morris, S. E. (1997). Rapid increases in cytokinin concentration in lateral buds of chickpea (*Cicer arietinum* L.) during release of apical dominance. *Planta, 202*(3), 271-276. doi: 10.1007/s004250050128

Umehara, M., Hanada, A., Magome, H., Takeda-Kamiya, N., & Yamaguchi, S. (2010). Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice *Plant Cell Physiol., 51*(7), 1118-1126

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., . . . Yamaguchi, S. (2008). Inhibition of shoot branching by new terpenoid plant hormones. [10.1038/nature07272]. *Nature, 455*(7210), 195-200. doi: http://www.nature.com/nature/journal/v455/n7210/suppinfo/nature07272_S1.html

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., & Shinozaki, K. (1999). A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *The Plant Cell Online, 11*(9), 1743-1754. doi: 10.1105/tpc.11.9.1743

van Hooijdonk, B. M. (2009). The physiological basis of vigour control by apple rootstocks-an unresolved paradigm: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Physiology at Massey University, Palmerston North, New Zealand.

van Hooijdonk, B. M., Woolley, D. J., Warrington, I. J., & Tustin, D. S. (2010). Initial alteration of scion architecture by dwarfing apple rootstocks may involve shoot-root-shoot signalling by auxin, gibberellin, and cytokinin. *Journal of Horticultural Science and Biotechnology, 85*(1), 59-65.

van Hooijdonk, B. M., Woolley, D. J., Warrington, I. J., & Tustin, D. S. (2011). Rootstocks modify scion architecture, endogenous hormones, and root growth of newly grafted ‘royal gala’ apple trees. *Journal of the American Society for Horticultural Science, 136*(2), 93-102.

Vančura, V., Přikryl, Z., Kalachová, L., & Wurst, M. (1977). Some quantitative aspects of root exudation. *Ecological Bulletins*(25), 381-386. doi: 10.2307/20112601

Vanstraelen, M., & Benkov´a, E. (2012). Hormonal interactions in the regulation of plant development *Annu. Rev. Cell Dev. Biol. , 28*, 463-487. doi: 10.1371/journal.pbio.0040226

Vattiprolu, N. M. R. G. (2012). *Vigour control in apple (Malus domestica) and kiwifruit ((Actinidia deliciosa and Actinidia chinensis): a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Physiology at Massey University, Palmerston North, New Zealand.*

Venkatachalam, P., Ezhili, N., & Thiyagarajan, M. (2012). I*n vitro* shoot multiplication of *Gloriosa superba* L.– An important anticancer medicinal herb. *International Conference on Biotechnology, Biological and Biosystems Engineering (ICBBBE'2012) December 18-19, 2012 Phuket (Thailand).*

Verdú, M., & Climent, J. (2007). Evolutionary correlations of polycyclic shoot growth in Acer (Sapindaceae). *American Journal of Botany, 94*(8), 1316-1320. doi: 10.3732/ajb.94.8.1316

Visser, J. (1989). Germination requirements of some root-parasitic flowering plants. *Naturwissenschaften, 76*(6), 253-261. doi: 10.1007/bf00368634

Vogel, J. T., Walter, M. H., Giavalisco, P., Lytovchenko, A., Kohlen, W., Charnikhova, T., . . . Klee, H. J. (2010). SlCCD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. *The Plant Journal, 61*(2), 300-311. doi: 10.1111/j.1365-313X.2009.04056.x

Von Arnold, S., & Tillberg, E. (1987). The influence of cytokinin pulse treatments on adventitious bud formation on vegetative buds of *Picea abies*. *Plant Cell, Tissue and Organ Culture, 9*(3), 253-261. doi: 10.1007/bf00040811

Vyas, S., Joshi, N., Tak, K., & Purohit, S. (2005). In vitro adventitious shoot bud differentiation and plantlet regeneration in *Feronia limonia* L. (Swingle). *In Vitro Cellular & Developmental Biology - Plant, 41*(3), 296-302. doi: 10.1079/ivp2004607

Wagner, E. B. a. B. M. (1994). Quantification of the daily cytokinin transport from the root to shoot of *Urtica dioca* L. *Bot. Acta, 107*, 342-348.

Waldie, T., McCulloch, H., & Leyser, O. (2014). Strigolactones and the control of plant development: lessons from shoot branching. *Plant J, 79*(4), 607-622. doi: 10.1111/tpj.12488

Walton, E., Fowke, P., Weis, K., & McLeay, P. (1997). Shoot axillary bud morphogenesis in kiwifruit (*Actinidia deliciosa*). *Annals of Botany, 80*(1), 13-21.

Wang, B., & Qiu, Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza, 16*(5), 299-363. doi: 10.1007/s00572-005-0033-6.

Ward, S. P., Salmon, J., Hanley, S. J., Karp, A., & Leyser, O. (2013). Using *Arabidopsis* to study shoot branching in biomass willow. *Plant Physiology, 162*(2), 800-811.

Wardlaw, I. F., & Mortimer, D. C. (1970). Carbohydrate movement in pea plants in relation to axillary bud growth and vascular development. *Canadian Journal of Botany, 48*(2), 229-237. doi: 10.1139/b70-035

Webb, C. J., Sykes, W. R., & Garnock-Jones, P. J. (1988). *Flora of New Zealand* (Vol. IV): Cataloguing in publication, Botany Division.

