HORMONAL CONTROL OF BRANCHING AND FLOWERING IN *ZANTEDESCHIA* SPECIES

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Abstract

Calla lilies (*Zantedeschia* sp. Family: Araceae) are perennial herbaceous geophytes, gaining commercial importance as a cut flower and potted flowering plant. Stimulating branching in *Zantedeschia* would equate to higher floral productivity via increasing tuber size/weight and/or via triggering the sympodial flowering cascade. Bud outgrowth is however controlled by an autonomous developmental programme, executed via different degrees of para- (apical dominance) and/or endodormancy.

Based on visual clues that represent underlying changes in the shoot apical meristem, the growth cycle of *Zantedeschia* was demarcated into three phases, which coincide with the transition of buds from apical dominance to endodormancy. Application of 6-benzylaminopurine (BAP; an aromatic cytokinin) was successful in stimulating branching in phase 1. This equated to an increase in tuber size/weight, which in turn resulted in increased floral productivity in the next growth cycle. . Efficacy of BAP alone to stimulate branching declined from phase 1 to phase 3, and the need for a sequential application of gibberellin (GA_3) increased concomitantly. GA_3 alone had no effect on branching. Efficacy of GA_3 alone to stimulate flowering declined from phase 1 to phase 3, and the need for a sequential application of BAP increased concomitantly. BAP alone had no effect on flowering. Stimulation of branching and enhanced flowering achieved by the reciprocal cross-talk between cytokinin and gibberellin may have major commercial implications.

When applied with unlabelled BAP, a significant decline in the uptake of [8-^{14}C] BAP ([8-^{14}C] BAP + BAP) was observed in phase 3, resulting in a decline in radioactivity available in the buds and upper region of the tuber. With unlabelled GA_3 ([8-^{14}C] BAP + GA_3) however, increased radioactivity was available in these parts in phase 3. *Meta*-topolin (mT) was identified as a metabolic product of BAP. Application of [8-^{14}C] BAP + BAP resulted in a decline in the amount of mT from phase 1 to phase 3. However, application of [8-^{14}C] BAP + GA_3 resulted in an increase in the amount of mT in phase 3. mT and 6-benzylaminopurine riboside (BAR) were also identified in natural plants.

Further studies on branching control in phase 1 involving topolins and strigolactones, elucidating the mechanisms of cross-talk between cytokinin and gibberellin in phase 3, reevaluating the relationship between branching and floral productivity, and corroborating the common mechanisms between dormancy and flowering are recommended.

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[$^{2}H_{7}$] BAP	Deuterated 6-benzylaminopurine
[$^{2}H_{7}$] BAR	Deuterated 6-benzylaminopurine riboside
[^{3}H]-iP	Tritiated isopentenyl adenine
[^{3}H]-Z	Tritiated zeatin
[8-^{14}C] BAP	Radioactive 6-benzylaminopurine
ABA	Abscisic acid
Ade	Adenine
Ado	Adenosine
AMP	Adenosine monophosphate
AP 1	*APETALA 1*
ARCK	Aromatic cytokinin
ATI	Auxin transport inhibitors
BAP	6-benzylaminopurine
BAR/BARP/BA9G	6-benzylaminopurine riboside/6-benzylaminopurine ribotide/6-benzylaminopurine 9-glucoside
BSA	Bovine serum albumin
CK	Cytokinin
CKX	*CYTOKININ OXIDASE/DEHYDROGENASE*
CO	*CONSTANS*
DAP	Days after planting
DMRT	Duncan's multiple range test
DPM	Disintegrations per minute
FLC	*FLOWERING LOCUS C*
FT	*FLOWERING LOCUS T*
FW	Fresh weight
GA_{n}	$Gibberellin_{respective\ number}$
HFCA	9–hydroxyfluorene–9–carboxylic acid
HPLC	High performance liquid chromatography

iP	Isopentenyl adenine
IPT	*ISOPENTENYL TRANSFERASE*
ISCK	Isoprenoid cytokinin
LC-MS	Liquid chromatography-Mass spectrometry
LD	Long day
LFY	*LEAFY*
LOG	*LONELY GUY*
LSD	Least significant difference
Min	Minutes
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
mT/mTR	*Meta*-topolin/ *Meta*-topolin riboside
NPA	1–N–Naphthalphthalamic acid
NSB	Non specific binding
NZ	New Zealand
oT/oTR/ oMeoT/ oMeoTR	*Ortho*-topolin/ *Ortho*-topolin riboside/ methoxy *Ortho*-topolin/ methoxy *Ortho*-topolin riboside
PAT	Polar auxin transport
PFT1	*PHYTOCHROME AND FLOWERING TIME 1*
pT/pTR	*Para*-topolin/ *Para*-topolin riboside
SAM	Shoot apical meristem
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
TBS	Tris buffered saline
TIBA	2, 3, 5–triiodobenzoic acid
Z/tZ	Zeatin/*trans*-zeatin

1. General introduction

1.1 Origin, taxonomy and morphological description

The genus *Zantedeschia* K. Spreng., native to South Africa, was originally referred to as *Calla aethiopica* by Linnaeus in his publication "Species Plantarum", and was later referred to as *Richardia* by Kunth (Traub, 1948). Since *Richardia* was already used for another genus, the current name *Zantedeschia* was proposed by Sprengel in 1826, in honour of an Italian botanist, Professor Giovanni Zantedeschi (Letty, 1973).

Zantedeschia, commonly known as Callas, Calla lilies, Arum lilies, Pig lilies, Cape Arums, Black-eyed Arums, Yellow Arums and Pink Arums (Letty, 1973), represents a relatively small genus of eight species (Singh et al., 1996), and belongs to the family Araceae, which comprises 107 genera and 2500 species of monocotyledonous herbs and vines (Grayum, 1990). A taxonomical re-assessment of the genus has been proposed by Singh et al. (1996), where *Z. albomaculata* subsp. *valida* has been promoted to species *Z. valida*. Further to this however, and of relevance to this thesis, two important groups of *Zantedeschia* have been recognized based on morphological characters, geographical distribution, and their horticulturally significant cyclic periodicity (Funnell, 1993; Singh et al., 1996):

I. The winter-flowering group, characterised by *Zantedeschia aethiopica*, in which the foliage does not die down during winter in the native habitat, and the stem is an underground storage organ, commonly called a rhizome.

II. The summer-flowering group (Aestivae), characterised by the remaining species that exhibit complete foliage senescence during winter, and flower during the summer months. The underground storage organ is a stem tuber.

The winter-flowering group is characterised by the presence of predominantly white coloured floral spathes, whereas the summer-flowering group, also commonly referred to as coloured calla lilies (Naor and Kigel, 2002; 2005a), produces floral spathes with a wide range of colours, including yellow, pink, brown, and red. *Zantedeschia* cv. 'Best Gold', used in the current study, is a commercially significant cultivar which belongs to the summer-flowering group, and produces yellow-coloured floral spathes. Given the differences evident in the morphology and cyclic periodicity of the two groups of

Zantedeschia, the focus of the following literature review and henceforth the entire thesis will primarily be associated with selections within the summer-flowering group, like 'Best Gold'.

A detailed morphological description of the genus and the corresponding species has been provided by Funnell (1993). Briefly, they are perennial herbaceous geophytes, with deciduous or evergreen leaves, long petioles and peduncles bearing the inflorescence, which comprises the spadix with pistillate and staminate flowers, subtended by sub-cylindrical to funnel-shaped spathes. In the current study, the spathe and spadix are collectively referred to as a flower (Fig. 1.3b). The buds, present in the underground tuber/rhizome, are the direct sites of floral initiation (Halligan et al., 2004; Fig. 1.3a).

1.2 Commercial significance

Production of *Zantedeschia* as a garden ornamental, potted flowering plant or cut flower is now established worldwide. In 2009, *Zantedeschia* ranked 13th among the most important cut flowers, based on sales value in international flower auctions (Benschop et al., 2009). The commercially significant parts of the plant comprise the flower and the tuber (Funnell, 1993). Since the size of the tuber and the number of buds present on the tuber (Fig. 1.3a) bear a direct relationship to the floral productivity of the plant (Corr and Widmer, 1991; Funnell et al., 1992; Naor et al., 2005a), any technology that increases these factors, and henceforth floral productivity, will be beneficial for the commercial cultivation of *Zantedeschia*. This motivation therefore sets the scene for the industry-oriented objectives directing the research presented within this thesis.

In New Zealand (NZ), *Zantedeschia* are the second most exported cut flower crop after orchids (Aitken and Hewett, 2009). Japan is the largest importer of cut flowers, and flower exports from NZ increased from 4.79 million stems in 2004-2005 to 5.13 million stems in 2005-2006 (Parminter et al., 2006). In 2000, NZ accounted for the production of 45% of the flowering sized tubers of *Zantedeschia* worldwide, with the United States and The Netherlands together supplying 50%, and the remaining 5% being produced by India, Sri Lanka and Central America (Kuehny, 2000). In 2009, the export value of *Zantedeschia* flowers and tubers stood at $NZ 4.7 million and $NZ 4.3 million, respectively (Aitken and Hewett, 2009). Therefore, research with *Zantedeschia*, targeted

either towards breeding of new cultivars, increasing floral productivity, and/or mitigating post harvest and pest/disease problems, has commercial potential.

1.3 Growth habit of Zantedeschia

Under conventional production systems it generally takes two growth cycles to obtain flowering-sized tubers of *Zantedeschia* (New Zealand Calla Council. www.callacouncil.org.nz). During the first growth cycle of *Zantedeschia* cv. 'Best Gold' (Fig. 1.1a-d), plants developed from either seed (Fig. 1.1a) or tissue-culture plantlets (Fig. 1.1b) produce a single shoot and exhibit a period of active growth characterized by visible leaf production and development. This is followed by the cessation of leaf production and subsequent natural senescence and withering of existing leaves (Fig. 1.1c), resulting in the formation of a small-sized tuber (Fig. 1.1d). Under natural conditions, flowering competence is not attained during the first growth cycle. This inherent inability to flower and the formation of only a single shoot, enabled the use of this single-shoot system with plants of 'Best Gold' as a cost effective and uniform experimental material to investigate bud dormancy and flowering in *Zantedeschia* (refer Chapters 4 - 8).

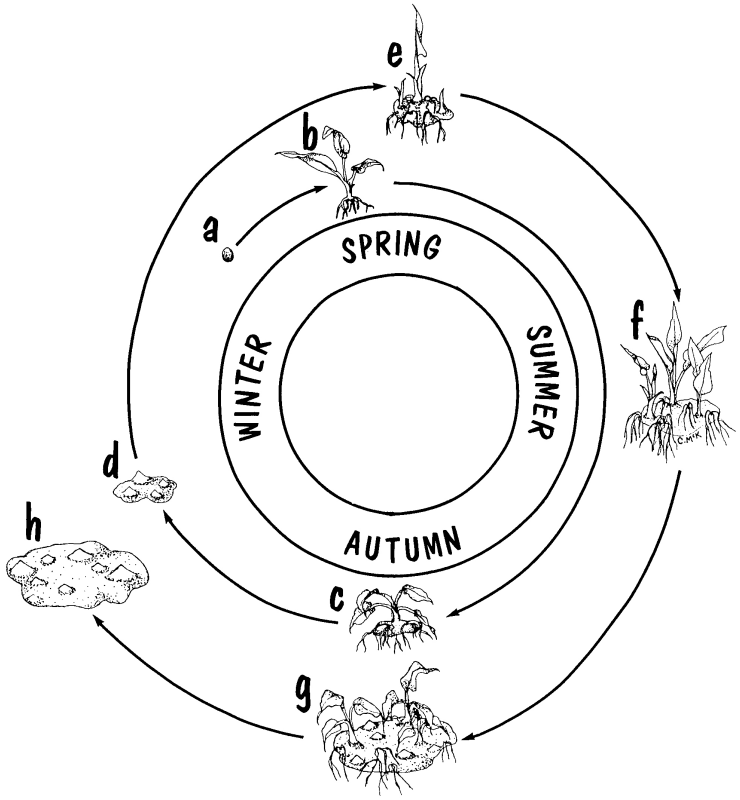


Fig. 1.1 Diagrammatic illustration of the two growth cycles of *Zantedeschia* cv. 'Best Gold'. First growth cycle comprising a single shoot from seed (**a**) or tissue culture plantlet (**b**) to a small-sized tuber (**d**); and second growth cycle comprising 3-4 primary shoots (**f**) from a small-sized tuber (**d**) to a flowering-sized tuber (**h**) (Image from New Zealand Calla Council, 2011. www.callacouncil.org.nz).

Upon planting the small-sized tuber, during the second growth cycle (Fig. 1.1d-h) 3-4 primary shoots are produced (Fig. 1.1f), and the same phenological events that occurred during the first growth cycle are observed in each shoot, resulting in the formation of a larger flowering-sized tuber (Fig. 1.1h). The second growth cycle is sometimes marked by flowering from the apical bud. Subsequent planting of the flowering-sized tuber (Fig. 1.1h) results in flowering of at least 2-3 primary shoots, followed by secondary and sometimes tertiary flowering in these shoots (Funnell, 1993). The size of the tuber therefore i.e., larger flowering-sized tuber (Fig. 1.1h) compared to small-sized tuber (Fig. 1.1d), is a key component that, amongst other factors such as the number of dominant buds (described below), also determines the floral productivity of *Zantedeschia* (Corr and Widmer, 1991; Naor et al., 2005a). Therefore, techniques that enhance tuber size are sought after by growers.

During both growth cycles, after the cessation of new leaf production, an atrophied (natural senescence before development) leaf primordium, occurs in the axil of the last-emerged leaf (Carrillo Cornejo et al., 2003; Halligan et al., 2004; Fig. 1.2). The role of this atrophied leaf primordium in the ontogeny of *Zantedeschia* has not been confirmed, albeit within this thesis it served as a feature that facilitated the demarcation of the growth cycle into distinct phases (refer Chapters 6 and 7).

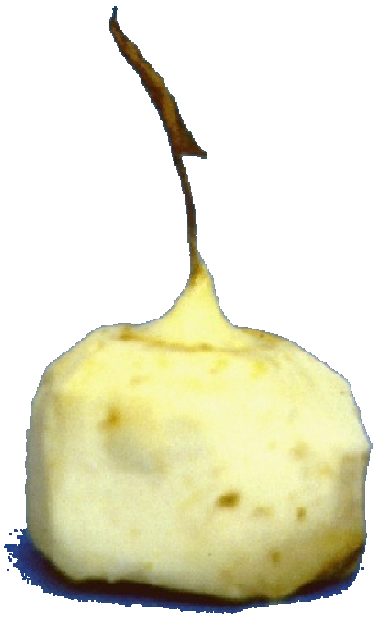


Fig. 1.2 An atrophied leaf primordium in the axil of the last-emerged leaf, during the growth cycle of *Zantedeschia* (Image from Carrillo Cornejo, 2001).

Within its growth cycle the dominant buds (Fig. 1.3a) on the tuber grow first to produce primary shoots, followed by secondary shoots located in the leaf axils of the primary shoot. This pattern can sometimes be followed by the growth of tertiary shoots from the axils of secondary shoots (Funnell, 1993). The sympodial growth habit (Reinhardt and Kuhlemeier, 2002) of *Zantedeschia*, therefore enables a cascade of growth and flowering, where floral transformation of the shoot apical meristem (SAM) (Naor et al., 2005a) of any primary shoot, initiates the growth and flowering of 2-3 axillary buds below it (Funnell, 1993; Fig. 1.3c). Floral transformation is preceded by bud outgrowth and, therefore, in *Zantedeschia*, under ideal circumstances the floral productivity is a direct consequence of the conversion rate of buds to shoots. Of horticultural relevance therefore, the greater the number of dominant buds on a tuber, the greater the number of primary shoots and, therefore floral productivity (refer Section 1.4).

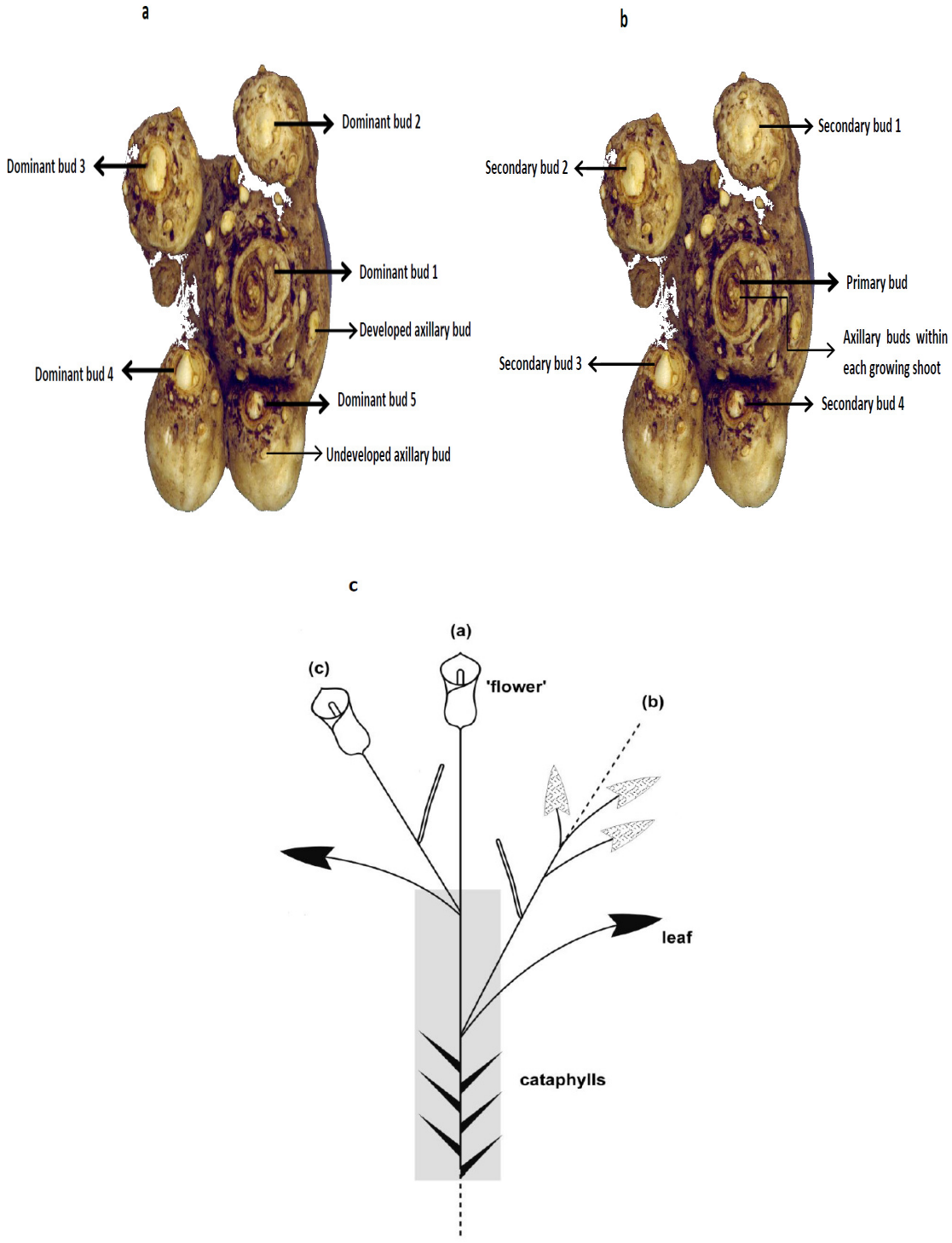


Fig. 1.3 (**a**) Flowering-sized tuber of *Zantedeschia* with variable population of buds as classified by Funnell and Go (1993); (**b**) flowering-sized tuber of *Zantedeschia* with variable population of buds as classified by Naor et al. (2005a); (**c**) shoot growth and flowering in one of the dominant buds, resulting in sympodial growth and flowering of secondary and/or tertiary shoots (Image from Halligan et al., 2004).

Flowering-sized tubers of *Zantedeschia* are characterised by a variable population of buds (Fig. 1.3). Based on their inherent ability to grow and/or flower in the presence/absence of GA, different systems of nomenclature of buds have been adopted: According to Funnell and Go (1993), dominant buds (Fig. 1.3a) initially emerge to produce primary shoots, and are capable of flowering under 'normal' conditions i.e., even in the absence of GA. Axillary buds are further classified as developed buds, which are capable of flowering in the presence of GA, and undeveloped buds, which are not prone to growth and/or flowering i.e., even in the presence of GA (Fig. 1.3a). Alternatively, Naor et al. (2005a) have defined the primary bud as the largest bud present on the tuber (Fig. 1.3b). The primary bud is the first to sprout and flower, followed by the growth and flowering of the secondary buds (Fig. 1.3b). Axillary buds are those located on the growing axis of each shoot, capable of sympodial flowering upon initiation of flowering in the primary/secondary buds (Fig. 1.3b). Due to inherent differences in the flowering potential of the variable population of buds, determining the response of the tuber/plant to different treatments has been a formidable task (Funnell and Go, 1993). Mapping the buds based on their flowering potentials is therefore recommended (refer Chapter 10). Due to prevalent usage of this system of nomenclature in literature and owing to the relative ease of understanding of this classification, the terminology adopted by Funnell and Go (1993) shall be used throughout this thesis (Fig. 1.3a).

1.4 Dormancy and floral productivity in Zantedeschia

The conversion rate of buds to shoots, which in part determines the floral productivity of *Zantedeschia* (Section 1.3), is primarily governed by an inherent developmental programme (Naor and Kigel, 2002), which in turn is executed via different degrees of dormancy. Dormancy is described as the temporary suspension of visible outgrowth of any structure containing a meristem and can be caused by: factors within the plant but outside the bud (para-), intrinsic factors within the bud (endo-) and/or, environmental factors (ecodormancy) (Lang, 1987). Year round commercial production of *Zantedeschia* is, therefore, constrained due to the alternating periods of growth and rest (Fig. 1.1) controlled by dormancy. In plant species exhibiting dormancy it is generally accepted that the three types of dormancy exist in a continuum and, at any given time, a bud may be simultaneously controlled by one or all types of dormancy (Faust et al.,

1997; Horvath et al., 2003). Research to date supports the notion that this hypothesis is equally applicable to *Zantedeschia* (Carrillo Cornejo et al., 2003; Halligan et al., 2004; Naor et al., 2005a).

As historically illustrated by Thimann and Skoog (1933), physical removal of the apical bud stimulated axillary bud outgrowth or branching, thereby demonstrating the existence of paradormancy/apical dominance in *Zantedeschia* (Clark et al., 1987). The strength of apical dominance differs between species and cultivars (Ngamau 2001a; Naor et al., 2005a), with *Zantedeschia* cv. 'Best Gold' used in this study being considered to possess strong apical dominance and, therefore, being less prone to branching (D'Arth et al., 2007). Also, within the growth cycle of a cultivar, the strength of apical dominance could decline with age (Shimizu-Sato et al., 2009). Therefore, the single-shoot system of *Zantedeschia* used for dormancy studies in this thesis (Chapters 4-7) potentially possess stronger apical dominance than the tubers used by Naor et al. (2005b). Since the floral productivity bears a direct relationship to the number of shoots or branching, a branched plant of *Zantedeschia* is desirable to increase the number of potential flowering sites (Clark et al., 1987; Funnell et al., 1992). This equation therefore provides additional focus to the underlying physiology to be explored within this thesis.

Growth of the *Zantedeschia* tuber is a function of leaf area and leaf area duration (Funnell et al., 2002). By alleviating apical dominance and, therefore, enhancing branching, the leaf area of the plant can be increased, resulting in the formation of a larger sized tuber, which in turn will potentially enhance the floral productivity of *Zantedeschia* (Corr and Widmer, 1991; Naor et al., 2005a). As subsequently developed further in this thesis, by elucidating the mechanisms that control apical dominance in 'Best Gold', it is hoped that strategies for increasing floral productivity of *Zantedeschia* may arise.

Though the ensuing establishment of endodormancy in *Zantedeschia* is acknowledged (Funnell, 1993; Naor et al., 2006), there are no visual clues that indicate the onset of endodormancy (Carrillo Cornejo et al., 2003). Halligan et al. (2004) suggested that endodormancy was likely to begin even before the cessation of new leaf production and, therefore, the cessation of new leaf production cannot be relied upon as a reliable indicator of the onset of endodormancy. A heat-unit accumulation model, using a base

temperature of 2.1°C was, therefore, developed to predict the onset and release of endodormancy in *Zantedeschia* (Carrillo Cornejo et al., 2003). According to this model, for two cultivars of the summer-flowering group, the duration of endodormancy occurred between 2145 and 2747 degree-days. While not previously applied to 'Best Gold', in the absence of any visual clues that indicate the onset of endodormancy, this heat unit model was used as a guideline in this thesis (refer Chapters 4 -7).

By alleviating endodormancy, the potential exists for the period of active growth to be extended during the two growth cycles of *Zantedeschia* (Fig. 1.1), and thereby enhancing floral productivity. The strength of endodormancy in *Zantedeschia* can be altered by the environmental conditions during the growth period. High temperature during the growth period produced small-sized tubers with increased dormancy, whereas exposure to lower temperatures produced larger tubers with less dormancy (Naor and Kigel, 2002). The demarcation of the growth cycle of *Zantedeschia* concomitant with the transition of buds from apical dominance to endodormancy, and the associated hormonal changes are topics that are subsequently explored in this thesis (refer Chapters 7 and 8).

Relaxation of endodormancy and/or imposition of ecodormancy can be achieved by storage of tubers at low temperatures (Jierwiriyipant and Tjia, 1988; Corr and Widmer, 1988; Funnell and MacKay, 1988b). Storage of tubers at 7 ± 3ºC is considered ideal to enforce ecodormancy by suppressing shoot growth (Funnell, 1993). While short-term storage may be required to break bud endodormancy (Corr and Widmer, 1988), long-term storage is employed by commercial growers to facilitate year round production of flowers (Funnell, 1993). However, concomitant with the increased durations of storage, a decline in the floral productivity of the tubers has been observed (Funnell and Go, 1993; Dennis et al., 1994). Though several reasons for this decline have been proposed (Funnell and Go, 1993), the exact mechanism of this reduction in flowering ability with increased storage duration is still largely unknown in *Zantedeschia*. As developed further in this thesis (refer Chapter 3), by elucidating the mechanisms that control this reduction, it was hypothesised that the decline in the flowering potential of *Zantedeschia* could be mitigated, thereby retaining the floral productivity.

1.5 Hormonal control of dormancy and flowering

Plant hormones are a group of naturally occurring, organic substances which influence physiological processes at low concentrations (Davies, 1995). The group of classical hormones is represented by auxin, gibberellins (GA), cytokinin (CK), ethylene and abscisic acid (ABA) (Kende and Zeevart, 1997). However, with the discovery and addition of several newer compounds into the paradigm of plant hormonal control (Gomez-Roldan et al., 2008) this group is expected to expand (Waldie et al., 2010).

Genomic information derived from *Arabidopsis* (*Arabidopsis thaliana* L.) (2000), as the first genome-sequencing effort of a flowering plant, and the subsequent sequencing of the rice genome (Goff et al., 2002; Yu et al., 2002), as the world's most important food crop, have heralded a better understanding of key physiological processes, previously unknown. This encompasses the identification of key genes involved in the biosynthesis, transport, metabolism and signal transduction of auxin (Woodward and Bartel, 2005), gibberellin (Schwechheimer, 2008), cytokinin (Kamada-Nobusada and Sakakibara, 2009; Kudo et al., 2010), ethylene (Bleecker and Kende, 2000) and ABA (Raghavendra et al., 2010).

While an optimum concentration of the hormone is crucial to evoke a physiological response (Cleland, 1983), Trewavas (1983) claimed that the sensitivity of the responding tissue was solely responsible for eliciting a response. A change in the sensitivity of the responding tissue can be measured by a change in the magnitude of an induced response to a known amount of exogenous hormone (Firn, 1986) and is, therefore, best demonstrated by dose-response curves (Weyers et al., 1995). The sensitivity status of the responding tissue is determined by several factors including uptake, transport, metabolism, receptor status and downstream signal transduction cascades (Firn, 1986). A general consensus has now emerged, that the concentration of the hormone and the sensitivity of the responding tissue are both essential to elicit a physiological response (Weyers and Paterson, 2001).

The physiological response initiated by hormonal action is further compounded by the pleiotropic nature of plant hormones (Davies, 1995). The advent of molecular/genetic approaches have aided in deducing interactions/cross-talk between the major hormones in the control of key developmental processes. Between CK and GA, meristem fate is regulated through reciprocal interactions between both of them, where CK inhibits GA

biosynthesis (Jasinski et al., 2005) and GA inhibits CK synthesis (Greenboim-Wainberg et al., 2005). Auxin up-regulates the genes involved in the rate limiting step of GA biosynthesis (Ross et al., 2001), and down-regulates the rate limiting step of CK biosynthesis (Nordström et al., 2004). Cross-talk between GA and other hormones (Weiss and Ori, 2007), auxin and other hormones (Chandler, 2009), as well as CKs and other hormones (Rashotte et al., 2005), have been described in detail.

In the control of apical dominance, root synthesis (Bangerth, 1994), local biosynthesis (Tanaka et al., 2006) and/or direct application of CK to the suppressed buds (Sachs and Thimann, 1967; Ali and Fletcher, 1970) is suggested to stimulate branching by playing an antagonistic role to terminally derived auxin (Dun et al., 2006). Recently, a new dimension has been added to our understanding of the control of branching, where a new group of hormones called 'strigolactones' have been identified to be transported acropetally and capable of inhibiting branching (Gomez-Roldan et al., 2008). Interaction between auxin, CK and strigolactone in the control of branching has, therefore, become an emerging area of investigation (Ferguson and Beveridge, 2009). While investigating the role of strigolactones in the control of branching was beyond the scope of this study, the recent findings in other plant species (Waldie et al., 2010) can provide an ideal platform for further exploration with *Zantedeschia*.

In the control of endodormancy, the antagonistic relationship between two different hormones, ABA and GA is widely implicated (Tanino, 2004). Exogenous application of synthetic ABA induced dormancy in woody perennials (El-Antably et al., 1967), and soaking of dormant potato tubers in a GA solution, resulted in the breaking of dormancy (Brian et al., 1955). Though CK have been shown to release buds from endodormancy (Turnbull and Hanke, 1985a), their role in endodormancy is still poorly understood (Horvath, 2009).

Expression of key flowering genes involved in floral transition i.e., *FT* and *LFY*, are regulated by the GA signalling pathway (Boss et al., 2004). While the role of GA in the control of flowering time is now established, the interface between the GA pathway and other flowering related pathways i.e., photoperiod, vernalization, and autonomous pathways, are beginning to be defined (Davis, 2009). Though molecular/genetic evidence for the control of flowering by CK is lacking, they probably play a role in the development of floral organs after floral initiation (Lejeune et al., 1994) by regulating

cell cycle genes involved in mitosis (Horvath et al., 2003). The photoperiod and vernalization moderated mechanisms that determine flowering time share common features with the onset of endodormancy (Horvath et al., 2003). Accordingly, the *FT* homolog of *Arabidopsis* in Poplar (*Populus* × *robusta*), the gene necessary for day length regulation of flowering, was also shown to control the day length dependent induction of endodormancy (Böhlenius et al., 2006). In *Zantedeschia*, day-neutral to flowering (Funnell, 1993), it is hypothesised that additional/exclusive mechanisms of flowering control may be in practice. Since the *FT* module is regulated by plant hormones, it is also possible that hormonal cross-talk mediates the common mechanisms between flowering and dormancy (refer Chapter 7).

Exogenous application of CK stimulated branching in tubers of *Zantedeschia* controlled by apical dominance (Naor et al., 2005b). However, the response of *Zantedeschia* to the application of CK after the inception of endodormancy has not been reported previously. Exogenous application of ABA and GA to *Zantedeschia* failed to impose and release buds from endodormancy, respectively, and the onset of endodormancy did not coincide with an increase in endogenous ABA levels (Naor et al., 2008). Also, the endogenous GA_3 content remained relatively low or constant throughout the growth period (Naor et al., 2008). Hence this raises the question as to whether the antagonistic relationship between ABA and GA established in other plant species, regulates endodormancy in *Zantedeschia* as well. In a few geophytes such as onion (*Allium cepa* L.) and gladiolus (*Gladiolus communis* L.), endodormancy release occurred only with the application of CK (Chrungoo, 1992). Though CK was effective in the release of endodormancy in potato, the sensitivity of the buds to CK was not consistent throughout the duration of endodormancy (Turnbull and Hanke, 1985a). Within this thesis, it was therefore suggested that the exogenous application of CK during the transition of buds from apical dominance to endodormancy, and subsequently after the establishment of endodormancy in *Zantedeschia*, may provide meaningful insights into the hormonal control of endodormancy.

In *Zantedeschia*, day-neutral to flowering (Funnell, 1993), exogenous application of GA promotes flower initiation (Corr and Widmer, 1987) irrespective of the meristem size and age (Naor et al., 2005a), and a spike in the endogenous GA concentration was observed during floral transition (Naor et al., 2008). The application of GA however, does not guarantee floral emergence. In shoots where floral initiation had occurred but

floral emergence had failed, development of the inflorescence had been arrested and there were signs of atrophy (Brooking and Cohen, 2002). This lack of conversion of floral initiation to floral emergence could possibly be due to the establishment of endodormancy. So far, the ability of GA to promote flowering in *Zantedeschia* after the establishment of endodormancy has not been reported. Long-term cold storage of *Zantedeschia* tubers, a strategy adopted by commercial growers to facilitate year round production of flowers (Funnell, 1993), is accompanied by a decline in flowering with increased durations of storage (Funnell and Go, 1993). Since GA is crucial for floral transition in *Zantedeschia* (Corr and Widmer, 1987), a change in the endogenous GA content and/or a decline in the sensitivity of the buds to GA were proposed by Funnell and Go (1993), but not examined. Within this thesis, it was suggested that investigating these hypotheses may provide fundamental knowledge on the decline in flowering associated with increased storage durations. Pursuing this line of research, based on the results obtained, may lead to the development of techniques that aid in mitigating this decline and, therefore, enhancing floral productivity.

Anomalies exist in the hormonal control of dormancy and flowering in *Zantedeschia*. While CKs were shown to alleviate apical dominance in *Zantedeschia*, and thereby stimulate branching (Naor et al., 2005b), their role in the control of endodormancy is poorly understood. The inability of ABA and GA to impose and release buds from endodormancy in *Zantedeschia* (Naor et al., 2008), respectively, further suggests a crucial role for CKs in the control of this process. Given that the number of shoots/branching bears a direct relationship to floral productivity in *Zantedeschia* (Funnell et al, 1992), and the degree of branching in turn is determined by the strength and type of dormancy, it is essential to have a better understanding of the hormonal control of these processes to achieve the commercial objective of increased floral productivity. The recent schema proposing common mechanisms between dormancy and flowering (Horvath, 2009) further underlines the need to elucidate the hormonal control of these two key developmental processes in *Zantedeschia*.

1.6 Aim and scope of the study

The scope of this study was primarily driven by the commercial objective to increase the floral productivity of *Zantedeschia*. The floral productivity of *Zantedeschia* is dependent on the conversion rate of buds to shoots, which in turn is controlled by an innate developmental programme (Naor and Kigel, 2002) executed via different degrees of dormancy during the growth cycle (Fig. 1). While the hormonal control of dormancy (Wareing and Saunders, 1971) and flowering (Davis, 2009) is undeniable, with regard to *Zantedeschia* the existing literature does not provide a clear understanding of these phenomena. The scope of this study was, therefore, formulated to deliver a better understanding of the physiological basis of branching and flowering in *Zantedeschia*, governed by hormonal control, with the aim of achieving the commercial objective.

Consequently, two major approaches were undertaken simultaneously to attain the commercial objective, with the aim of pursuing just one approach further, based on the results obtained.

1. To analyse and alleviate the decline in flowering observed with increased durations of storage. A key component of the commercial production process, as described in Section 1.4, is the cold storage of tubers for long durations. This long-term storage is accompanied by a decline in the flowering ability of the tubers in the next growth cycle. Funnell and Go (1993) suggested the following reasons for the decline in flowering observed with extended durations of storage;

    a) Low endogenous GA level.

    b) Change in sensitivity of buds to exogenous GA application.

    c) Reduced uptake of GA.

    To determine which of these factors was involved in the decline in flowering with storage, it was viewed that the model proposed by Firn (1986), wherein changes in dose-response curves are interpreted, would provide some answers and directions for further research. Therefore, to explore this facet further, longer durations of storage than those used by Funnell and Go (1993) were proposed to be tested (refer Chapter 3).

2. Stimulation of branching by alleviating apical dominance. As detailed in Section 1.4, this approach was considered warranted because branching in *Zantedeschia* bears a direct relationship to floral productivity (Clark et al., 1987; Funnell et al., 1992). Stimulation of axillary bud outgrowth or branching has traditionally involved the impedance of polar auxin transport (PAT) and/or the enhanced supply of CK (Dun et al., 2006). As developed in Chapters 4 and 5 the impedance of PAT was attempted by both manual and chemical methods. Pilot studies involving the exogenous application of CK was carried out simultaneously (Chapter 6), and if successful was planned to be explored further so as to determine its role in the control of endodormancy and, thereby, flowering.

2. Review of literature

2.1 Definition and types of dormancy

Dormancy is the temporary suspension/absence of visible outgrowth of any structure containing a meristem, and can be caused by factors outside the bud but within the plant (paradormancy), intrinsic factors within the bud (endodormancy) and/or environmental factors (ecodormancy) (Lang, 1987). However, growth within a meristem is not readily 'visible' and the term 'absence' of growth is ambiguous because dormancy constitutes an inability to initiate growth. Therefore, drawing references from a seed perspective, bud dormancy can be defined as the inability to initiate growth from meristems under favourable conditions (Rohde and Bhalerao, 2007). While the former definition illustrates the result of dormancy, the latter does not adequately describe the parameters of favourable conditions, and moreover lacks a time factor. Surely, visible growth is a non-definitive term, and in geophytes means growth above the soil surface. The inability to initiate growth, on the other hand, represents a characteristic feature of the dormant state. Therefore, collating the salient features of both these definitions, in a more strict sense, bud dormancy could be described as a state/condition of the bud characterized by a period of inability to initiate physical growth.

Though buds and seeds exhibit periods of dormancy, the respective mechanisms of control could be largely different due to the inherent nature of these organs (Olsen, 2003). Since the present study in *Zantedeschia* pertains to bud dormancy, this will be the focus throughout the thesis. Dormancy exists in a continuum (Tanino, 2004) where the induction, maintenance and release of the three types of dormancy overlap each other (Faust et al., 1997) (Fig. 2.1). Apical dominance exists in *Zantedeschia* (Ngamau, 2001a), where scooping/decapitation of the apical bud resulted in the growth of 3-4 axillary buds (Clark et al., 1987). The strength of apical dominance varies between cultivars (Naor et al., 2005a), and *Zantedeschia* cv. 'Best Gold' used in the current study is considered to possess strong apical dominance (D'Arth et al., 2007). Moreover, within a species/cultivar, the degree of

apical dominance could decline with age (Shimizu-Sato et al., 2009). In our experience, seedlings of *Zantedeschia* cv. 'Best Gold' produce only a single-shoot (the single-shoot system used for dormancy studies in this thesis) during their first growth cycle (Fig. 1.1a-d), whereas at least 2-3 primary shoots are produced during their second growth cycle (Fig. 1.1d-h), thereby depicting a decline in the strength of apical dominance with the age of the plant.

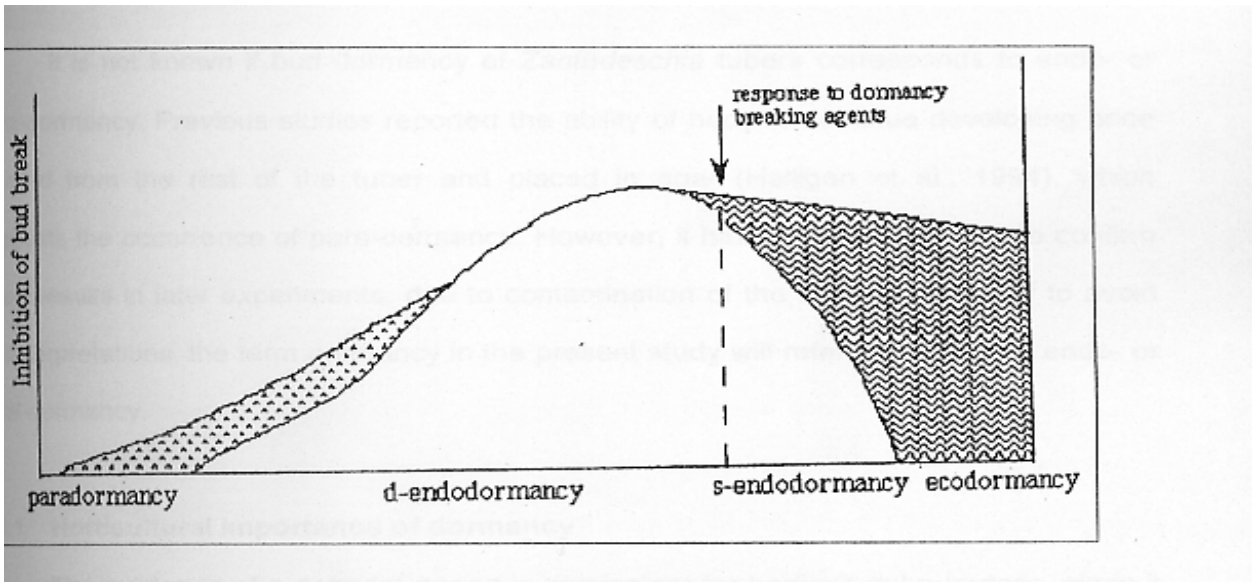


Fig. 2.1 Diagrammatic representation of the continuum of dormancy, marked by overlapping phases of para-, endo- and ecodormancy, differentiated based on the response of buds to dormancy breaking agents (Image from Faust et al., 1997).

Though the ensuing establishment of endodormancy is acknowledged (Carrillo Cornejo et al., 2003), there are no visual clues to the onset of endodormancy (Halligan et al., 2004). Unlike other plant species where endodormancy is induced by environmental signals (Tanino, 2004), summer-flowering *Zantedeschia* have an autonomous, predetermined developmental cycle, in which the induction of endodormancy and the associated changes, such as the cessation of new leaf production and leaf senescence, do not necessarily require any external signals (Naor and Kigel, 2002). Therefore, the cessation of new leaf production cannot be relied upon as an indicator of the onset of endodormancy, and

endodormancy could commence even before this process (Halligan et al., 2004). While short term cold storage of tubers is required to mitigate bud endodormancy (Corr and Widmer, 1988), long term storage via imposed ecodormancy is capable of inhibiting subsequent shoot growth (Funnell, 1993). This method is employed by commercial growers for scheduled planting and flowering (Funnell and Go, 1993).

2.2 The plant hormone concept

Plant hormones are a group of naturally occurring, organic substances which influence physiological processes at low concentrations (Davies, 1995). The plant hormone concept was initially based on the animal model where the hormone concept fulfilled the fundamental elements of synthesis in one organ, transport, and action in a second organ (Weyers and Paterson, 2001). It was soon discovered that plant hormones do not always follow these tenets derived from the animal model, because synthesis could occur in several organs and action could occur in the synthesized organ (Davies, 1995). Since then, the plant hormone concept has been considered to be unique in its own respect, and a distinct set of criteria have been proposed for an organic substance to qualify as a plant hormone (Jacobs, 1959).