Westwood, J. H., Yu, X., Foy, C. L., & Cramer, C. L. (1998). Expression of a defense-related 3-hydroxy-3-methylglutaryl CoA reductase gene in response to parasitization by *Orobanche* spp. *Molecular Plant-Microbe Interactions, 11*(6), 530-536. doi: doi:10.1094/MPMI.1998.11.6.530

Weyers, J. D. B., & Paterson, N. W. (2001). Plant hormones and the control of physiological processes. *New Phytologist, 152*(3), 375-407. doi: 10.1046/j.0028-646X.2001.00281.x

Weyers, J. D. B., Paterson, N. W., A'Brook, R., & Peng, Z.-Y. (1995). Quantitative analysis of the control of physiological phenomena by plant hormones. *Physiologia Plantarum, 95*(3), 486-494. doi: 10.1111/j.1399-3054.1995.tb00867.x

White, J. C., & Mansfield, T. A. (1977). Correlative Inhibition of Lateral Bud Growth in Pisum sativum L. and Phaseolus vulgaris L.: Studies of the Role of Abscisic Acid. *Annals of Botany, 41*(6), 1163-1170.

Wickson, M., & Thimann, K. V. (1958). The Antagonism of Auxin and Kinetin in Apical Dominance. *Physiologia Plantarum, 11*(1), 62-74. doi: 10.1111/j.1399-3054.1958.tb08426.x

Wigchert, S. C. M., Kuiper, E., Boelhouwer, G. J., Nefkens, G. H. L., Verkleij, J. A. C., & Zwanenburg, B. (1999). Dose−response of seeds of the parasitic weeds *Striga* and *Orobanche* toward the synthetic germination stimulants GR24 and Nijmegen 1. *Journal of Agricultural and Food Chemistry, 47*(4), 1705-1710. doi: 10.1021/jf981006z

Wilson, B. F. (2000). Apical control of branch growth and angle in woody plants. *Am. J. Bot., 87*(5), 601-607.

Woolley, D. J., & Wareing, P. F. (1972). The role of roots, cytokinins and apical dominance in the control of lateral shoot form in *Solanum andigena*. *Planta, 105*(1), 33-42.

Xie, X., Kusumoto, D., Takeuchi, Y., Yoneyama, K., Yamada, Y., & Yoneyama, K. (2007). 2′-Epi-orobanchol and solanacol, two unique strigolactones, germination stimulants for root parasitic weeds, produced by tobacco. *Journal of Agricultural and Food Chemistry, 55*(20), 8067-8072. doi: 10.1021/jf0715121

Xie, X., Yoneyama, K., Kisugi, T., Uchida, K., Ito, S., Akiyama, K., . . . Nomura, T. (2013). Confirming stereochemical structures of strigolactones produced by rice and tobacco. *Mol Plant, 6*(1), 153-163.

Xie, X., Yoneyama, K., & Yoneyama, K. (2010). The Strigolactone Story. *Annual Review of Phytopathology, 48*(1), 93-117. doi: doi:10.1146/annurev-phyto-073009-114453

Yamazaki, H., Nishijima, T., Koshioka, M., & Miura, H. (2002). Gibberellins do not act against abscisic acid in the regulation of bulb dormancy of *Allium wakegi* cv. Araki. *Plant Growth Regulation, 36*(3), 223-229. doi: 10.1023/a:1016577529378

Yokota, T., Sakai, H., Okuno, K., Yoneyama, K., & Takeuchi, Y. (1998). Alectrol and orobanchol, germination stimulants for *Orobanche minor*, from its host red clover. *Phytochemistry, 49*(7), 1967-1973. doi: Doi: 10.1016/s0031-9422(98)00419-1

Yoneyama, K., Awad, A. A., Xie, X., Yoneyama, K., & Takeuchi, Y. (2010). Strigolactones as germination stimulants for root parasitic plants. *Plant and Cell Physiology, 51*(7), 1095-1103. doi: 10.1093/pcp/pcq055

Yoneyama, K., Sato, D., Takeuchi, Y., Sekimoto, H., Yokota, T., & Sassa, T. (2006). Search for Germination Stimulants and Inhibitors for Root Parasitic Weeds *Natural Products for Pest Management* (Vol. 927, pp. 88-98): American Chemical Society.

Yoneyama, K., Takeuchi, Y., Ogasawara, M., Konnai, M., Sugimoto, Y., & Sassa, T. (1998). Cotylenins and fusicoccins stimulate seed germination of *Striga hermonthica* (Del.) *Benth* and *Orobanche minor* Smith. *Journal of Agricultural and Food Chemistry, 46*(4), 1583-1586. doi: 10.1021/jf970964i

Zhang, J., & Davies, W. J. (1987). Increased synthesis of ABA in partially dehydrated root tips and ABA transport from roots to leaves. *Journal of Experimental Botany, 38*(12), 2015-2023. doi: 10.1093/jxb/38.12.2015

Zhang, K., Letham, D. S., & John, P. C. L. (1996). Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinaselike H1 histone kinase. *Planta, 200*(1), 2-12. doi: 10.1007/bf00196642

Zhang, R., Zhang, X., Wang, J., Letham, D. S., McKinney, S. A., & Higgins, T. J. V. (1995). The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expressing an *ipt* gene. *Planta, 196*(1), 84-94. doi: 10.1007/bf00193221

Zwanenburg, B., Mwakaboko, A. S., Reizelman, A., Anilkumar, G., & Sethumadhavan, D. (2009). Structure and function of natural and synthetic signalling molecules in parasitic weed germination. *Pest Management Science, 65*(5), 478-491. doi: 10.1002/ps.1706