Though several compounds have been added to the list of plant hormones (Santner and Estelle, 2009), auxin, gibberellins (GA), cytokinin (CK), abscisic acid (ABA) and ethylene are still considered to be the classical hormones (Kende and Zeevart, 1997; Fig. 2.2). Though each of these classical hormones has a characteristic effect on plant growth, a single physiological process could be affected by several hormones and a single hormone may affect several physiological processes (McCourt, 1999). Cross-talk between hormones could be direct, indirect or co-regulated (Chandler, 2009), and new insights into this intricate network of hormonal cross-talk are being postulated (Jasinski et al., 2005; Rashotte et al., 2005; Weiss and Ori, 2007). An understanding of this cross-talk has been further compounded by the discovery of new plant hormones, such as strigolactones (Gomez-Roldan et al., 2008) and their induction into the existing hormonal network (Ferguson and Beveridge, 2009). Nevertheless, though it may seem complex in the

beginning, these studies will eventually provide a better understanding of the hormonal control of key developmental processes (Ross and O'Neill, 2001). Cross-talk between CK and GA in the regulation of dormancy and flowering progressively became a key aspect of the current study in *Zantedeschia*.

In the hormonal control process, two factors determine the scale of physiological response:

1. Concentration of the hormone.

   Again, in line with thinking derived from the original animal model, the concentration of the hormone was considered to be the sole determinant of a physiological response (Cleland, 1983). However, correlations between the concentration of the hormone and the corresponding physiological response, were not always proportional (Weyers and Paterson, 2001). This inconsistency in the correlation can be caused by the:

   a) Lack of accurate measurement of hormone concentrations.

      Endogenous hormones exist in very low concentrations i.e., cytokinins are present at less than 30 pmol/g fresh weight (Mok and Mok, 2003) and, therefore, the ability to successfully extract and identify such small quantities of metabolites could be a potential cause for inaccurate measurements (Hedden, 1993). However, the latest state-of-the-art techniques (Birkemeyer et al., 2003; Pan and Wang, 2009) have provided a better understanding of the identification and quantification of hormones.

   b) Several forms of plant hormones.

      The number of identified gibberellins has now crossed over one hundred (Richards et al., 2001), and naturally occurring cytokinins could belong to the isoprenoid or aromatic group (Mok and Mok, 2003), with several isomers present within each group. The bioactivity of these forms could be spatially and temporally different, which further exacerbates the correlation between the concentration of a particular isoform and the physiological response.

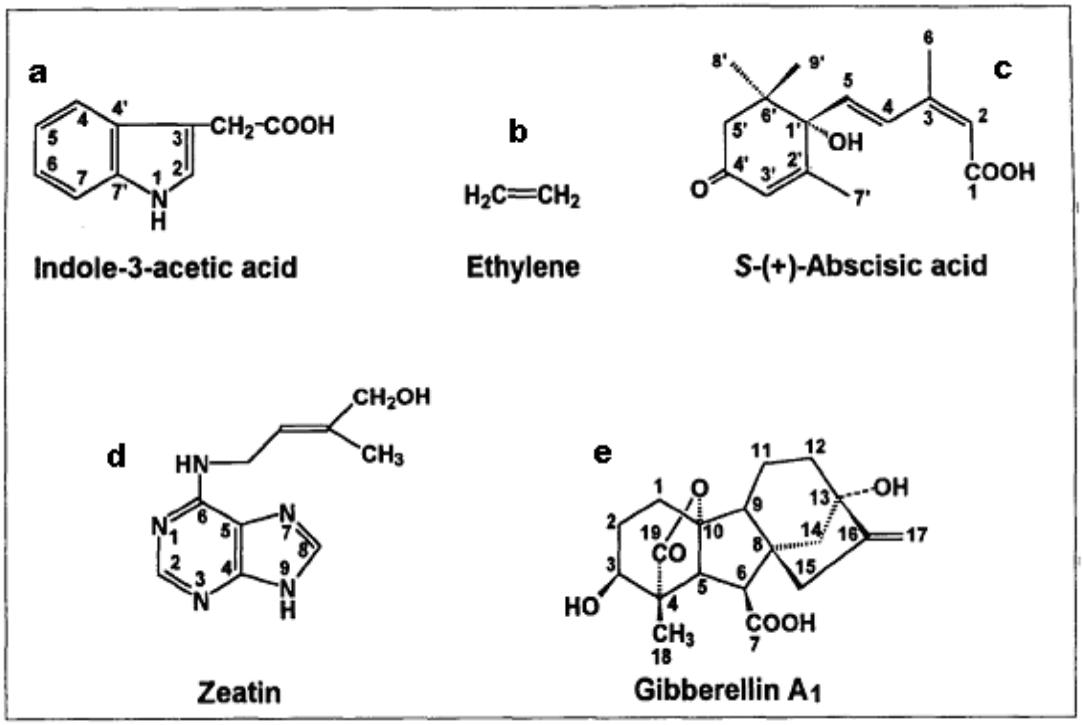


Fig. 2.2 Chemical structures of the five classical plant hormones represented by **a**) auxin (Indole-3-acetic acid) **b**) ethylene **c**) ABA (Abscisic acid) **d**) CK (Zeatin) and **e**) GA (Gibberellin A_1) (Image from Kende and Zeevart, 1997).

c) Metabolism of plant hormones

Plant hormones are capable of rapid metabolism. For example, exogenously applied cytokinins were rapidly metabolized to reversible and irreversible metabolites (Jameson, 1994). Similar to the presence of several natural forms, the metabolism of plant hormones is also spatially and temporally regulated. This rapid metabolism of the active form and the presence of several metabolites further hinder the establishment of a correlation between the hormone concentration and the physiological response elicited.

2. Sensitivity of the responding organ/tissue

While the concentration of the hormone is indispensable to elicit a physiological response, Trewavas (1983) challenged the notion that concentration was the sole determinant of hormonal control. The lack of correlation between hormonal

concentration and the corresponding physiological response further supported his proposal that the sensitivity of the responding tissue was solely responsible to stimulate a response (Davies, 1995). In a bid to resolve this stand-off between these divergent opinions, Firn (1986) attempted to develop the concept of sensitivity changes more fully.

Consequently, a change in the sensitivity of the responding cell or organ was described as a change in the magnitude of an induced response to a known amount of exogenous hormone (Firn, 1986). Therefore, sensitivity changes are best demonstrated by dose-response curves (Weyers et al., 1995), and Firn (1986) provided a modelling approach suggesting factors that contribute to sensitivity changes (Fig. 2.3). According to this approach, sensitivity changes can be caused by changes in

a) Receptivity – number of receptors
b) Affinity of the receptors
c) Response capacity of the signal transduction chain
d) Endogenous hormone concentration
e) Uptake efficiency
f) Metabolism
g) Transport *en route* to the site of action

2.2.1 Sensitivity changes and Firn's graphs

According to Firn's (1986) approach (Fig. 2.3), with an ideal dose-response curve (Fig. 2.3A), where a known concentration of a hormone binds to the receptor, the physiological response follows a sigmoidal curve. A change in the affinity of the receptor, compared to the standard (Fig. 2.3A), would show as a displacement of the curve along the x-axis, with the shape of the curve remaining the same (Fig. 2.3B). A change in the response capacity of the receptor system or signal transduction, as depicted in Fig. 2.3C, would show a similar response as the standard curve (Fig. 2.3A), up to a certain concentration of the hormone, after which the system is saturated. Fig. 2.3D is exhibited when the endogenous hormone

concentration of the responding tissue is higher than that in the standard (Fig. 2.3A), at the beginning of the experiment. Finally, Fig. 2.3E depicts a decline in the number of receptors or receptivity, where a change in the slope of the response curve occurs compared to the standard curve.

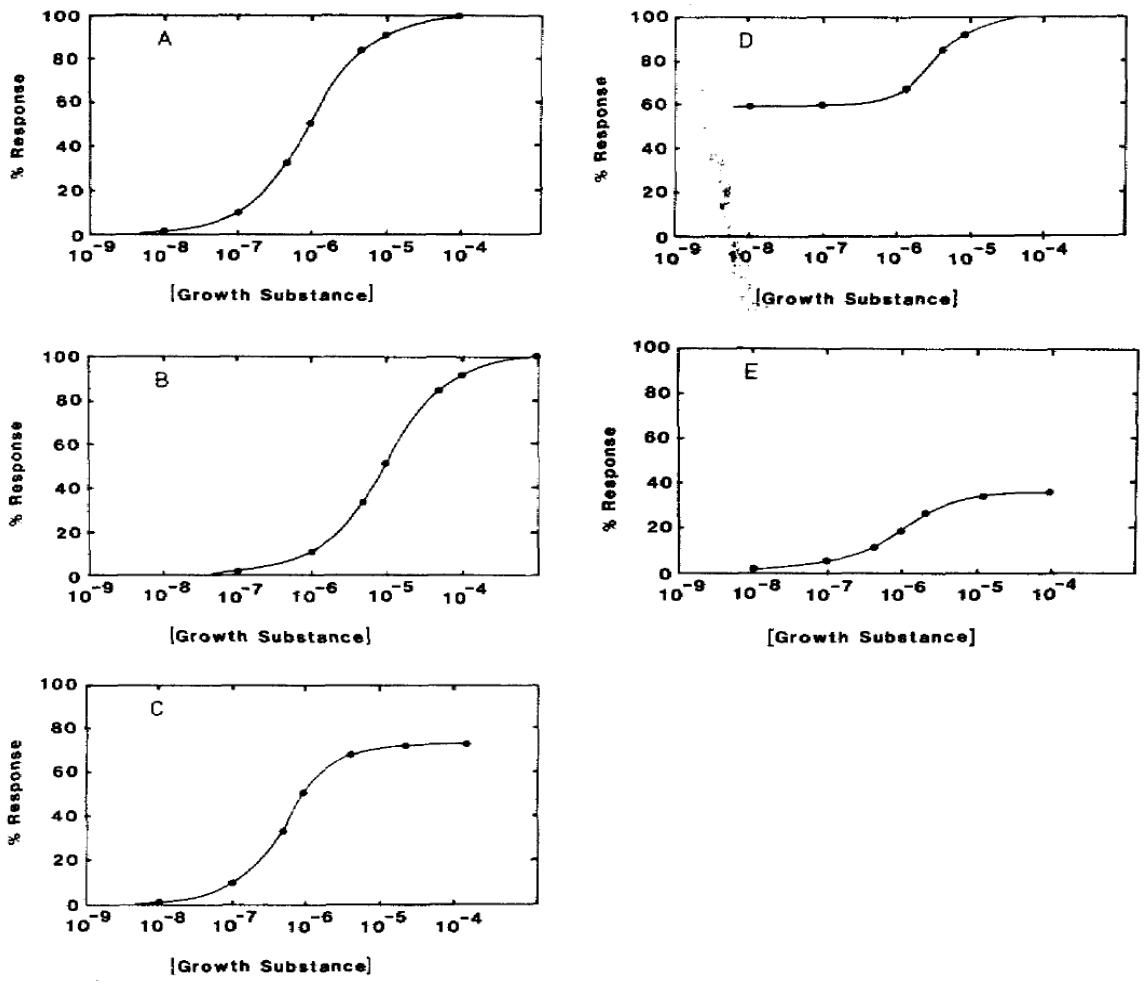


Fig. 2.3 Five forms of dose-response curves corresponding to changes in sensitivity within a responsive cell, as proposed by Firn (1986) representing: **A** the standard, **B** a change in the affinity, **C** a change in the response capacity of the signal transduction system, **D** high endogenous hormonal content and, **E** a change in the receptivity or the number of receptors (Image from Firn, 1986).

Following the identification of the receptors of several plant hormones (Chang et al., 1993; Ueguchi-Tanaka et al., 2005; Heyl and Schmulling, 2003), there is now a greater scope to corroborate the sensitivity concept. Currently however, a general consensus has now been arrived at, where the concentration of the hormone and the sensitivity of the responding tissue are both responsible to elicit a physiological response (Weyers and Paterson, 2001). The present study in *Zantedeschia* explores the roles of the concentration of the hormones (CK and GA) and the changes in the sensitivity of the buds to exogenous hormone applications, during the transition of buds from apical dominance through to ecodormancy via endodormancy (Chapters 3, 7 and 8).

2.3 Hormonal control of dormancy

Due to their sessile nature, plants need to maintain a strict control of growth processes, where plant hormone interactions play a pivotal role (Galston and Davies, 1969). In apical dominance, the communication between two distal organs (apical and axillary buds) is mediated through hormonal interactions (Ferguson and Beveridge, 2009). Changes in day-length and temperature are the primary regulators of the induction, maintenance and release of endodormancy in several plant species (Tanino, 2004). These environmental signals are translated into physiological and/or morphological responses through the action of plant hormones (Santner and Estelle, 2009). Likewise, inhibition of bud outgrowth induced by ecodormancy was found to correlate with changes in endogenous hormone concentrations (Naor et al., 2008). The following sections deal with the hormonal control of the three types of dormancy in detail.

2.3.1 Apical dominance

The basic architecture of a plant is laid down during embryogenesis (McSteen and Leyser, 2005). This architecture which determines the degree of branching is plastic enough to be modified by environmental signals, but is also genetically controlled (Shimizu-Sato and Mori, 2001). This allows for a diverse range of branching capabilities in different plant species (McSteen and Leyser, 2005). However, these developmental changes in the shoot system are different episodes in the life of a single organ, the SAM (Poethig, 1990). The

inhibitory control of the shoot apex over the outgrowth of axillary buds is broadly termed as apical dominance (Napoli et al., 1999). Therefore, since its formation, axillary buds may remain in one of the three stages (Shimizu-Sato and Mori, 2001; Dun et al., 2006): a stage of dormancy, a transitional stage where the bud may proceed to growth or revert to dormancy, and a stage of sustained growth (Fig. 2.4).

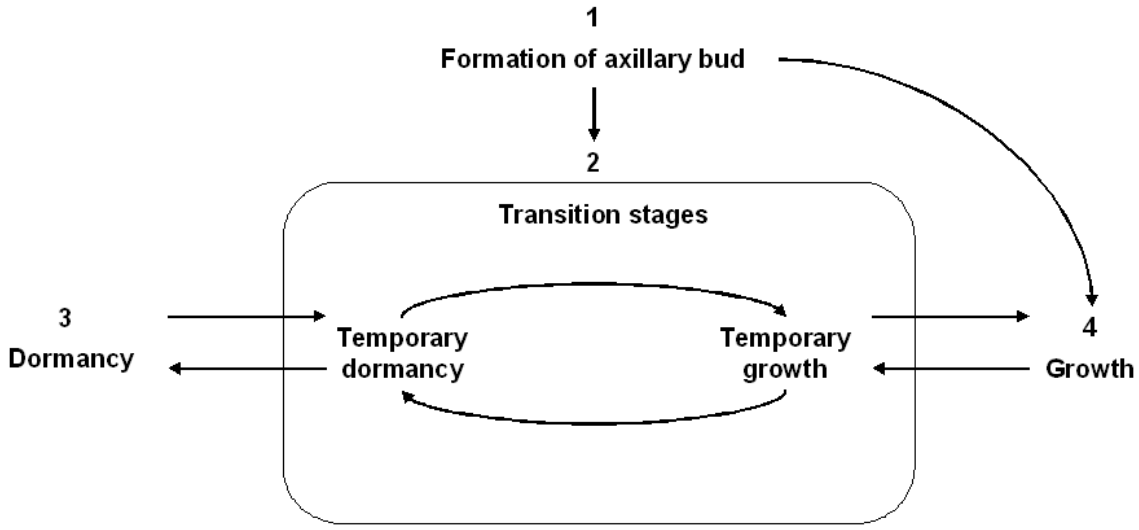


Fig. 2.4 Diagrammatic representation of different stages of growth of axillary buds involving **1**) formation of the axillary bud **2**) transitional stage either towards **3**) sustained dormancy or **4**) sustained growth (Image from Shimizu-Sato and Mori, 2001).

Apical dominance has been examined through different approaches i.e., physiological, genetic and molecular (Shimizu-Sato and Mori, 2001). Considering the scope of this study, the physiological approach shall be dealt with in detail. The physiological approach is mainly concerned with the role of hormones (Shimizu-Sato and Mori, 2001) and the bud transition hypothesis (Dun et al., 2006).

2.3.1.1 Auxin

Auxin biology is among the oldest fields of plant research (Woodward and Bartel, 2005). That auxin synthesized in the apical bud was responsible for the inhibition of axillary bud outgrowth was demonstrated by the classical decapitation and auxin replacement

experiments (Thimann and Skoog, 1933; 1934). While the basipetal transport of auxin was acknowledged (Thimann and Skoog, 1934), the ‘direct theory’ of correlative inhibition proposed that auxin moved up the axillary bud to impose inhibition of its growth (Le Fanu, 1936). Snow (1929) reported that rather than the apical bud itself, the young leaves in the apical region were responsible for the export of auxin. This was corroborated by Thimann and Skoog (1934), who showed that the amount of auxin produced, was roughly in inverse proportion to the age of the leaf. Albeit, auxin produced by mature leaves also contributes to the overall auxin economy of the plant (Jager et al., 2007).

The ‘direct theory’ of inhibition by auxin was refuted, mainly because auxin is not capable of acropetal transport into the suppressed axillary bud (McSteen and Leyser, 2005), and radiolabeled auxin was not found to enter the axillary buds (Hall and Hillman, 1975). However, a relatively new theory highlights the ability of an axillary bud to export auxin to the mainstream auxin transport system in the stem, which in turn determines its growth potential (Leyser, 2005). In an intact plant, auxin loaded onto the polar auxin transport (PAT) system is transported to its full capacity in the basipetal direction, thus limiting the export of auxin from the axillary bud. A decline in this capacity of auxin flow in the mainstream PAT system, caused by decapitation or by the application of auxin transport inhibitors (ATI), enables the export of auxin from the axillary bud, and thereby initiates axillary bud outgrowth (Leyser, 2005; McSteen and Leyser, 2005; Leyser, 2009). The enhanced auxin flow in the PAT system of the highly branched *rms* mutants of pea further corroborated the auxin flow hypothesis (Dun et al., 2006). However, further studies are required to validate this hypothesis.

The dismissal of the ‘direct theory’ obviously led to the hypothesis that a second messenger, capable of acropetal transport, was involved in the control of apical dominance (Le Fanu, 1936; Leyser, 2005). CK, GA, ABA and ethylene were probable candidates for the second messenger (Chatfield et al., 2000). Among these options, the case was strongly in favour of CKs, because the direct application of CK to the suppressed axillary buds, stimulated outgrowth (Sachs and Thimann, 1967), and decapitation led to an increase in the export of CK from the roots (Bangerth, 1994). This antagonistic relationship between auxin

and CK was further corroborated by the finding that auxin regulates CK biosynthesis (Nordstrom et al., 2004). Snow (1929) had suggested that the inhibitory signal was able to move upwards in the inhibited shoot, at least for a short distance. Recently, auxin has also been suggested to up-regulate the synthesis of strigolactones (Ferguson and Beveridge, 2009), which are capable of branching inhibition (Gomez-Roldan et al., 2008). A clear picture on the interaction between cytokinin and strigolactones is still not available, though both are primarily synthesized in the roots (Waldie et al., 2010). Strigolactones could most likely fit the description of the upward, mobile, and inhibitory signal proposed by Snow (1929).

2.3.1.2 Cytokinins

CKs are ubiquitous hormones (Stirk and Van Staden, 2010) with a characteristic trait of promoting cell division (Miller et al., 1955). In plants, they regulate diverse developmental and physiological processes such as the regulation of root and shoot growth, branching, chloroplast development, leaf senescence, stress response and pathogen resistance (Heyl and Schmülling, 2003). The roots are considered to be the primary site of biosynthesis (Letham, 1994), though synthesis in the stem adjacent to the axillary bud is also possible (Tanaka et al., 2006). Natural CKs are adenine derivatives that belong to either the isoprenoid group (ISCKs), which possess an aliphatic N^6-side chain or the aromatic group (ARCKs), which possess an aromatic side chain (Mok and Mok, 2003). The ISCK group is mainly represented by zeatin (Z) and isopentenyl adenine (iP)-type of CKs, whereas the ARCKs primarily comprise 6-benzylaminopurine (BAP) and its hydroxylated analogues called topolins (Strnad, 1997). Topolins have been identified naturally in *Zantedeschia* (das Neves and Pais, 1980a), and in this study, their transport and metabolism in relation to dormancy changes, evolved into a topic of detailed study (refer Chapters 8 and 9). A review of literature exclusively on the current research status and applications of topolins was therefore deemed vital for this thesis, and subsequent research in *Zantedeschia* (refer Section 2.3.1.2.1).

CKs are traditionally used to promote branching in several plant species, by alleviating apical dominance (Miller, 1961; Bubán, 2000; Shimizu-Sato et al., 2009). This involves an antagonistic relationship between auxin and CKs, where the ratio between the two hormones rather than their absolute amounts, determines the degree of branching (Leyser, 2005). The antagonistic relationship between auxin and CKs has been corroborated by studies where decapitation led to an increased CK export from the roots (Bangerth, 1994) and subsequent delivery to the axillary buds (Turnbull et al., 1997). Further, auxin directly regulates the expression of *isopentenyl transferase* (*IPT*) genes (Nordstrom et al., 2004) which encode a key enzyme in the CK biosynthetic pathway (Kakimoto, 2003). The presence of auxin is also required to mediate CK induced cell division (Miller, 1961). CKs did not stimulate branching when the axillary bud was completely isolated from the stem (Peterson and Fletcher, 1973), suggesting that CK must interact with a stem factor, probably auxin, before bud outgrowth.

Initial studies on branching advocated the direct application of CKs to the suppressed axillary buds to stimulate their outgrowth (Sachs and Thimann, 1967; Ali and Fletcher, 1970). Subsequently, both ^{14}C-zeatin (Van Staden, 1982) and ^{14}C-BAP (Abo-Hamed et al., 1984) were shown to be transported from the subtending leaf to the corresponding axillary bud, and thereby promote branching indirectly. In *Zantedeschia*, the buds are present on an underground tuber (Funnell, 1993). Therefore, direct application of CKs to the buds is impossible after planting. A foliar application of BAP however stimulated branching (Naor et al., 2005b). As mentioned earlier, the strength of apical dominance varies between cultivars (Naor et al., 2005a) and with age within a cultivar (Shimizu-Sato et al., 2009). Previous studies have used tubers or plantlets *in vitro* (Naor et al., 2005b) to analyse the effect of BAP. However, *Zantedeschia* cv. ‘Best Gold’ is considered to possess strong apical dominance (D’Arth et al., 2007) and this strength could vary depending on the growth cycle (Fig. 1.1). Therefore, the role of CKs in alleviating apical dominance in this cultivar of *Zantedeschia*, during the first growth cycle, could provide additional information on the control of apical dominance in *Zantedeschia*. The knowledge generated on the underlying mechanisms of apical dominance control in *Zantedeschia*, may in turn

direct further research towards devising strategies to alleviate apical dominance and thereby enhance floral productivity by stimulating the degree of branching.

2.3.1.2.1 Topolins

Refer to the attached paper (With kind permission from Puspa Publishing House: International Journal of Bio-resource and Stress Management (IJBSM), Topolins: Current research status and applications, Volume 2 (1), 2011, Pages 10-25, Arvind Kumar Subbaraj).

2.3.1.3 Other hormones

Among the hormones hypothesized as second messengers to auxin in the control of apical dominance, ethylene is unlikely to be a candidate because ethylene production failed to correlate with axillary bud outgrowth after decapitation (Li and Bangerth, 1992). Though ABA is suggested to play a significant role in the control of endodormancy (Tanino, 2004), the rate of axillary bud outgrowth in excised nodal sections of *Arabidopsis* was not significantly affected by ABA, either alone or in the presence of auxin (Chatfield et al., 2000).

When GA was applied along with the auxin indole-3-acetic acid (IAA) to decapitated stumps of pea, the strength of apical dominance was more pronounced than when IAA was applied alone (Jacobs and Case, 1965). GA probably caused more IAA to be present and effective far from the site of application. In English Ivy, application of GA stimulated enhanced branching compared to the application of CK (Lewnes and Moser, 1976). Whether GA acts as a second messenger to auxin is ambiguous. However, auxin promotes GA biosynthesis (Ross and O'Neill, 2001) and decapitation resulted in reduced GA content in the stem (Ross et al., 2001). A negative interaction between CK and GA has also been proposed, where CK inhibits GA synthesis and vice-versa (Weiss and Ori, 2007). Albeit, in 16-days old seedlings of soybean that had ceased cell division, CK alone was not effective, and a sequential application of GA was required to stimulate axillary bud outgrowth (Ali and Fletcher, 1970). In *Zantedeschia*, the simultaneous application of CK and GA as Promalin^{TM} (Valent BioSciences, USA), did not enhance branching significantly compared to the application of CK alone (Funnell et al., 1992). In soybean, Ali and Fletcher (1971) noted that a sequential application of CK followed by GA was more effective in stimulating branching, compared to their simultaneous application. Hence the role of GA in the control of branching in *Zantedeschia*, and its interaction with CK in this process, is considered worthy of further investigation.

Recently, a new model for bud outgrowth that incorporates strigolactones and the yet to be identified feedback signal has been outlined (Ferguson and Beveridge, 2009) (Fig. 2.5).

According to this model, the feedback signal that rapidly triggers axillary bud outgrowth upon decapitation is auxin-independent, and is most likely to be a physical response such as changes in turgor pressure or electric potential. Growing axillary buds then affect the auxin status, which in turn positively regulates strigolactone synthesis and negatively regulates CK synthesis (Ferguson and Beveridge, 2009). Subsequent growth into a shoot is facilitated by the export of auxin from the axillary bud to the mainstream PAT system, which ultimately establishes vascular connections for nutrient transport (Leyser, 2005; 2009).

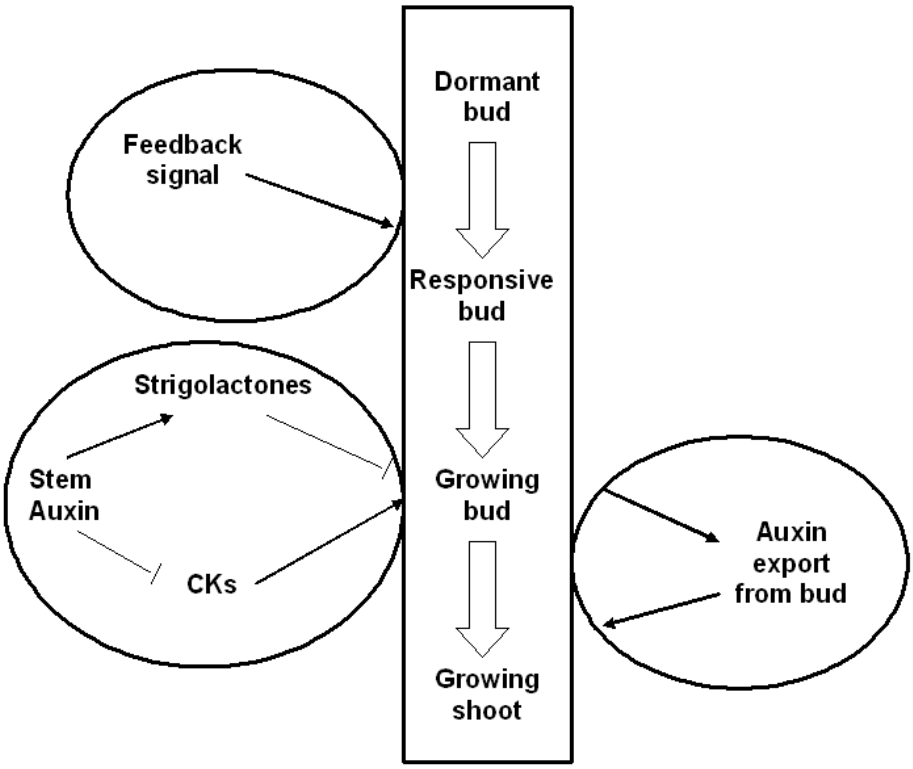


Fig. 2.5 Recent model of bud outgrowth incorporating the role of the feedback signal, strigolactones, auxin, CKs and the auxin export hypothesis (Leyser, 2005) in the control of different stages of axillary bud outgrowth (Image modified from Ferguson and Beveridge, 2009).

2.3.1.4 Bud transition hypothesis

As mentioned earlier (Fig. 2.4), different stages exist in the process of bud outgrowth (Shimizu-Sato and Mori, 2001). Several factors such as the ontogeny of the plant, nodal position, age, genotype, light and temperature determine the developmental stage of the bud (Horvath et al., 2003). Therefore, the sensitivity of the buds to hormones also differs between these stages. The action of auxin on the buds in the transition stage (Fig. 2.5) could result in the re-imposition of apical dominance (Dun et al., 2006). The application of CK, probably during this stage, in soybean plants failed to stimulate axillary bud outgrowth, and a sequential application of GA was required to induce sustained growth (Ali and Fletcher, 1970; 1971). The purpose of these developmental stages could be to modulate the overall architecture of the plant, thereby balancing the endogenous and environmental signals (Dun et al., 2006). Whether a similar change in sensitivity of the buds of *Zantedeschia* was associated with shifts between different types of dormancy was subsequently explored in Chapters 7 and 8 of this thesis.

2.3.2 Endodormancy

Endodormancy is an intrinsically controlled process (Lang, 1987), which involves changes in growth hormones, respiratory substrates, carbohydrate metabolism, nucleoproteins and gene regulation (Chrungoo, 1992; Horvath et al., 2003). Environmental signals, especially light and temperature play a critical role in the imposition and release of endodormancy (Horvath et al., 2003). The initial cue for the induction of endodormancy is derived from the exposure of leaves to short-day conditions, which has been proved in 60 species of 35 genera of woody perennials (Wareing, 1956). Temperature also affects the duration and strength of endodormancy, though limited studies have investigated this aspect (Olsen, 2003). To translate these environmental signals to physiological and/or morphological responses in the bud, the role of hormones is inevitable.

Due to the lack of visual clues, it is difficult to predict the onset of endodormancy during the growth cycle of *Zantedeschia* (Halligan et al., 2004). Therefore, a model based on heat-unit accumulation was developed. According to this model, using a base temperature of

2.1ºC, endodormancy was proposed to span between 2145 and 2747 degree days (Carrillo Cornejo et al., 2003). The onset of endodormancy in *Zantedeschia*, unlike other plant species (Wareing, 1956), is not activated by external factors (Funnell, 1993), meaning that it is autonomously controlled (Naor et al., 2008). Irrespective of low or high temperatures and short or long day photoperiodism, and even with an adequate supply of water, the periodic pattern of development, marked by the inception of endodormancy was observed (Funnell, 1988). Though shifts in the temperature regime are not responsible for the induction of endodormancy, temperature during the current growth cycle affected the duration and strength of endodormancy during the next growth cycle (Naor and Kigel, 2002). High temperature during plant growth caused strong endodormancy, and accelerated the initiation of endodormancy-associated processes, such as senescence. Longer periods of high temperature also reduced the tuber size and increased the strength of endodormancy (Naor and Kigel, 2002).

Albeit, cyclic periodicity represented by alternating periods of growth and rest is exhibited by *Zantedeschia* (Funnell, 1993). Similar to the control of endodormancy in other plant species (Wareing, 1956), where hormones mediate the onset and release of endodormancy imposed by environmental signals (Wareing and Saunders, 1971), it is logical to assume that endogenous signals including hormones should play a pivotal role in the control of the autonomously controlled cyclic periodicity in *Zantedeschia*. Early studies with other plant species suggested that an inhibitory concentration of auxin was responsible for endodormancy induction. However, subsequent identification of a correlation between short days and a dormancy inducing mobile factor, followed by studies that revealed the involvement of GA, CKs and ethylene in the control of endodormancy, have emphasized the paramount role of hormones in the control of endodormancy (Wareing and Saunders, 1971). Now, with the addition of analyses using genetic (Horvath et al., 2003), mutant (Olsen, 2003) and quantitative trait loci (QTL) (Bielenberg, 2011), greater insights into the control of endodormancy are being furnished. However, in plants such as *Zantedeschia*, where fundamental knowledge on the control of endodormancy is lacking, it is logical to commence with physiological studies, before proceeding to the molecular/genetic aspects

of control. This, therefore, forms part of the underlying research strategy presented within this thesis.

2.3.2.1 Abscisic acid

The dormancy inducing mobile factor, initially termed ‘dormin’, which was suggested to be transported from leaves exposed to short days to the apical bud of birch (*Betula* spp.), a woody perennial, was later identified as ABA (Eagles and Wareing, 1964). Exogenous application of ABA to the leaves of several plant species grown under long day conditions also induced the onset of bud endodormancy (El-Antably et al., 1967). In potato, an herbaceous perennial, sustained synthesis and action of ABA was required for the induction and maintenance of endodormancy (Suttle and Hultstrand, 1994), whereas a declined level of endogenous ABA did not correlate with the release of endodormancy (Suttle, 1995). ABA is thought to induce the onset of endodormancy through the regulation of cell-cycle related genes (Anderson et al., 2001; Horvath et al., 2002). However, the effect of ABA in the induction of endodormancy is not universal (Perry and Hellmers, 1973).

In *Zantedeschia*, where endodormancy is autonomously controlled (Naor and Kigel, 2002), an exogenous application of ABA failed to impose endodormancy, and an increase in the endogenous ABA content was also not observed during the onset of endodormancy (Naor et al., 2008). This lack of correlation between the ABA content and the induction of endodormancy can possibly be attributed to the different growth conditions maintained during experimentation (Naor et al., 2008), differences in the sensitivity of the buds to ABA (Suttle, 1995), discrepancies in the distribution and compartmentalisation of ABA, and the type of bud tissue tested (Tanino, 2004). However, in contrast to the observation in potato (Suttle, 1995), a decline in the endogenous ABA content was observed in *Zantedeschia*, towards the period of release of endodormancy and subsequent sprouting (Naor et al., 2008). Since earlier studies (Naor et al., 2008) failed to establish a correlation between ABA and the onset and release of endodormancy in *Zantedeschia*, this line of investigation was not pursued in this thesis. Alternative modes of endodormancy control in *Zantedeschia* were therefore hypothesized, and based on the observations made by Turnbull

and Hanke (1985a) in potato, a role for CKs was postulated in the control of endodormancy (Chapters 7 and 8).

2.3.2.2 Gibberellin

As mentioned above (refer Section 2.3.2.1) in the case of *Zantedeschia*, the release of endodormancy, unlike apical dominance, does not merely involve the removal of the conditions that impose it (Wareing, 1956). Therefore, the role of growth promoting hormones such as GA and CKs, in facilitating endodormancy release was hypothesized (Wareing and Saunders, 1971). Among other factors, the antagonistic role of GA to ABA in the control of bud dormancy, has received widespread support (Tanino, 2004) and GA is suggested to act on the same cell-cycle-related genes involved in the imposition of endodormancy (Horvath et al., 2002). Initial evidence was provided by Brian et al. (1955), where dormant potato tubers treated with an exogenous application of GA sprouted earlier than untreated tubers (control). An increase in endogenous GA content was also observed at the time of bud burst during spring/summer in woody (Eagles and Wareing, 1964) and herbaceous (Smith and Rappaport, 1961) perennials. Since the release of endodormancy is followed by bud outgrowth, and a characteristic trait of GA is the enhancement of cell elongation (Stowe and Yamaki, 1959), it is difficult to ascertain the role of GA in the control of endodormancy (Suttle, 2004). This is further compounded by the presence of more than a hundred forms of GA (Richards et al., 2001).

As observed with ABA, where an exogenous application failed to induce endodormancy (Naor et al., 2008), the exogenous application of GA also failed to release buds from endodormancy in *Zantedeschia* (Naor et al., 2005b). GA_3 is the predominant GA found naturally in *Zantedeschia* (Naor et al., 2008), and during the growth period following the release of endodormancy, the total GA content was low and constant. High levels were observed only immediately after the storage of tubers concomitant with shoot growth prior to planting (Naor et al., 2008). However, in another cultivar of *Zantedeschia*, branching was stimulated by an exogenous application of GA, though at the time of application, the buds were presumed to be at the end of the growth cycle (Carrillo Cornejo, 2001) and

therefore under shallow endodormancy (Fig. 2.1). Such anomalies in the correlation between the endogenous GA concentration and the control of endodormancy have also been observed in potato, wherein GA was suggested to play a critical role in sprout outgrowth following the termination of endodormancy, rather than its induction (Suttle, 2004). It is possible therefore, that temporal changes in the sensitivity of buds to GA, determine the degree of bud outgrowth (Suttle, 2004).

2.3.2.3 Cytokinins

The induction and release of endodormancy cannot always be attributed to the antagonistic relationship between ABA and GA (Shih and Rappaport, 1970). In some plant species, such as *Allium cepa* L. (Onion), *Freesia sp.* (Jacquin) Klatt. (Freesia) and *Gladiolus communis* L. (Gladiolus), endodormancy release was achieved only with the application of CKs, while GA had no effect (Chrungoo, 1992). The role of CKs in the control of endodormancy has however received little attention (Turnbull and Hanke, 1985a). A significant dose of an exogenous application of radiolabeled CKs, injected into potato tubers, was translocated to the buds which resulted in subsequent outgrowth (Turnbull and Hanke, 1985b). A 20-50 fold increase in endogenous CK content was also observed, coinciding with the natural break of endodormancy (Turnbull and Hanke, 1985b), and this increase was mostly due to the *de novo* biosynthesis of CKs (Suttle, 1998). The metabolism of CKs and the relative sensitivity of the responding tissues to CKs (Turnbull and Hanke, 1985b; Suttle, 2001), combined with their transport (Chrungoo, 1992), also influence the release of buds from endodormancy. Moreover, ABA was found to increase the expression of *cytokinin oxidase/dehydrogenase* (*CKX*) genes (Brugiere et al., 2003) which encode a key enzyme that irreversibly degrades CKs (Mok and Mok, 2003). Since CKs and GA exhibit a reciprocal interaction (Weiss and Ori, 2007), it is logical to assume that the regulation of bud endodormancy involves an intricate network of hormonal mechanisms.

Studies involving the exogenous application of CKs in *Zantedeschia* have been undertaken in a bid to stimulate branching by alleviating apical dominance and, therefore, enhance the floral productivity (Naor et al., 2005b). Their role in the control of endodormancy however,

has not previously been reported. Exogenous application of CKs therefore, and the concomitant changes in the branching response observed during different types of dormancy, formed a critical component of the thesis (refer Chapters 6-8).

Attempts to quantitatively describe the endogenous CK content within *Zantedeschia* reported a high content in sprouting buds than endodormant buds, and the CKs were predominantly present in the form of nucleotides (D'Arth et al., 2007). The quantification of CKs in the study by D'Arth et al. (2007) was however confined to only the ISCK group, and the ARCKs were not taken into account. Given that the structural differences between the ISCK and ARCK groups have far reaching physiological implications determined by differences in their perception, activity and metabolism (refer Section 2.3.1.2.1), and due to the natural occurrence of ARCKs in *Zantedeschia* (das Neves and Pais, 1980a), further investigation pertaining to this group of CKs was considered a valid research strategy to pursue (refer Chapters 8 and 9).

Since the different types of dormancy exist in a continuum (Faust et al., 1997) (Fig. 2.1), it is difficult to precisely predict the transition of buds from a state of apical dominance to endodormancy (Halligan et al., 2004). Decapitation/scooping of the apical bud (Thimann and Skoog, 1933; 1934) can be used to verify the existence of apical dominance, where removal of the apical bud stimulates axillary bud outgrowth. The onset of endodormancy can also be diagnosed by decapitation, where the removal of the apical bud would fail to stimulate axillary bud outgrowth. Likewise, the response of the buds to an exogenous application of CK can be used as a diagnostic tool to predict the transition of buds from apical dominance to endodormancy. While the application of CKs would stimulate branching in plants under apical dominance (Shimizu-Sato et al., 2009), endodormant buds would fail to respond to an application of CKs. In the current study, this diagnostic tool was used to determine the transition of buds from apical dominance to endodormancy, which vindicated in the division of the growth cycle of *Zantedeschia* into three phases (refer Chapters 6 and 7).

2.3.2.4 Other hormones

Ethylene has been implicated in both the induction (Chao et al., 2007) and release of endodormancy (Chrungoo, 1992). The exact mechanism (cause or effect) of ethylene in endodormancy control is, however, not known. Since the senescence process precedes the induction of dormancy, and ethylene is involved in the control of senescence (Abeles, 1987), it is possible that ethylene expedites the induction of endodormancy (Horvath et al., 2003). However, in *Zantedeschia*, the onset of endodormancy probably occurs before the cessation of leaf production (Halligan et al., 2004) and, therefore, the control of ethylene in this process in *Zantedeschia* remains unclear.

Early studies hypothesized similarities between apical dominance and endodormancy, where a high concentration of auxin was hypothesized to inhibit bud outgrowth (Smith and Kefford, 1964). Accordingly, high endogenous auxin content was found in shoots of *Pyrus communis* L. (pear) at the time of endodormancy induction, and this concentration declined continuously towards the release of endodormancy. These results were contradicted by Bennett and Skoog (1938), whose results with pear failed to correlate the endogenous auxin concentration and the inception of endodormancy.

With the subsequent identification of ABA as a prime controller of endodormancy, auxin was suggested to be positively regulated by the ABA mechanism or vice-versa (reviewed in Samish, 1954). Corroborative evidence supporting this interaction has been obtained through molecular studies, where auxin (Grossman and Hansen, 2001) and ethylene (Sharp et al., 2000) are suggested to trigger ABA biosynthesis.

2.3.3 Ecodormancy

Ecodormancy is imposed by environmental factors (Lang, 1987) such as cold or drought stress (Horvath et al., 2003). Temperate plant organs require a period of chilling to overcome endodormancy (Shirazi, 2003), and subsequent maintenance of this chilling period mediates the transition of buds from endodormancy to ecodormancy (Faust et al., 1997) (Fig. 2.1). The duration of the chilling period and temperature required to overcome

endodormancy depend on the plant species (Samish, 1954). Normally the duration ranges between 250 and 1000 hours, and the optimum temperature ranges between 0ºC and 10ºC (Shirazi, 2003). For geophytes with commercial significance, low temperature storage is employed to delay flower and leaf emergence, which allows for shipping and long-term storage (Dole, 2003).

In *Zantedeschia*, tubers planted immediately after leaf removal i.e., without a storage period, remained endodormant and failed to grow, whereas short-term storage of tubers (approximately 6 weeks) at 9ºC resulted in endodormancy release with increased shoot growth and maximum overall growth (Corr and Widmer, 1988). The increasing popularity of *Zantedeschia* as a cut flower and pot plant (Benschop et al., 2009), means that commercial production of *Zantedeschia* requires scheduled planting and flowering by the growers (Funnell and Go, 1993). This is best achieved by long-term cold storage of the tubers at 7 ± 3ºC (Funnell and MacKay, 1988b). A major drawback of this long-term storage is that the flowering potential of the tubers declined with extended durations of storage (Jierwiriyipant and Tjia, 1988; Funnell and Go, 1993; Dennis et al., 1994). The storage of tubers involves changes in the endogenous hormone levels (Keren-Paz et al., 1989; Naor et al., 2008), carbohydrate metabolism (Shin et al., 2002; Naor et al., 2008), water status (Robinson et al., 2000; Van der Toorn et al., 2000) and the rate of respiration (Hosoki, 1984). As hypothesized by Funnell and Go (1993), the role of hormones and the concurrent changes in the sensitivity of the buds associated with the decline in flowering in *Zantedeschia* were subsequently developed as a topic for further evaluation in this thesis (refer Chapter 3).

2.3.3.1 Hormones

Plant hormones can partially or fully replace the requirement for a cold treatment to release endodormancy (Dole, 2003). GA and ethylene have been predominantly implicated in this process (Boonekamp, 1996). Though a spike in endogenous GA concentration was observed immediately after storage of tubers of *Zantedeschia* at 20ºC (Naor et al., 2008), an exogenous application of GA failed to release buds from endodormancy (Naor et al.,

2005a). Exogenous application of ethylene prior to the storage of tubers had no significant effect on the growth and floral productivity of *Zantedeschia* (Funnell and MacKay, 1988b). Though, in this study, the buds were not truly endodormant at the time of investigation, ethylene has been shown to release the buds of some geophytes from endodormancy (Dole, 2003).

The decline in the flowering potential observed with long-term cold storage of tubers in *Zantedeschia*, was restored with an exogenous application of GA (Funnell et al., 1988; Funnell and Go, 1993). Therefore, the decline in the flowering potential could be due to a:

- Change in the sensitivity of buds to gibberellins
- Decline in the concentration/activity of endogenous levels of gibberellins
- Change in the plants ability of buds to take up liquid/gibberellin

Spatial and temporal changes in the sensitivity of the responding tissues (Weyers and Paterson, 2001) can be caused by several factors (Firn, 1986). In *Zantedeschia* the endogenous GA content was low and constant throughout the growing period, and there was a 10 fold increase at the commencement of flowering (Naor et al., 2008). Brooking and Cohen (2002) reported that the floral productivity of *Zantedeschia* was higher in tubers treated with GA for 16 hours than those treated for 5 minutes. Therefore, changes in the permeability of tissues during cold storage (Dole, 2003), which determines the uptake of GA, could influence the floral productivity of *Zantedeschia*. As detailed in Chapter 3, the first two hypotheses proposed by Funnell and Go (1993) were planned to be investigated, using the modelling approaches postulated by Firn (1986).

2.4 Strategies to stimulate branching

Modifications in the developmental morphology of the plant, either through environmental factors or genetic factors, are moderated through the action of plant hormones (Davies, 1995). Historically the strategies used to stimulate branching in plants, have mostly relied on the antagonistic relationship between auxin and CK (Dun et al., 2006), where the inhibition of apically derived auxin and/or the direct application of CK to the suppressed

buds have been traditionally practiced to stimulate branching.

2.4.1 Decapitation

The influence of the apex is evident in the near-universal phenomenon of apical dominance, in which removal of the apical bud results in release of axillary buds below it. In fact, the discovery of apically derived auxin as the primary source for the control of apical dominance was identified using decapitation and auxin replacement experiments in *Vicia faba* L. (Thimann and Skoog, 1933; 1934). Excision/decapitation of the apical bud and hence depletion of the auxin pool in the control of apical dominance, was used to develop the famous PESIGS rules to describe the hormonal nature of a chemical substance (Jacobs, 1959). Since then, the use of decapitation studies have been undertaken in several plant species, mainly to elucidate the role of auxin in the control of shoot branching and to stimulate axillary bud outgrowth (reviewed in Le Fanu, 1936; Snow, 1937; Phillips, 1975; Napoli et al., 1999; McSteen and Leyser, 2005).

The rate of axillary bud development as a result of decapitation may differ between species. In some species, the elongation of axillary buds occurs in few hours, whereas in others the lag period could be longer, depending on the degree of dominance exerted. Cline (1997) divided the development of axillary buds before and after decapitation into four stages (Fig. 2.6), i.e. axillary bud formation (Stage 1), imposition of inhibition or apical dominance (Stage 2), initiation of axillary bud outgrowth following decapitation (Stage 3) and, subsequent elongation and development of the axillary bud into a branch (Stage 4). Though the stages could be overlapping, they can be considered to be separate, depending on the degree of inhibition present inherently in the studied plant species.

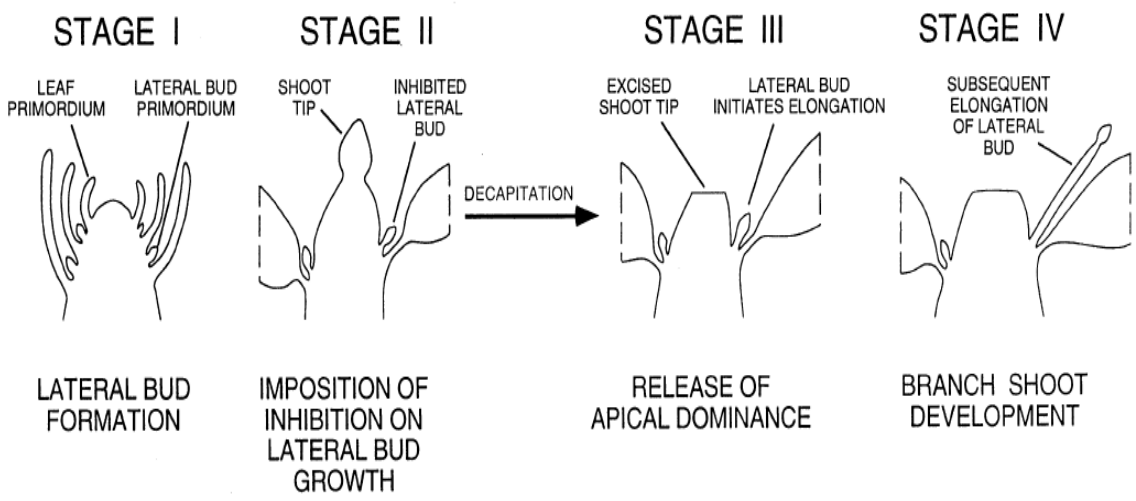


Fig. 2.6 Stages of apical dominance before and after release by decapitation of the shoot apex (Image from Cline, 1997).

Though decapitation studies have led to a better understanding of apical dominance in plants, its adoption as a strategy to stimulate branching has to be dealt with caution. This is because, in addition to the stress induced by wounding, decapitation also induces a number of specific responses, e.g., xylem sap flow rate changes, sink strength alterations, as well as changes in assimilate and hormone transport. Moreover, decapitation removes a region of hormone synthesis, including auxin and gibberellin and probably other unidentified signals which could be detrimental to plant growth (Napoli et al., 1999; Dun et al., 2006).

Due to the deleterious effects described above, though decapitation has been found to induce axillary bud outgrowth in *Zantedeschia* (Clark et al., 1987), the use of decapitation as a commercial practice is not recommended. This is further compounded by the logistics of decapitation in geophytes, where the buds are present on an underground storage organ. Moreover, since the apical bud is also a site of flower initiation, decapitation can remove a potential flowering site and, thereby, reduce the floral productivity in *Zantedeschia*. Hence, while decapitation was used as a tool to diagnose the existence of apical dominance in the current study, it was not advocated as an experimental treatment.

2.4.2 Defoliation

Snow (1929) determined that the young leaves in close proximity to the apical bud in *Pisum sativum* L. (pea) were an active site of auxin biosynthsis, and played an important role in the control of axillary bud outgrowth. Even after the removal of the apex, the leaves prevented the growth of the buds in the corresponding leaf axils. These inhibited buds showed accelerated growth when the subtending leaf was removed. It was then found that a chemical substance from the leaf was involved in the inhibition of the axillary bud. The content of this chemical substance, probably auxin, in the internodes of pea plants was reduced after defoliation (Jager et al., 2007). Subsequently, defoliation has been used, as a non-chemical approach to induce branching in several plant species (Ericsson et al., 1980 in *Pinus sylvestris* L. (Scots pine); Hilton et al., 1987 in *Quercus robur* L. (Oak); Volz et al., 1994 in *Malus domestica* Borkh. (Apple); and Collin et al., 2000 in *Fraxinus excelsior* L. (Common ash)).

In common ash, the scale of axillary bud burst depended on the degree of defoliation (partial or complete) and the stage of the plant at the time of defoliation (before cessation of stem elongation and after cessation of stem elongation). When seedlings were defoliated 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 days after emergence, renewal of stem elongation occurred only up to 70 days. After 90 days, defoliation did not promote axillary bud outburst. Partial defoliation (50% & 75%) seemed to be less effective than complete defoliation (100%), both before and after the completion of stem elongation. It was suggested that removal of leaves could alter the nutrient assimilate level and bring about hormonal changes that result in a modification of paradormancy or apical dominance (Collin et al., 2000).

While the role of young leaves as a potential source of auxin synthesis, and hence as a controlling factor of apical dominance, has been suggested (Snow, 1929; Cline, 1997), the older/mature leaves were also suggested to contribute to the auxin economy of the plant (Jager et al., 2007). In pea, they showed that defoliation reduced the endogenous auxin content in the apical bud and the stem internodes. Though defoliation reduced endogenous

auxin content in both the young and mature leaves, substantial amounts of extractable auxin remained in the mature leaf, suggesting that auxin from the mature leaf could also contribute to the total auxin economy of the plant. In line with these findings therefore, in the current thesis it was hypothesized that both young and old/mature leaves contribute to the total auxin content of *Zantedeschia*, and hence the degree of apical dominance. Further to this, it was hypothesised that the effect of defoliation could also change with the stage of development of the plant. As explored within chapter 4, removal of leaves could therefore be attempted as a technique to inhibit the auxin content, thereby resulting in stimulation of branching, by alleviating apical dominance in *Zantedeschia*.

2.4.3 Auxin transport inhibitors

Since the leaves are a key source of nutrients to the corresponding axillary buds (Dun et al., 2006), removal of leaves by defoliation may sometimes result in the absence/slow growth of axillary buds (Thimann and Skoog, 1934). Moreover, due to the disadvantages associated with the practice of decapitation and the need to maintain an intact plant during studies into apical dominance, the use of auxin transport inhibitors (ATI) was proposed as an alternative strategy to explore the involvement of auxin in the control of apical dominance (Napoli et al., 1999) and, if successful, can be employed as a commercial tool to promote branching. ATIs perform the physiologically intended objective of decapitation, i.e., inhibition of polar auxin transport, and subsequently have been used in several studies to stimulate axillary bud outgrowth in other plant species (Mitchell et al., 1965; Chatfield et al., 2000).

Though ATIs perform the physiologically intended objective of decapitation, the underlying mechanism of action could be different in ATIs. While decapitation of pea led to a concomitant increase of endogenous CK content, the application of ATI failed to influence the CK content (Koukourikou-Petridou and Bangerth, 1997). Based on the auxin export hypothesis (refer Section 2.3.1.1) however, an alternative mode of action for ATIs has been proposed, wherein blocking the PAT stream in the stem using ATIs facilitates the export of auxin from the corresponding axillary buds, thereby enhancing bud activity

(McSteen and Leyser, 2005).

ATIs are classified into several groups based on their chemical structure (Katekar and Geissler, 1980):

- Phytotropins – 1 – N – Naphthylphthalamic acid (NPA)
- Morphactins – 9 – hydroxyfluorene – 9 – carboxylic acid (HFCA) and derivatives
- Compounds not in either class – 2, 3, 5 – triiodobenzoic acid (TIBA)

All three classes of ATI have been used to inhibit polar auxin transport in several plant species to stimulate axillary bud outgrowth, and to investigate the underlying mechanism of apical dominance (Snyder, 1949; Mitchell et al., 1965; White and Hillman, 1972). The role of TIBA and NPA in the reduction of apical dominance is however, not universal. In *Malus domestica* Borkh. (apple) seedlings, TIBA actually appeared to increase apical dominance of the compact trees (Lee and Looney, 1977). Other ATIs have also been reported to be effective in plant species (Leopold and Klein, 1951; Katekar and Geissler, 1977; Nakajima et al., 2001).

TIBA has a marked tendency for premature death and abscission of the terminal meristem, as well as of many of the axillary buds of the trifoliate leaves in *Phaseoulus vulgaris* L. (bean) (Snyder, 1949). In bean plants, application of TIBA and morphactin (CFM) as a ring around the second internode, had several undesirable effects on the plants such as, abscission of young leaflets and nodes, limited outgrowth of axillary buds followed by their abscission and, inhibition of main bud and leaf growth (White and Hillman, 1972). There was no evidence of inhibitory or formative effects on axillary buds of bean when treated with phthalamic acid derivatives (NPA included) (Mitchell et al., 1965). NPA was less phytotoxic than TIBA and had successfully released axillary buds from apical dominance in apple, below the point of application (van Hooijdonk, personal communication). Since NPA is considered to be less phytotoxic than TIBA, its application as an ATI was considered worthy of being evaluated with *Zantedeschia* (Chapter 5).

2.4.4 Cytokinins

Auxin and CKs have been shown to interact in several physiological and developmental processes, including apical dominance, control of the cell cycle, axillary root initiation, regulation of senescence and vasculature development (Shimizu-Sato and Mori, 2001). The interaction of these two hormones can be synergistic, as is the case for the regulation of cell cycle, or antagonistic, as is the case for the regulation of axillary bud meristems and the formation of axillary roots (Rashotte et al., 2005).

As mentioned earlier (refer Section 2.3.1.2), in a diverse range of plants, direct application of CKs to the suppressed buds resulted in the outgrowth of buds via alleviating apical dominance (Sachs and Thimann, 1967; Ali and Fletcher, 1970; McSteen and Leyser, 2005). As a result, foliar sprays of the synthetic CK, 6-benzylamino-9-(tetrahydropyran-2-yl)-9H-purine (PBA), increased branching in carnation, chrysanthemum, poinsettia, petunia and fuchsia (Jeffcoat, 1977). In addition to a number of dicotyledons, such as *Gerbera jamesonii* Bolus. (Gerbera), *Fragaria* × *ananassa* Duchesne. (Strawberry) and *Malus domestica* Borkh. (Apple), *in vitro* branching by CK in a number of monocotyledons, such as Gladiolus, *Iris germanica* L. (Iris) and *Narcissus sp.* L. (Narcissus) was demonstrated by Hussey (1976). *In vitro* plantlets cultured on a basic medium containing 6-benzylaminopurine (BAP) caused the usual single *in vitro* shoots of all species to branch. In *Zantedeschia*, since the buds are present on an underground tuber, direct application of CKs to the suppressed buds is impossible after planting. Therefore, foliar application of CKs has been employed for the successful stimulation of branching in *Zantedeschia* (Naor et al., 2005b).

The synergistic effect of CKs and gibberellic acid (GA), in the release of axillary buds of soybean has been highlighted (Ali and Fletcher, 1970). The sequential application of CK followed by GA was suggested to be more effective compared to their simultaneous application (Ali and Fletcher, 1970; Volz et al., 1994). CK application was suggested to induce cell division and a subsequent GA application was suggested to induce cell elongation. The response of the plant to the application of CK and GA, either individually

or in sequential combination, however, depended on the stage of the plant. CK applied alone was effective in the release of axillary buds, 8 days after seedling emergence in soybean. However, 16 days after seedling emergence, a sequential application of CK and GA was required for the release of these buds (Ali and Fletcher, 1970). Therefore, the use of CKs to induce multiple branching in several monocots and dicots can be considered to be a near-universal strategy (Shimizu-Sato et al., 2009). As detailed already in Section 2.3.1.3, the application of CK alone and its interaction with GA, in stimulating axillary bud outgrowth in *Zantedeschia* warrants further investigation. Since BAP was already effective in stimulating branching in *Zantedeschia* (Naor et al., 2005b), its usage was advocated throughout the current study. Also, since BAP belongs to the aromatic group of CKs (Mok and Mok, 2003), the analyses exclusive to this group of CKs, such as their extraction and purification (Hoyerová et al., 2006), metabolism (Strnad, 1997), chromatographic separation (Strnad et al., 1990) and immunodetection (Strnad, 1996) were used (refer Chapters 8 and 9).

2.5 Flowering

2.5.1 Floral transition in plants – An overview

While a molecular genetic analysis of flowering was not the theme of the current study in *Zantedeschia*, an overview of the recent developments in identifying key genes involved in floral transition, and a summary of the key pathways that lead to floral transition was considered fundamental. A major portion of this knowledge has been generated from experiments in the model plant *Arabidopsis thaliana* L. Given that hormones play an obligatory role in the control and convergence of these pathways (Davis, 2009), and the recent pool of evidence drawing common mechanisms between dormancy and flowering in plants (Horvath, 2009), this overview seemed more relevant for this thesis, and would aid in directing further research on flowering in *Zantedeschia*.

The transition to flowering, which marks the beginning of reproductive development and leads to the formation of fruits and seeds, is an important phenological event in the ontogeny of plants (Zeevart, 1962), and is of commercial significance for agricultural and

horticultural crops (Bernier et al., 1993). The time to flower, which is critical for successful completion of the reproductive phase, is determined by an integration of environmental and endogenous cues (Putterill et al., 2004), which in turn are orchestrated by several genetic and biochemical routes called pathways, which operate alone or in unison (Amasino, 2010) (Fig. 2.7).

In general, the most prominent environmental cues are changes in photoperiod and temperature (Amasino, 2010). The photoperiod pathway (also known as long day pathway), exclusive to *Arabidopsis* (Fig. 2.7), synchronises itself with the circadian clock apparatus and the light sensing/photoreceptor system (Boss et al., 2004), and directly affects the activity of the key flowering-time gene *CO* (Putterill et al., 2004). *CO* directly upregulates the expression of the floral pathway integrators (such as FT, SOC and LFY) resulting in floral transition (Boss et al., 2004). Not surprisingly therefore, *Arabidopsis* mutants deficient in CO expression were late flowering (Putterill et al., 2004). A light quality pathway may be integrated with the photoperiodic pathway (Fig. 2.7), where phytochromes and cryptochromes which perceive red/far-red and blue light respectively, produce a signal that is required by CO to activate FT (Levy and Dean, 1998). An exclusive light-quality pathway is also possible, in which a nuclear protein (PFT1) was shown to induce flowering as a shade avoidance strategy, by regulating FT without involving CO (Cerdán and Chory, 2003).

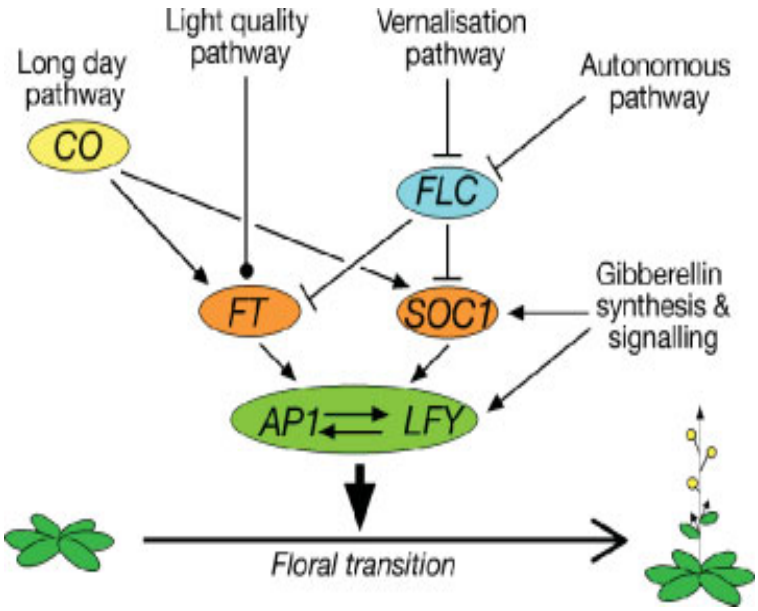


Fig. 2.7 Depiction of different pathways and key genes involved in floral transition of the model plant *Arabidopsis* (Image from Putterill et al., 2004).

Some plants require vernalisation or a prolonged period of cold temperature during winter to ensure flowering in spring (Putterill et al., 2004). A few other plants may be less sensitive to environmental cues or act autonomously in the transition to flowering (Levy and Dean, 1998). Autonomous flowering is suggested to be indirectly determined by internal cues such as plant size or the number of nodes (Sachs, 1999) and the competence to flower administered by a phase change in the SAM (Poethig, 1990). The vernalisation and autonomous pathways act through the floral repressor FLC, which is antagonistic to CO and FT (Putterill et al., 2004) (Fig. 2.7). Vernalisation turns off FLC which enables floral competence in the SAM, which subsequently becomes susceptible to the action of CO and FT gene products (Amasino, 2010).

It has been difficult to provide an exclusive position for hormones in the floral transition process, because hormones play a critical role in the control of all flowering pathways (Fig. 2.7). However, GA appears to play a promotive role in the flowering of *Arabidopsis* (Fig. 2.7), where application of GA accelerated flowering in non-inductive photoperiods and late-flowering mutants (Levy and Dean, 1998). GA acts in promoting flowering, in part by

up-regulating the floral pathway integrators (Blázquez et al., 1998). The role of other hormones i.e., both classical and recent, have also been implicated in the floral transition pathway (Davis, 2009). While the genetic control of flowering was not the focus of this thesis, a basic understanding of the different flowering pathways was established from the literature, so as to provide a potential explanation of the results obtained with experiments pertaining to flowering in *Zantedeschia*, which is day-neutral to flowering (Funnell, 1993), and initiates flowering with the application of GA (Naor et al., 2005a) (Described in Section 2.5.3).

2.5.2 Hormonal control of flowering

Historically, the involvement of a chemical substance of hormonal nature i.e., florigen in the transition to flowering has been hypothesized (Hamner and Bonner, 1938). Though the chemical identity of florigen has been tentatively revealed as the FT protein (Notaguchi et al., 2008), other hormones, both classical and recent have a crucial role in the control of flowering (Davis, 2009). Accordingly, classical hormones (Kende and Zeevart, 1997; Fig. 2.2) and the so-called 'new' hormones, such as jasmonic acid, salicylic acid, brassinosteroids etc., have been postulated to play key roles in the floral transition process (Davis, 2009). Of the classical hormones, GA and CKs deserve special mention due to their prevalent occurrence in flowering literature.

2.5.2.1 Gibberellin

Though GA can enact the role of florigen in LD plant species (King and Evans, 2003), it did not initiate flowering in several SD species (Lang, 1957) and, therefore, was ruled out as the chemical identity of florigen (Chailakhyan, 1968). Nevertheless, for the first time since its discovery (Stowe and Yamaki, 1959), GA was postulated in a role beyond the control of shoot growth by cell elongation (Lang, 1957). The chemical control of flowering by GA, and thereby several aspects of reproductive development, has provided a plethora of practical applications for agricultural and horticultural crops (Pharis and King, 1985).

Activating the floral switch by exogenous application of GA in many plant species (Pharis and King, 1985), and the lack of flowering in mutants impaired in terms of GA biosynthesis (Wilson et al., 1992), have established GA as an undeniable flowering-time promoter (Davis, 2009). Further studies using pharmacological, genetic and transgenic assay tools have suggested an exclusive flower-promoting pathway for GA, though cross-talk with other pathways is also possible (Davis, 2009) (Fig. 2.7). The LD induction of GA synthesis in the leaves of *Lolium* sp., and the subsequent rise of endogenous GA levels in the apex (King and Evans, 2003), highlight the significance of the interaction between the photoperiodic pathway and GA. *FLC*, a flowering repressor and a key gene in the vernalisation and autonomous flowering pathways (Fig. 2.7) blocked GA action in the SAM (Sheldon et al., 1999). The replacement of vernalisation by GA is species specific (Sheldon et al., 2000), and the exact mode of action of GA in the vernalisation pathway remains unclear (Davis, 2009). It is more likely that vernalisation predisposes the SAM to GA action (Levy and Dean, 1998).

GA participates in the key steps i.e., floral competence, induction and development, that ultimately lead to flower formation (Mutasa-Göttgens and Hedden, 2009). How exactly GA mediates this transition has been a topic of longstanding discussion (Pharis and King, 1985). Analyses using the modern genetic tools, as mentioned earlier, have revealed that GA acts by the up-regulation of the floral pathway integrators (Boss et al., 2004). Exogenous application of GA up-regulated *LFY* gene expression and GA deficient mutants had very low LFY expression (Blázquez et al., 1998). Autonomous pathway mutants also showed low LFY gene expression (Sheldon et al., 2000). Until now, more than one hundred GAs have been identified in plants (Richards et al., 2001), and all the GA tested do not possess a common promotive role in flowering (Mutasa-Göttgens and Hedden, 2009). GA_4 however, has been found to possess high florigenic activity (Davis, 2009). Given that in *Zantedeschia*, the presence of GA mediates floral transition in buds irrespective of their size and age (Naor et al., 2005a), their role in the control of flowering during different phases of the growth cycle formed a critical component of this thesis (refer Chapter 7). In this context, though analyses pertaining to the molecular/genetic control of GA in flowering

were beyond the scope of this study, a basic understanding of the significance of GA in the scheme of flowering was considered essential in this section.

2.5.2.2 Cytokinins

In contrast to GA, clear genetic evidence on the role of CK in the control of flowering is lacking (Boss et al., 2004). In early studies, the endogenous content of nucleic acids (from which CKs are derived) was high during the inductive photoperiod (Chailakhyan, 1968), and an exogenous application of CK promoted flowering in a few plant species (Hansen et al., 1988; Machackova et al., 1993). However, reasonable evidence supporting the role of CKs in the control of flowering has been derived from the long-day (LD) plant *Sinapis alba* L., where endogenous CK was suggested to be transported from the mature leaves and/or the roots towards the SAM upon photoperiodic induction (Bernier et al., 1993; Lejeune et al., 1994). CKs are capable of being transported in both the xylem and phloem (Hirose et al., 2007) as different isoforms (Romanov, 2009). In *S. alba*, an exogenous application of CK also promoted flowering, whereas the application of GA was unable to evoke flowering (Bernier et al., 1993).

Whether CKs are directly involved in floral transition or indirectly by facilitating cell division (Miller et al., 1955) after floral determination, remains ambiguous. While a transient increase in the mitotic activity of the SAM was observed upon photoperiodic induction in *S. alba* (Bernier et al., 1993), the timing of this increase was not coherent with induction of the signal in the leaves (Lejeune et al., 1994). Therefore, at this stage it is difficult to confirm if CKs are involved in the floral evocation process *per se*, or in the organogenesis that follows (Dewitte et al., 1999). The critical role of the *FLC* gene in the flowering pathway (Fig. 2.7) and its predominant expression in the roots, suggests a possible modulation of its expression by CKs (Davis, 2009). The apparent lack of mutants that implicate CKs in flowering however, have protracted the timeline for any analysis of CKs in flowering (Levy and Dean, 1998). Within the premises of employing CKs to stimulate branching in *Zantedeschia* (Chapter 6), and due to the direct relationship between the degree of branching and floral productivity (refer Section 1.4), the influence of CK

alone and in combination with GA on floral productivity was investigated (Chapter 7).

2.5.2.3 Other signals

Sucrose either applied alone (Corbesier et al., 1998) or in combination with either CK (Bernier et al., 1993) or GA (Blázquez et al., 1998), has been found to be effective in mediating floral transition in *Arabidopsis* and *S. alba*. In addition, other classical hormones such as ethylene, ABA and auxin, and several other 'new' hormones such as jasmonic acid and brassinosteroids have also been implicated in the control of flowering under certain circumstances and/or in some species (Levy and Dean, 1998; Davis, 2009).

2.5.3 Flowering in *Zantedeschia*

Floral transition in *Zantedeschia* is independent of a photoperiodic induction (day-neutral) (Corr, 1988) and/or a vernalisation requirement (Funnell, personal communication). Provided bud outgrowth is not limited by the influence of dormancy, floral differentiation occurs under conditions ideal for vegetative growth (Funnell, 1993). In *Zantedeschia*, visible manifestation of the floral transition in the SAM (Fig. 2.8) is usually preceded by the formation of 1-6 cataphylls and 2 leaves prior to floral (spathe and spadix) differentiation (Halligan et al., 2004). Due to its sympodial growth habit (Funnell, 1993), floral transition in the primary shoot (Fig. 2.8a), is normally followed by a flowering cascade in the secondary (Fig. 2.8b) and tertiary (Fig. 2.8c) shoots.

Based on microscopic observations in plantlets *in vitro*, Naor et al. (2004) observed 6 stages in the process of floral transition and development in *Zantedeschia*. Upon transition to flowering the flat-shaped vegetative SAM is converted to a swollen, dome-shaped apex. Subsequently, a smooth-surfaced, elongated apex (spadix primordia) is formed, surrounded by the spathe primordia. In the spadix of the summer-flowering species, female florets are formed in the base and male florets above them, which in the final stage of flower development (Stage 6) are manifested visually.

In *Zantedeschia*, where floral transition appears to be immune to external/environmental signals (Funnell, 1993), internal signals are more likely to play a pivotal role in moderating

the flowering process.

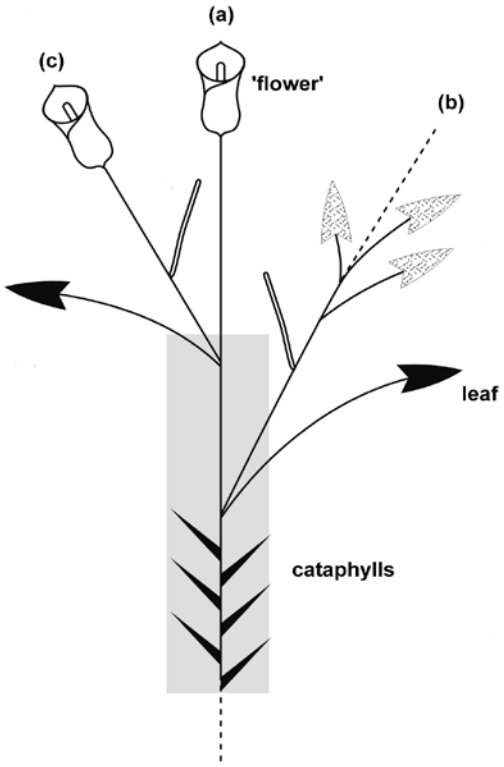


Fig. 2.8 Sympodial flowering habit of *Zantedeschia* depicting cataphylls and leaves formed prior to flowering in the (**a**) primary shoot followed by flowering in the (**b**) secondary and (**c**) tertiary shoots (Image modified from Halligan et al., 2004).

Hormonal inputs are instrumental in orchestrating these internal signals, and GA is capable of catalysing floral transition in *Zantedeschia* (Cohen, 1981; Corr and Widmer, 1987), irrespective of meristem size and age (Naor et al., 2005a). GA acts by increasing the proportion of shoots that flower and the number of flowers per shoot (Corr and Widmer, 1987; Funnell et al., 1991), which is directly related to the concentration and treatment duration of GA (Brooking and Cohen, 2002). Endogenous GA content in the bud and adjoining tuber tissue also increased at least 10 folds prior to floral differentiation (Naor et al., 2008). While the apical meristem remained flat during its vegetative state, a significant change in apex morphology was observed at 15 days after GA application. At approximately 30 days after GA application, a distinct dome shaped apex was observed

characterised by an elongating peduncle lifting the apex above the tuber surface (Naor et al., 2005a). While GA_3 was the most abundant endogenous GA in *Zantedeschia* (Naor et al., 2008), no significant differences on flowering were observed between the application of GA_3 and GA_{4+7} (Brooking and Cohen, 2002; Naor et al., 2005b). However, GA_3 was not as effective as Promalin (GA_{4+7} + BAP; Valent BioSciences, USA) in the conversion of dominant buds to their floral state (Funnell et al., 1992). The presence of GA does not always guarantee visible manifestation of floral induction. In plants grown *in vitro* (Naor et al., 2004) or *in vivo* (Brooking and Cohen, 2002), inflorescence development ceased at later stages despite the application of GA. Plants treated with a dose of BAP, following the treatment with GA, showed an increased percentage of plantlets that reached the advanced stage (Stage 6) of floral development. BAP alone had no effect on floral differentiation, but in the presence of GA, enhanced floral emergence (Naor et al., 2005b). The application of BAP, following GA treatment had no significant effect on the flowering of the primary shoot, but significantly increased flowering in the secondary shoot, compared to the application of GA alone (Naor et al., 2005b). It has been hypothesised that BAP possibly acts by mitigating apical dominance and thereby increasing the number of shoots available for floral induction by GA (Naor et al., 2005b). A similar response was noted by Funnell et al. (1992) with the application of BAP + GA_{4+7} as Promalin^{TM} (Valent BioSciences, USA), albeit both primary and secondary shoots were induced to flower.

In addition to the number of dominant buds/branching (refer Section 1.3), tuber size is also a key determinant of floral productivity in *Zantedeschia*, where increased flowering was observed with increasing tuber diameter (Corr and Widmer, 1991) and weight (Naor et al., 2004a). Since the tuber comprises a commercially significant part of *Zantedeschia* (refer Section 1.2), they are commercially graded and marketed by diameter (Corr and Widmer, 1991). Larger sized tubers produced a greater number of flowers, especially from the secondary shoots (Naor et al., 2005a), which has been hypothesised to be achieved by relaxing the strength of dormancy (Borochov et al., 1997) and/or by changes in carbohydrate metabolism (Corr, 1988).

Growth of the tuber is a function of leaf area and leaf area duration (Funnell et al., 2002). Therefore, an increase in tuber size can be hypothetically achieved by increasing the number of axillary shoots which in turn would enhance the leaf area of the branched plant, compared to the control (unbranched plant). In theory therefore, two approaches were postulated to accomplish the commercial objective of increasing floral productivity in *Zantedeschia* (refer Section 1.6). While one approach was aimed at mitigating the decline in floral productivity with increased durations of storage, the second approach was based on devising strategies to stimulate branching in *Zantedeschia*, with the dual motives of increasing the number of dominant buds/shoots and thereby generating larger sized tubers.

2.6 Common mechanisms between dormancy and flowering

Given that, both endodormancy and flowering are governed by the same environmental signals i.e., photoperiod and vernalisation, it is not unreasonable to imagine that both these key developmental processes are controlled by the same mechanisms (Horvath et al., 2003). Accordingly, the CO/FT module (refer Section 2.5.1), a vital component of the photoperiodic flowering pathway (Fig. 2.7) was found to mediate the short-day induced growth cessation/endodormancy in poplar. Over-expression of the FT gene bypassed the onset of endodormancy under short-days and interference of FT gene expression immediately imposed endodormancy (Böhlenius et al., 2006). Comparative studies in other crops have also yielded similar results (Horvath, 2009). Since in these photoperiodic responsive plant species the photoperiodic pathway of flowering (Fig. 2.7) involves the entrainment of the circadian clock as well, further studies investigating the liaison between the circadian clock and the onset of endodormancy are being undertaken (Rohde and Bhalerao, 2007). In *Zantedeschia*, where in contrast the onset of endodormancy is autonomously controlled (Chapter 2.3.2) and flowering is not stimulated by external/environmental signals (Chapter 2.5.2), the intricate network of internal signals have to be relied upon to determine the common features between dormancy and flowering. Hormonal cross-talk is likely to be an integral part of this network. However, GA which triggers the floral switch in *Zantedeschia* (Corr and Widmer, 1987; Funnell, 1993; Brooking and Cohen, 2002; Naor et al., 2005a), is unable to release buds from

endodormancy (Naor et al., 2008), thereby indicating additional factors of control. Among these, based on personal observation, it is likely that the number of nodes/leaves put forth by the SAM (Sachs, 1999), prior to the onset of endodormancy during the vegetative growth cycle, and prior to flowering during the flowering growth cycle could be a common feature between dormancy and flowering in *Zantedeschia*.

3. Identification of factors that contribute to the decline in flowering with long-term storage of *Zantedeschia* tubers

3.1 Introduction

The increasing commercial significance of *Zantedeschia* (refer Section 1.2), and its inherent cyclic periodicity (refer Section 1.4), have persuaded growers to adopt a scheduled planting of tubers, which often requires long-term cold storage of tubers to achieve successive plantings (Dennis et al., 1994). While short-term cold storage is capable of relaxing endodormancy (Corr and Widmer, 1988), long-term storage controls the timing of floral emergence by inhibiting vegetative shoot growth (Funnell and Go, 1993), possibly by imposing ecodormancy (Naor et al., 2006). To facilitate year round production of flowers, the current commercial practice in New Zealand involves storing tubers dry for up to 4 months (Dennis et al., 1994), and the inhibition of shoot outgrowth is ideally achieved at a temperature of 7 ± 3°C (Funnell and MacKay, 1988b).

A major drawback associated with this long-term storage is a decline in the floral productivity observed with increasing durations of storage (Funnell et al., 1988; Funnell and Go, 1993). This decline in the flowering ability was not due to chilling injury and/or meristem abortion, because the decline occurred irrespective of storage temperature (5, 12, 18 or 24ºC; Funnell et al., 1988) and dissection of meristems revealed an active apex (Funnell, 1993; Funnell and Go, 1993). As detailed within the following paragraph, other factors were, therefore, hypothesized to be involved in this decline of flowering associated with long-term tuber storage.

The application of Promalin™ (Valent BioSciences, USA), comprising the gibberellins (GA), GA_{4+7} and the cytokinin 6-benzylaminopurine (BAP), partially compensated for this decline in flowering (Funnell and MacKay, 1988a), and higher concentrations of Promalin™ were required to maintain an increased level of floral productivity with increasing durations of storage (Funnell and Go, 1993). Since GA induces floral transition in *Zantedeschia* (Cohen, 1981; Corr and Widmer, 1987), and since the concentration of the hormone and the sensitivity of the responding tissue are both key factors in determining a

physiological response (refer Section 2.2), the decline in flowering associated with long-term storage was hypothesized to be due to one or more of the following (Funnell and Go, 1993):

- Reduction in concentration/activity of endogenous GA
- Change in sensitivity to exogenous GA
- Reduction in uptake efficiency of liquids/GA

Trewavas (1983) argued that a spatial and/or temporal change (Weyers and Paterson, 2001) in the sensitivity of the responding tissue (in this case the bud/meristem) to the hormone (in this case GA) is often ignored as a factor that contributes to the manifestation of a physiological response. In a bid to demonstrate possible factors that could contribute to sensitivity changes, Firn (1986) proposed a modelling approach based on dose-response curves (refer Section 2.2.1). Based on the factor that contributes to the change in sensitivity, a corresponding change in the dose-response curve is observed. These model curves can, therefore, be used as a template to diagnose sensitivity changes.

Within this thesis, the commercial objective of increasing floral productivity in *Zantedeschia* was proposed to be achieved through two approaches (Section 1.6). As addressed within this chapter, one of the approaches involved mitigating the decline in flowering observed with long-term storage of tubers. To establish an initial assessment of the factors that contribute to this decline, the first two hypotheses proposed by Funnell and Go (1993) were sought to be tested, with the aim of pursuing this approach further, based on the results obtained. Therefore, the objectives of the current study were:

1. To identify changes in the endogenous GA content concomitant with increasing durations of storage and,
2. To identify changes in the sensitivity of the buds to exogenous GA application and, thereby, tentatively identify the factors that contribute to these sensitivity changes.

3.2 Materials and methods

3.2.1 Plant material and culture

Flowering-sized tubers (Fig. 1.1h) (30 ± 2 cm circumference) of *Zantedeschia* cv. 'Best Gold' were lifted from a field in Palmerston North, New Zealand (40º20'S) during March 2006. Tubers with 8 ± 2 dominant buds (Fig. 1.3a) were used for this experiment. After the respective treatment application (refer section 3.2.3), tubers were planted at a uniform height in 3.5 l polythene planter bags containing a commercial bark-based medium (Daltons; New Zealand) (Appendix 11.1) and slow-release fertilizer (Appendix 11.2). After planting, the bags were topped up with the same medium to a uniform height. The bags were transferred to a 50% shaded glasshouse (minimum temperature 15°C; vented at 20°C) at the Plant Growth Unit, Massey University, Palmerston North, New Zealand (40º20'S), and watered through capillary matting on drained benches using drippers which supplied between 50 and 60 ml of water per plant per day. Overhead watering was provided immediately after planting and subsequently once every week until 120 days after planting to minimise any increase in salinity on the medium's surface. A fungicide, Thiram (Tetramethyl thiuram disulfide; Nufarm Ltd., AUS) @ 1.5 g/l) was applied as a growing medium drench, 8-10 days after planting.

3.2.2 Storage treatment durations

Tubers selected using the criteria stated above (Section 3.2.1) were stored for 4, 6 or 9 months, until they were planted. The cool store was maintained at 7 ± 2 °C and 70 ± 10% relative humidity, for the respective storage durations.

3.2.3 GA treatment

In previous experiments that have studied the effect of GA on flowering (Corr and Widmer, 1987; Brooking and Cohen, 2002), GA_3 has been applied at a concentration range of 0 to 3 mM. Since the current experiment involved the storage of tubers, a higher range was postulated so as to achieve satisfactory flowering. Therefore, GA_3 (Molecular weight = 346.38 g/mole) (OlChemIm Ltd., Czech Republic) at one of the following concentrations *viz*. 0 (control), 0.003, 0.009, 0.03, 0.09, 0.3, 0.9, 3 or 9 mM (equivalent to 0, 1, 3, 10, 30,

100, 300, 1000 or 3000 mg l^{-1}) was prepared by dissolving appropriate quantities of GA_3 in 5 ml of 95% ethanol, and the final volume was made up using distilled water. Tween 20 (Sigma CAS No. 9005-64-5; 0.2%) was added as a surfactant. The control treatment (0 mM) comprised all components except GA_3.

After each storage duration (i.e., 4, 6 and 9 months), tubers were removed from the cool store and maintained at room temperature for 6-7 hours. Tubers were randomly allocated to one of the GA_3 treatment concentrations, with the respective treatments applied by soaking the tubers in a plastic tray of the treatment solution for one hour. Since the upper part of the tuber was shown to be the primary region of uptake (Brooking and Cohen, 2002), the tubers were soaked with the upper part facing downwards into the GA_3 solution. A control treatment for each storage duration comprised tubers soaked in a solution of distilled water with Tween 20. After soaking, tubers were removed from the trays and allowed to dry overnight at room temperature. The following morning, tubers were planted as described above (Section 3.2.1).

3.2.4 Experimental design and statistical analysis

Following the 3 different storage durations, 12 tubers (replicates) were allocated randomly to one of each concentration of GA_3 in a 3 × 9 factorial structure (3 storage durations × 9 GA_3 concentrations × 12 replicates = 324 tubers). The planter bags containing the tubers were placed on drained benches with capillary matting, arranged along the width of the glasshouse. Prior measurement of air temperature at different locations within the glasshouse did not reveal significant differences, hence within each storage duration, individual replicates were placed on benches at random locations within the glasshouse, at a density of 36 replicates per bench.

Analysis of variance, means ± standard errors, mean separation tests and contrasts for the number of flowers and shoots were carried out using PROC GLM of SAS version 9.2 (SAS Institute., USA) and MS Excel (Microsoft Corp., USA). A test of proportions was performed using a Z-test (Polaris Marketing Research Inc., USA).

3.2.5 Observations

Shoots were tagged and numbered as they emerged. The number of shoots per tuber and the number of flowers per shoot and, therefore, the tuber, differentiated as primary and secondary flowers (Fig. 2.7) were counted every 15 days from when flowers appeared (i.e., presence of spathe and spadix evident) until 120 days after planting (DAP), after which floral emergence ceased. Primary flowers were those that emerged as a result of floral transition in the shoot apical meristem (SAM) of the primary shoot (Fig. 2.7a), and were normally the first to emerge. Secondary flowers were those that emerged as a result of the sympodial shoot growth habit, following flowering in the primary shoot (Fig. 2.7b). Data on the time to floral emergence and the quality of flowers were not considered crucial for corroborating the stated objectives, and were therefore not measured.

Since the number of shoots bears a direct relationship to the floral productivity of *Zantedeschia* (refer Section 1.3), it was hypothesized that the number of shoots may provide an indirect estimate of the floral productivity of *Zantedeschia*. Therefore, the effect of different concentrations of GA_3 on branching after different storage durations and between different storage durations was measured at the end of the experiment i.e., 120 days after planting.

Due to an inherent variability in the population of buds, measurement of the floral productivity at the whole plant/tuber level presents difficulties in determining the response of the individual bud/shoot (Funnell and Go, 1993). Therefore, in the current experiment and previous studies (Funnell and Go, 1993; Brooking and Cohen, 2002), the flowering response has been measured as a proportion of the number of shoots (Fig. 3.1; 3.2). For each storage duration (4, 6 or 9 months), the effect of different concentrations of GA_3 on the floral productivity of the tuber was determined as a proportion of the total number of flowers per tuber to the total number of shoots per tuber (i.e. total flowering percentage), and the mean number of flowers per tuber. The ability of GA_3 to induce floral transition in each shoot was calculated as the proportion of the number of primary flowers per tuber to the total number of shoots per tuber (i.e. primary flowering percentage).

3.3 Results

3.3.1 Overview

Maximum number of shoots was obtained at around 90 DAP, and this number was retained through to the end of the experiment (120 DAP). Visible manifestation of flowering was not observed beyond 120 DAP, and so flowering data was not recorded beyond this time. At 120 DAP, the production of new leaves had ceased, probably suggesting the onset of endodormancy (refer Section 1.3).

3.3.2 Number of shoots

Among the main effects comprising storage durations and concentrations of GA_3, GA_3 had no effect on the number of shoots ($P > 0.1$) irrespective of increasing concentrations. A significant decline ($P < 0.0001$) in the number of shoots was however observed after 9 months of storage (3.5 ± 0.2 shoots) compared to the 4 (4.2 ± 0.2 shoots) and 6 (4.8 ± 0.2 shoots) month storage durations (Table 3.1). An interaction between the two factors was not evident ($P > 0.1$).

3.3.3 Flowering

3.3.3.1 Number of flowers

A significant decline in the total number of flowers (primary and secondary) per tuber was not observed ($P > 0.1$) after 4 (1.8 ± 0.2 flowers), 6 (1.8 ± 0.2 flowers) or 9 (1.5 ± 0.2 flowers) months of storage (Table 3.2). Irrespective of the storage durations, an increase in the total number of flowers per tuber was observed with increasing concentrations of GA_3 ($P < 0.0001$; Table 3.2). Interaction between the two main factors was significant ($P < 0.0005$), suggesting an interdependence of the number of flowers on both the storage durations and concentrations of GA_3. The significant interaction was caused by a significant decline and increase in the number of flowers caused by 0.09 mM and 9 mM GA_3 respectively, after 6 months of storage compared to 4 and 9 month storage durations (Table 3.2). This was considered to be a random event.

Table 3.1 Number of shoots (± SE) per tuber of *Zantedeschia* cv. 'Best Gold' obtained following the application of increasing concentrations of GA_3 (mM) after 4, 6 or 9 months of tuber storage

Storage Durations (months)	**GA_3 Concentration (mM)**									**Mean number of shoots**
	0	**0.003**	**0.009**	**0.03**	**0.09**	**0.3**	**0.9**	**3**	**9**	
4	4.1 ab (0.6)	4.5 a (0.4)	4.6 a (0.5)	3.8 a (0.4)	4.3 a (0.5)	4.9 a (0.6)	4.3 ab (0.4)	4.0 a (0.6)	3.7 b (0.6)	4.2 (0.2) b
6	5.0 a (0.6)	4.7 a (0.6)	4.3 a (0.6)	4.6 a (0.5)	4.5 a (0.6)	4.3 a (0.4)	5.3 a (0.7)	5.2 a (0.4)	5.3 a (0.4)	4.8 (0.2) a
9	2.8 b (0.6)	4.7 a (0.6)	3.5 a (0.7)	4.3 a (0.6)	2.5 b (0.3)	3.4 a (0.7)	3.4 b (0.5)	3.8 a (0.6)	2.6 b (0.6)	3.5 (0.2) c

Within the same column, different *small letters* indicate significant differences ($\alpha = 0.05$) between the mean number of shoots after 4, 6 or 9 months of storage. Mean separation by Least Significant Difference (LSD) (*n = 12*)

Table 3.2 Total number of flowers (primary and secondary) (± SE) per tuber of *Zantedeschia* cv. 'Best Gold' obtained following the application of increasing concentrations of GA_3 (mM) after 4, 6 or 9 months of tuber storage

Storage Durations (months)	**GA_3 Concentration (mM)**									**Mean number of flowers**
	0	**0.003**	**0.009**	**0.03**	**0.09**	**0.3**	**0.9**	**3**	**9**	
4	0.3 a (0.2)	0.1 a (0.1)	0.2 a (0.1)	0.4 a (0.2)	2.2 a (0.2)	2.9 a (0.3)	3.5 a (0.5)	3.7 a (0.4)	3.2 b (0.3)	1.8 a (0.2)
6	0.5 a (0.3)	0.2 a (0.2)	0.3 a (0.2)	0.4 a (0.2)	0.6 b (0.2)	2.1 a (0.4)	3.5 a (0.7)	3.8 a (0.3)	5.0 a (0.4)	1.8 a (0.2)
9	0.1 a (0.1)	0.4 a (0.2)	0.2 a (0.2)	1.2 a (0.4)	1.4 a (0.3)	1.6 a (0.5)	2.9 a (0.6)	3.3 a (0.4)	2.5 b (0.8)	1.5 a (0.2)

Within the same column, different *small letters* indicate significant differences ($\alpha = 0.05$) between the mean number of total flowers (primary and secondary) after 4, 6 or 9 months of storage. Mean separation by Least Significant Difference (LSD) (*n = 12*)

3.3.3.2 Total flowering percentage

After 4, 6 or 9 months of storage, the floral productivity of the tubers across all the concentrations of GA_3, expressed as the total flowering percentage (comprising primary and secondary flowers), was interpreted as a sigmoidal dose-response curve (Fig. 3.1). Between 4 and 6 month storage durations, a significant difference in the total flowering percentage was not observed ($P > 0.1$) between 0 mM and 0.03 mM concentrations of GA_3 (Fig. 3.1). However, between 0.09 mM and 3 mM, a decline in the total flowering percentage was observed after 6 months of storage, though the difference was significant ($P = 0.05$) only at 0.09 mM, where 51.1% and 12.9% flowering was observed after 4 and 6 months of storage, respectively (Fig. 3.1).

Between 0 and 0.009 mM GA_3, the response curve after 9 months of storage was similar to that observed after 4 and 6 months of storage (Fig. 3.1). Between 0.03 mM and 3 mM however, the total flowering percentage after 9 months of storage was higher than that after 6 months, though the difference was significant ($P = 0.05$) only at 0.09 mM GA_3 (Fig. 3.1). The total flowering percentage after 9 months of storage was similar ($P > 0.1$) to that after 4 months of storage (Fig. 3.1).

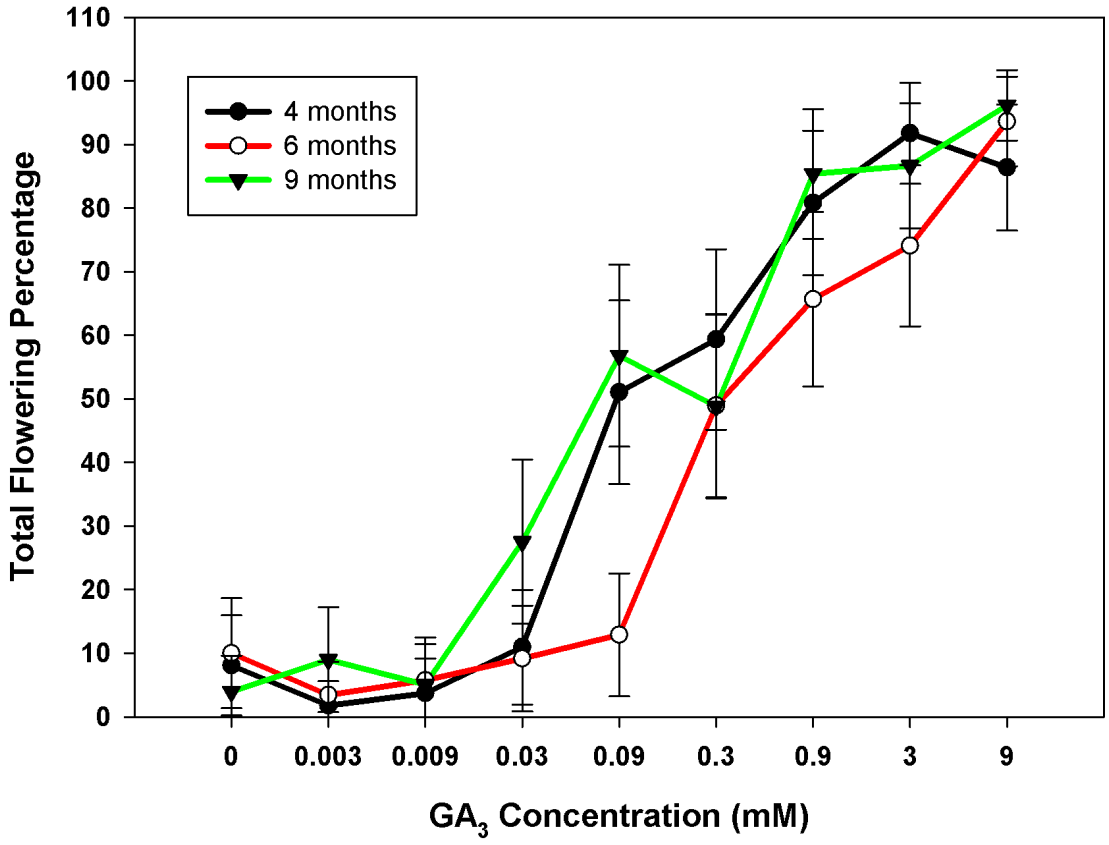


Fig. 3.1 Total flowering (Primary and secondary) percentage (± SE) of *Zantedeschia* cv. 'Best Gold', obtained following the application of increasing concentrations of GA_3 (mM) after 4, 6 or 9 months of storage of tubers.

3.3.3.3 Primary flowering percentage

The change in the ability of GA_3 to induce floral transition in the shoots with increased durations of storage was expressed by the primary flowering percentage (Fig. 3.2). The dose-response curves for the primary flowering percentage (Fig. 3.2) showed a similar pattern to that illustrated by the total flowering percentage (Fig. 3.1) for all storage durations. Between 4 and 6 month storage durations, though not significant ($P > 0.1$), a decline in the primary flowering percentage was observed at 0.09 mM, 0.3 mM and 3 mM concentrations of GA_3 (Fig. 3.2). Between 6 and 9 months storage durations, an increase in the primary flowering percentage was observed at 0.03 mM, 0.09 mM and 3 mM concentrations of GA_3, though the difference was significant ($P = 0.1$) only at 0.09 mM,

where 43.2% and 11.1% of the shoots flowered after 6 and 9 months of storage, respectively (Fig. 3.2). The lower magnitude of this variable was due to the exclusion of secondary flower data.

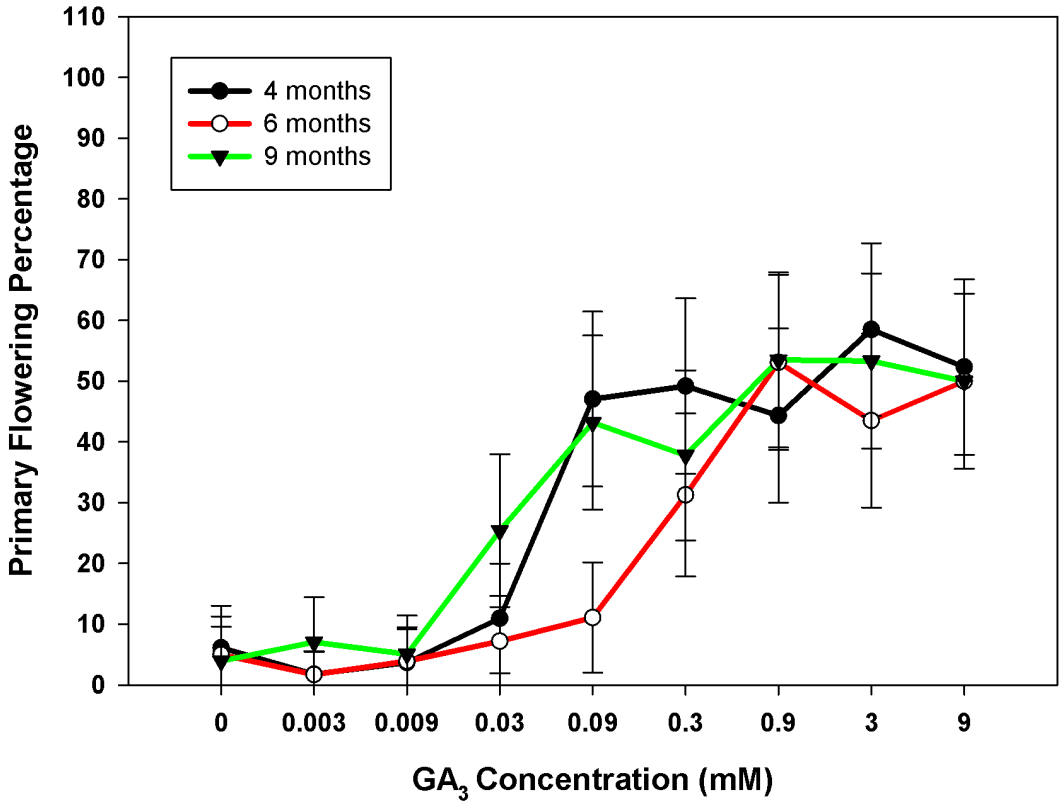


Fig. 3.2 Primary flowering percentage (± SE) of *Zantedeschia* cv. 'Best Gold', obtained following the application of increasing concentrations of GA_3 (mM) after 4, 6 or 9 months of tuber storage.

3.4 Discussion

Flowering-sized tubers of *Zantedeschia* have a variable population of buds comprising dominant (Fig. 1.3a) and axillary buds, which are further classified as developed and undeveloped axillary buds (Funnell and Go, 1993). The occurrence of this variable population of buds is further compounded by the different terminology adopted to describe these buds, where Naor et al. (2005a) termed 'Dominant bud 1' as the primary bud (Fig. 1.3a), the remaining dominant buds (2-5; Fig. 1.3a) as secondary buds and the buds located

along the axis of each growing shoot as axillary buds. Previous experience has shown that a counting of dominant buds, prior to planting, does not always equate to the number of emerging shoots. In the current study itself, while tubers with an average count of 8 dominant buds were selected at the beginning of the experiment, a maximum of only 5.3 ± 0.7 shoots emerged (Table 3.1).

Concomitant to the variable population of buds is an inherent variability in the ability of the respective shoots to flower (Funnell and Go, 1993). The apical bud i.e., dominant bud 1 (Fig. 1.3a) is always the first to emerge and flower, and the percentage of floral stems per shoot declines basipetally along the tuber axis (Naor et al., 2005a). While the presence of GA_3 had no discernible effects on the number of shoots, the storage duration of 9 months caused a significant decline in the number of shoots, compared to the 4 and 6 months storage (Table 3.1). These limited numbers of shoots i.e., apical shoot and a few dominant shoots, were therefore naturally prone to flowering than the variable population of shoots that emerged after 4 and 6 months of storage.

The decline in the total (Fig. 3.1) and primary (Fig. 3.2) flowering percentages observed between the 4 and 6 month storage durations demonstrated a shift along the x-axis which, according to Firn (1986; Fig. 2.3B) was interpreted to represent a change in the sensitivity conferred by a change in the affinity of the receptors to GA_3. Therefore, between 4 and 6 months storage of the tubers, a change in the affinity of the buds to GA_3 was most likely to contribute to the change in sensitivity, which in turn resulted in a reduced flowering percentage. These results reiterate the results previously obtained by Funnell and Go (1993), where a shift along the x-axis was also observed for a flowering response with *Zantedeschia* 'Best Gold' concomitant with a logarithmic concentration of Promalin™, between 0, 3 and 6 months of storage. Therefore, combining the results of both these studies with *Zantedeschia*, although the experimental material and the GA source were different, it can be concluded that a decline in the floral productivity observed between 0 and 6 months storage of tubers is contributed by a decline in the affinity of the receptor/responding tissue to GA.

Brooking and Cohen (2002) also reported a difference in the dose-response curves with different treatment durations of GA. In their research, the dose-response curves between tubers treated with GA for increasing soak durations demonstrated a progressive shift in the x-axis of the flowering response with increasing duration. This change in the dose response suggests a change in the affinity of the buds to GA. However it is also possible that a change in the uptake efficiency of the tubers/buds could have contributed to this difference in response. Though Firn (1986) has listed a change in the uptake efficiency of the hormone amongst possible factors that could contribute to changes in the sensitivity of the responding tissue (refer Section 2.2.1), the proposed model graphs (Fig. 2.3) do not accommodate a change in the uptake efficiency. It therefore becomes impossible to predict the changes in the dose-response curves conferred by a change in the uptake efficiency. In the current experiment, all tubers from the three storage durations were subjected to 1 hour soak duration, irrespective of GA_3 concentration. Though a decline in affinity is suggested to contribute to the sensitivity changes observed between tubers stored for 4 and 6 months, a decline in uptake efficiency could also be hypothesized. Further studies are required to investigate if in fact uptake efficiency declines with increasing storage durations.

While the decline in the total (Fig. 3.1) and primary (Fig. 3.2) flowering percentages between the 4 and 6 month storage durations was attributed to a decline in sensitivity contributed by a decline in the affinity of the receptors to GA, a further decline in the sensitivity of the buds to GA_3 and, therefore, a decline in the response depicted by the flowering percentage, was expected after 9 months of storage of the tubers. Within the current experiment however, between 0.03 and 9 mM GA_3 the total flowering percentage was higher after 9 months of storage than after 6 months of storage (Fig. 3.1). Since the response between this range of GA_3 concentrations was similar to that obtained after 4 months of storage, it is easy to assume that the sensitivity of the buds to GA_3 was restored after 9 months of storage. However, as mentioned earlier, it is more likely that the natural tendency of the limited shoots that emerged after 9 months of storage to flower, which was ultimately manifested as an increase in the total and primary flowering percentages between the 6 and 9 month storage durations (Fig. 3.1; 3.2). Given the variability in the ability of different shoots to flower, a bud to bud comparison of the factors that determine floral transition needs to be studied. Without a clear mapping of the buds based on their capacity

to flowering, it is difficult to make a sensible interpretation of flowering data. Moreover, the flowering-sized tubers used in the current experiment are naturally prone to flowering, revealed by the flowering of the control treatment (Table 3.2). Therefore, the effect of GA_3 on flowering in the current experiment was inclusive of the innate flowering potential of a shoot, and consequently the tuber. In future research, utilising an experimental system that exclusively reports the effect of GA on flowering would indeed be beneficial to overcome this inherent variability in flowering.

In other geophytes such as potato (*Solanum tuberosum* L.), post harvest storage of tubers resulted in a decline in the endogenous GA content (Suttle, 2004). Likewise in *Zantedeschia*, a decline in the endogenous content of GA was hypothesized by Funnell and Go (1993), and the current experiment, to be amongst several other factors responsible for the decline in the floral productivity observed with increasing durations of tuber storage. According to Firn's (1986) approach (refer Section 2.2.1), the dose-response curve obtained by an increase in the endogenous hormone concentration would be expressed by an increase in the corresponding response, even at the lower concentrations of the hormone (dose) (Fig. 2.3D). In the current experiment however, after any of the storage durations, both the total flowering percentage (Fig. 3.1) and the primary flowering percentage (Fig. 3.2) failed to exhibit a dose-response curve similar to that suggested by Firn (1986), with regard to a change in the endogenous hormone concentration (Fig. 2.3D). It is therefore concluded that the endogenous GA concentration was not high after 4 months of storage, to decline further after 6 and 9 months of storage. Comparing the dose-response curves obtained by Funnell and Go (1993) with the model graph predicting changes in the endogenous hormone levels proposed by Firn (1986; Fig. 2.3D), the endogenous GA content was not high, even after 0 months of storage, to decline further. Naor et al. (2008) also reported that the endogenous GA concentration remained constant throughout the storage period. However, the dose-response curves obtained from the current experiment, and that from Funnell and Go (1993), were based on the exogenous application of GA_3 and GA_{4+7} (as Promalin™), respectively and, therefore, represent endogenous changes with regard to these two types of GA alone. However, more than 100 types of GA have been identified in plants so far (Richards et al., 2001). As a future research strategy, quantification of other GAs, crucial for the biosynthesis of GA_3 in *Zantedeschia*, may

provide a greater insight into the changes in the endogenous GA concentration with increasing durations of storage.

3.5 Conclusions

Based on the modelling approach proposed by Firn (1986), the decline in the flowering percentage between 4 and 6 month storage durations was attributed to a decline in the sensitivity conferred by a concomitant decline in the affinity of the receptors to GA_3. The increase in the flowering percentage between 6 and 9 month storage durations was attributed to the natural tendency of the limited shoots to flower, rather than a restoration of sensitivity. Finally, a decline in the endogenous GA concentration was not observed with increasing durations of storage.

As planned earlier (refer Section 1.6), due to the successful stimulation of branching using the second approach to achieve the commercial objective (refer Section 1.6), this approach was extensively investigated throughout the rest of this thesis. The first approach as explored within the current chapter was therefore not pursued further within this thesis.

4. Methods to stimulate branching in *Zantedeschia* - Defoliation

4.1 Introduction

The sympodial growth habit of *Zantedeschia* (Funnell, 1993; refer Section 1.3) dictates a direct relationship between the number of shoots and floral productivity (refer Section 1.4). Since shoot emergence precedes floral initiation, a branched plant of *Zantedeschia* is considered to possess a higher flowering potential (Funnell, 1993), and is therefore desired by the grower community (Clark et al., 1987). In Section 1.6, the commercial motivation of the thesis and the two strategies devised to achieve this commercial objective was outlined. Both approaches were planned to be undertaken simultaneously, with the aim of subsequently undertaking more extensive investigations using one approach, based on the results obtained. As explored within the current chapter, based on the second approach (refer Section 1.6), experiments were conducted to evaluate methods of stimulating branching. In this thesis, it is hypothesised that the stimulation of branching in *Zantedeschia* will influence floral productivity via two mutually inclusive/exclusive mechanisms:

- As mentioned above, the sympodial growth habit of *Zantedeschia* allows floral initiation in the shoot apical meristem (SAM) of the primary shoot to trigger a cascade of flowering in the secondary, and subsequently the tertiary, shoots (Funnell, 1993). Therefore, an increase in the number of shoots should manifest as an increase in the overall floral productivity of the tuber.

- Floral productivity of *Zantedeschia* is directly related to the size of the tuber (Corr and Widmer, 1991; Naor et al., 2005a), which in turn is a function of the leaf area and leaf area duration (LAD) during the growth cycle (Funnell et al., 2002). A branched plant with an increased leaf area allows an increased rate of net photosynthesis, which in turn influences the biomass and source-sink partitioning of the plant (Sakamoto, 2006). In *Zantedeschia*, this could equate to a larger sized tuber and, therefore, increased floral productivity.

While the apical region as a whole was considered to be responsible for the correlative inhibition of axillary bud outgrowth in other plant species, the specific part(s) of that region that contributed to this inhibition were at that time unknown (Snow, 1929). In a few tree species, Goebel (1880) as cited by Snow (1929), is suggested to have stimulated branching by defoliating the shoots while maintaining an intact apical bud. The role of leaves in the control of branching was further supported by Snow (1929), who showed that the young leaves residing in the apical region, rather than the whole region *per se*, was responsible for correlative inhibition of the axillary buds. The chemical identity of this inhibition was subsequently revealed as auxin by Thimann and Skoog (1933; 1934).

The classical decapitation and auxin replacement experiments (Thimann and Skoog, 1933; 1934) also provided sufficient evidence that the apical region and the adjoining immature tissues were a primary source of auxin synthesis. However, other growing regions are also capable of auxin synthesis (Woodward and Bartel, 2005). To facilitate auxin distribution throughout the plant system, two modes of auxin transport have been proposed (Friml and Palme, 2002):

- the slower, polar auxin transport (PAT), system, where auxin loaded onto this system moves in a cell-to-cell manner strictly in a basipetal direction and,
- the relatively faster, transport in the phloem, where transport occurs in both acropetal and basipetal directions. Auxin flow within the phloem may also later be integrated into the PAT system.

The export of auxin from the leaves was corroborated by the transport of radiolabeled auxin applied exogenously on the leaves of *Pisum sativum* L. (pea), and the subsequent identification of radioactivity in the PAT system (Cambridge and Morris, 1996). While the auxin export was thought to be confined to the immature tissue/young leaves alone (Snow, 1929; Davies, 1995), Jager et al. (2007) showed that the mature leaves are also capable of auxin export, and contribute to the overall auxin economy of the plant. Auxin exported from the leaves, irrespective of young or mature is considered partly responsible for the inhibition of the subtending axillary buds because, in the presence of leaves, the subtending axillary buds failed to grow even after decapitation (Dostál, 1926 cited in Snow, 1929).

Therefore, defoliation has been used as a non-chemical approach to induce branching in several plant species (Ericsson et al., 1980 in *Pinus sylvestris* L. (Scots pine); Hilton et al., 1987 in *Quercus robur* L. (Oak); Volz et al., 1994 in *Malus domestica* Borkh. (Apple); and Collin et al., 2000 in *Fraxinus excelsior* L. (Common ash)).

The potential to stimulate branching in *Zantedeschia* has been demonstrated by the decapitation/scooping of the apical bud (Clark et al., 1987). However, as described in Section 2.4.1, the adoption of decapitation as a commercial practice has several drawbacks and, therefore, was not explored further in the current research. Given this response to decapitation however, as part of the current thesis, pilot studies ($n = 10$) were conducted to provide a preliminary assessment of the defoliation treatments, which comprised:

- removal of the two immature/young leaves,
- removal of the two mature/old leaves,
- removal of all existing leaves and,
- the control treatment, where the plants were left intact.

Significant stimulation of branching was not achieved by any of the defoliation treatments applied ($P > 0.1$; Data not presented). Given that, when treatments were applied in this pilot study the plants had accumulated 2000 ± 100 degree-days, it was considered likely that the onset of endodormancy (Carrillo Cornejo et al., 2003) may have rendered the defoliation treatments ineffective. Therefore, as detailed within this chapter, it was proposed to evaluate the effect of the same and additional defoliation treatments when applied during the active growth period i.e., prior to the establishment of endodormancy, with the specific objectives of:

1. Evaluating the efficacy of defoliation during the active growth period in stimulating branching in *Zantedeschia* and,
2. Quantifying any effect of these treatments on the size of the resulting tuber.

4.2 Materials and Methods

4.2.1 Plant material and culture

Seedlings of *Zantedeschia* cv. 'Best Gold', during their first growth cycle (Fig. 1.1b), were used as the experimental material. To conduct the experiment prior to the establishment of endodormancy, seedlings that had accumulated 1300 ± 100 degree-days were selected (Carrillo Cornejo et al., 2003). This was achieved approximately 80 ± 5 days after sowing and, at this stage, the seedlings typically comprised a single shoot with 4-5 expanding(ed) leaves (Single-shoot system; Fig. 4.1a). Under the same environmental conditions, seedlings would be expected to produce 7 ± 1 leaves before the cessation of new leaf production (refer Chapter 7). Therefore, the seedlings selected with 4-5 leaves were considered to be in their active growth period.

The seedlings were grown in 2.8 l polythene bags in a commercial bark-based medium (Appendix 11.1) with a slow-release fertilizer (Appendix 11.2) and automated irrigation, as described in Section 3.2.1. The temperature and irrigation conditions in the glasshouse were also as described in Section 3.2.1.

4.2.2 Defoliation treatments

The defoliation treatments were devised to analyse the effect of young and/or old leaves on the inhibition of the corresponding axillary buds. Therefore, the following treatments were applied in the current experiment:

- Removal of the two existing youngest/immature leaves at the commencement of the experiment (Fig. 4.1b)
- Removal of the two existing oldest/mature leaves at the commencement of the experiment (Fig. 4.1c)
- Removal of all existing leaves at the commencement of the experiment, followed by the continuous removal of all subsequently emerging leaves (Fig. 4.1d)
- Removal of all existing leaves at the commencement of the experiment (Fig. 4.1e)
- No removal of leaves (Control) i.e., intact plant (Fig. 4.1a)

Fig. 4.1 Single-shoot system of *Zantedeschia* cv. 'Best Gold' during its active growth period after the application of different defoliation treatments where **a**) no leaves were removed (control), **b**) the two youngest leaves were removed, **c**) the two oldest leaves were removed, **d**) all existing leaves were removed at the commencement of the experiment, followed by the continuous removal of subsequently emerging leaves and, **e**) all existing leaves were removed at the commencement of the experiment only.

4.2.3 Observations

As mentioned above (Section 4.2.1), no new leaf production was observed after the emergence of 7 ± 1 leaves. The cessation of new leaf production was, therefore, regarded as an indicator of the end of the active growth period (refer Chapter 7). The number of visible axillary shoots was counted at fortnightly intervals until the cessation of new leaf production (approximately 140 ± 5 days after sowing). Any visible axillary shoot with height > 1 cm was counted.

Following the cessation of new leaf production, senescence and wilting of existing leaves were subsequently observed, thereby marking the end of the growth cycle. Once foliage senescence was complete (approximately 255 ± 5 days after sowing) the fresh weight of the tuber was measured, after the tubers were lifted following the withdrawal of irrigation.

4.2.4 Experimental design and statistical analysis

Thirteen single-plant replicates per treatment were arranged in a completely randomized design. As implemented in Chapter 3, the replicate plants were grown in a glasshouse with no significant differences in the temperature and light conditions observed at different locations. In light of this, blocking of the treatments was not adopted.

Analysis of variance, means ± standard errors and mean separation tests for the number of shoots and fresh weight of tubers, between different defoliation treatments, were carried out using PROC GLM of SAS version 9.2 (SAS Institute., USA) and MS Excel (Microsoft Corp., USA).

4.3 Results

4.3.1 Overview

At the commencement of the experiment, all plants essentially comprised only one primary shoot (single-shoot system; Fig. 4.1a). An advantage of using this system compared to the tubers (multi-shoot systems), especially for branching studies, is that the subsequent emergence of axillary shoots can be easily attributed to the single-shoot (Fig. 4.2), whereas this becomes a formidable task in the multi-shoot system. Under the growing conditions described above (refer Section 4.2.1) the control (intact) plants produced new leaves with a plastochron of 10 days until the formation of 7 ± 1 leaves. This was followed by the cessation of new leaf production and the subsequent onset of the senescence process. Senescence started in the outermost cataphylls, followed by the mature/old leaves at the periphery of the single-shoot.

4.3.2 Branching

Apart from the removal of the two older leaves, which stimulated branching by an additional 0.4 axillary shoots per plant (Fig. 4.2) compared to the control ($P < 0.05$; Table 4.1), none of the defoliation treatments were successful in stimulating branching ($P > 0.1$; Table 4.1). From when the treatment applications commenced, through to the cessation of new leaf production i.e., the time when the counting of shoots was stopped, the control

(intact) plants (Fig. 4.1a) and those treated with the continuous removal of emerging leaves (Fig. 4.1d), had only one primary shoot i.e., these treatments failed to stimulate any axillary bud outgrowth throughout the experimental period (Table 4.1). However, the removal of the two youngest leaves (Fig. 4.1b), or removal of all existing leaves (Fig. 4.1e), stimulated the outgrowth of a few axillary shoots compared to the control (0.2 ± 0.1 and 0.1 ± 0.1 axillary shoots, respectively).

Table 4.1 Number of axillary shoots/branching produced per plant ± SE and fresh weight of the tuber ± SE following the application of different defoliation treatments on single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' during their active growth period.

Defoliation Treatment	**Number of axillary shoots per plant**	**Fresh weight of tuber (g)**
Removal of 2 oldest leaves	0.4 ± 0.2 a	37.9 ± 2.6 a
Removal of 2 young leaves	0.2 ± 0.1 ab	29.6 ± 3.1 b
Removal of all existing leaves at commencement	0.1 ± 0.1 b	28.5 ± 3.0 b
Continuous removal of leaves	0.0 b	0.0 c
Control (Leaves intact)	0.0 b	32.6 ± 1.3 ab

Within the same column different s*mall letters* indicate significant differences (α = 0.05) between different defoliation treatments. Mean separation by LSD (*n = 13*)

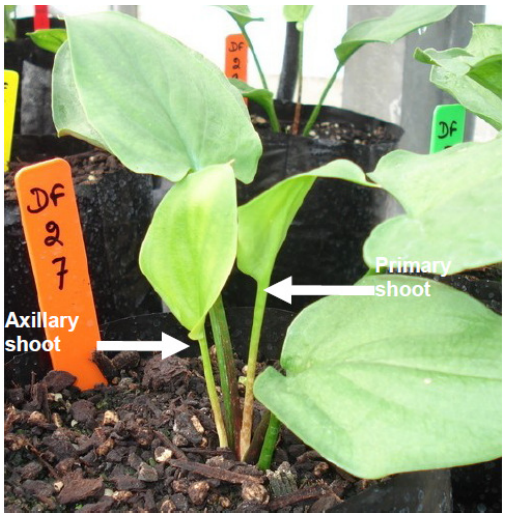


Fig. 4.2 Emergence of a new axillary shoot in the single-shoot system of *Zantedeschia* cv. 'Best Gold' following the application of the defoliation treatment where the two oldest leaves were removed.

4.3.3 Tuber weight

The treatment where the leaves were continuously removed after emergence (Fig. 4.1d) failed to produce any visible tuber at the end of the growth cycle (Table 4.1). The treatment where all the existing leaves were removed at the commencement of the experiment (Fig. 4.1e), as well as when only the two youngest leaves were removed (Fig. 4.1b), produced tubers with 12.6% and 9.2% lesser fresh weight than the control, respectively (Table 4.1). Overall, amongst the treatments with some kind of defoliation applied, the removal of the two oldest leaves produced tubers with the greatest fresh weight ($P < 0.06$; Table 4.1).

4.4 Discussion

4.4.1 Branching

The growth potential of axillary buds is correlated with their size, age and position along the stem axis, and is influenced by hormonal and nutritional gradients (Cline, 1997). In *Zantedeschia*, the older/mature leaves are physically located at the periphery of the primary shoot. This means that the corresponding axillary buds present on an underground tuber are relatively farther away from the SAM compared to the buds in the axils of the youngest leaves. Considering that the axillary bud outgrowth is controlled by the influence of auxin

from the SAM and the corresponding leaves, the defoliation treatment where the two youngest leaves were removed (Fig. 4.1b) could have potentially removed the influence of auxin from the corresponding leaves, but the buds in this position are more likely to be exposed to the influence of auxin from the SAM to a much greater degree, compared to the buds in the axils of the oldest leaves. This impact of auxin from the SAM could be responsible for the lack of significant branching observed with this defoliation treatment compared to the control (Table 4.1). On the other hand, in the treatment where the two oldest leaves were removed (Fig. 4.1c), while the removal of the leaves could have removed the inhibitory effect of auxin from the corresponding leaves, the increased physical distance of the buds from the SAM could have mitigated the inhibitory influence of auxin from the SAM. This cumulatively could have resulted in the increased branching produced by this treatment compared to the control (Table 4.1). The dynamics of auxin distribution in the tubers of *Zantedeschia* has not been explored before. However, a difference in the flowering potential of buds along the axis of the tuber has also been observed in *Zantedeschia* (Naor et al., 2005a), which deems this topic of different growth potentials of the buds at different positions on the tuber, worthy of further investigation.

Prior to the establishment and vindication of the hormonal control of apical dominance (Thimann and Skoog, 1933; 1934), the diversion of nutrients from the apical region to the axillary buds, or the lack of it, was thought to be the mechanism of control of correlative inhibition (McCallum, 1905). Though this theory became less eminent with the demonstration and validation of the hormonal theory, the nutrient diversion theory cannot be entirely neglected because nutrients can control hormonal gradients (Phillips, 1975). In the current experiment, the defoliation treatments where all the existing leaves were removed at the commencement of the experiment (Fig. 4.1e), and also where the leaves were continuously removed as and when they emerged (Fig. 4.1d), failed to produce a significantly higher degree of branching than the control (Table 4.1). This could probably be due to the lack of sufficient nutrients to facilitate axillary bud outgrowth, owing to the removal of the leaves (source). Such a phenomenon has also been noted in pea, where axillary bud outgrowth was slow in the absence of leaves (Thimann and Skoog, 1934). The leaves also act as a source/sink for growth promoting hormones (Thomas, 1983) such as gibberellin (GA) (Phillips, 1975) and cytokinins (CK) (Bangerth, 1994). In the current

experiment, the removal of all the existing leaves, or the continuous removal of leaves, could both make the corresponding axillary buds devoid of these growth promoting hormones. If so, this could probably lead to the absence of bud outgrowth. Therefore, the presence of a few leaves in the treatments where the two young/old leaves were removed, could have supplied the required nutrients and/or growth promoting substances, ultimately resulting in a branching response (Table 4.1). To further investigate the role of different leaves, differentiated by position and/or age on the growth of the corresponding axillary buds, the tracking of radiolabeled auxin applied to the leaves can be advocated.

4.4.2 Tuber weight

The greater tuber weight obtained with the defoliation treatment where only the two oldest leaves were removed, compared to all other treatments except the control, concomitant with the highest degree of branching stimulated by this treatment (Fig. 4.2), supported the hypothesis framed at the beginning of the experiment (refer Section 4.1), that the degree of branching in *Zantedeschia* does indeed positively influence the size of the tuber (Table 4.1). The floral productivity of *Zantedeschia*, amongst other factors, is also dependent on the size of the tuber (Corr and Widmer, 1991; Naor et al., 2005a). Tubers of *Zantedeschia* weighing less than 20 g had a lower flowering percentage (4%) compared to 80-100% flowering observed with tubers greater than 40 g weight (Naor et al., 2005a). The increase in the size of the tuber achieved via increasing the degree of branching in the current experiment, may ultimately equate to an increased floral productivity of *Zantedeschia*. To test this hypothesis would, however, require the replanting of the resultant tubers with the application of GA, which was beyond the scope of the current experiment.

Tuber weight is directly proportional to the leaf number, area and duration (Humphries, 1958; Funnell et al., 2002). Even in major food crops such as wheat, grain yields were proportional to their leaf area duration during grain development (Welbank et al., 1966). In the current study, the increase in tuber weight is most likely to have been achieved by the increased leaf area stimulated by branching, and the consequential increase in the net assimilation rate of this leaf area (Funnell et al., 2002). While in the current experiment leaf area was not recorded, the stimulation of branching by this treatment was visibly noted to increase the total leaf area of the plant. Alternatively, since the photosynthetic capacity of

the leaves declines with age (Marty et al., 2010), the replacement of the old/mature leaves (by removal) with a new flush of leaves from the axillary shoots, could also have been the cause of the enhanced growth of the tubers. Likewise, the removal of the two youngest leaves combined with the lesser degree of branching stimulated by this defoliation treatment, could have resulted in the lower tuber weight observed with this treatment, compared to the treatment where the two oldest leaves were removed (Table 4.1).

The defoliation treatment where the leaves were continuously removed, as and when they emerged, failed to produce any visible tuber at the end of the growth cycle (Table 4.1). While the plastochron of the control plants was 10 days until the cessation of new leaf production, the plants with this treatment exhibited a plastochron extended by at least 2 days compared to that evident within the control. Since tuber growth in *Zantedeschia* is a direct function of the leaf area and LAD (Funnell et al., 2002), the prevention of complete formation of leaves imposed by this treatment could either have resulted in the lack of tuber formation, or the abortion of tubers during the growth cycle. The same logic can be used to explain the inability of the treatment where all the existing leaves were removed at the commencement of the experiment, to induce a higher tuber weight than the control (Table 4.1). The removal of all the existing leaves clearly abolished the photosynthetic source, thereby affecting the overall source-sink relationship.

The increase in tuber weight observed with the removal of the two oldest leaves in the current experiment, was obtained by an increase of only 0.4 ± 0.2 axillary shoots (or less than one axillary shoot) induced by this treatment compared to the control (Table 4.1). Provided alternative strategies can achieve further increases in the degree of branching, greater scope exists to further increase the size (weight) of the tuber in *Zantedeschia*. As illustrated in the current experiment, clearly the leaves play a significant role in both branching and tuber weight in *Zantedeschia*. Replanting the tubers obtained with different defoliation treatments would elucidate the connection between branching, tuber weight and floral productivity. Given the limited extent to which branching was increased by the defoliation treatments, this was not conducted during the current study, in lieu of investigating such alternative strategies. While further exploration of the effects of different combinations of defoliation treatments on the degree of branching may have elucidated this

relationship better, other strategies were attempted simultaneously to tap the branching potential of *Zantedeschia*. Description of these strategies and the experiments conducted towards this objective were the focus of Chapters 5 and 6.

4.4.3 The stage of growth during experimentation

As described in Section 2.3.1.4, different stages exist in the process of bud outgrowth (Shimizu-Sato and Mori, 2001), which determine the efficacy of the branching inducing treatments. Integration of the results of the pilot study with that of the current experiment, illustrated that the stage of growth during which the defoliation treatments were applied was crucial for the visible manifestation of a branching response. While the removal of the two oldest leaves produced the highest number of axillary shoots amongst all defoliation treatments tested, both in the current experiment conducted during the active growth period (1300 ± 100 degree-days; 4-5 leaves) and in the pilot study conducted after the cessation of new leaf production (2000 ± 100 degree-days; 6-7 leaves; data not presented), the difference was significant ($P < 0.06$) only when the experiment was conducted during the active growth period. A similar observation was made in seedlings of common ash, where defoliation treatments applied prior to the cessation of stem elongation were effective in stimulating branching, whereas the same treatments applied beyond 80 days after the cessation of stem elongation proved to be ineffective in the stimulation of branching (Collin et al., 2000). Prior to the cessation of stem elongation, the buds were considered to be under the influence of paradormancy, so that the removal of the inhibitory influence (defoliation) resulted in branching, whereas in the latter case, the buds were considered to be endodormant. The same logic can be used to explain the significant increase in branching following the application of defoliation treatments during the active growth period as described in the current chapter (Table 4.1) and the lack of the same during the pilot study. Therefore, it can be concluded that the stage of growth of the seedling is crucial in determining the branching response. The stimulation of branching achieved by the application of defoliation treatments during the active growth period of the growth cycle, underlined the significance of identifying the active growth phase when applying branch-inducing treatments in future experiments (refer Chapters 6 and 7).

4.5 Conclusions

The defoliation treatment where the two oldest leaves were removed produced a significant increase in the number of shoots/branching, which resulted in a concomitant increase in tuber weight. However, a significant increase in branching was achieved only when the experiment was conducted during the active growth period of the growth cycle, thereby highlighting the importance of the stage of growth during which the branching inducing treatments should be applied. The increase in tuber weight, achieved by stimulating less than one additional axillary shoot per plant by the defoliation treatment where the two oldest leaves were removed, provided additional inspiration to pursue this physiological approach to achieve the commercial objective of increasing floral productivity in *Zantedeschia*, compared to the alternative approach described in Chapter 3.

5. Methods to stimulate branching in *Zantedeschia* – Auxin Transport Inhibitors

5.1 Introduction

In pursuing the evaluation of strategies to stimulate branching in *Zantedeschia* based on the second approach (refer Section 1.6), the use of auxin transport inhibitors (ATI) was postulated for examination within the current experiment. While an increase in branching and an associated increase in tuber size/weight was achieved by the removal of the two oldest leaves (Table 4.1), it was hypothesized that a greater potential to stimulate branching existed in *Zantedeschia* (refer Section 4.5). Consequently, alternative strategies to stimulate branching in *Zantedeschia* were contemplated.

Within the strategies employed to stimulate branching by the inhibition of auxin (refer Section 2.4), the drawbacks associated with the adoption of decapitation, such as stress induced by wounding, elimination of the region of synthesis of growth promoting hormones such as gibberellin (GA), and the removal of a potential flowering site in *Zantedeschia*, have already been elaborated (refer Section 2.4.1). ATI perform the physiologically intended objective of decapitation i.e., inhibition of polar auxin transport (PAT), however, with the advantage of retaining an intact apex. Several classes of synthetic (Katekar and Geissler, 1977; 1980) and natural (Nakajima et al., 2001) ATI are known. The synthetic types are predominantly divided into 3 groups (Katekar and Geissler, 1980) as:

- Phytotropins – e.g. 1–N–Naphthalphthalamic acid (NPA),
- Morphactins – e.g. 9–hydroxyfluorene–9–carboxylic acid (HFCA) and derivatives,
- Compounds not in either class – e.g. 2, 3, 5–triiodobenzoic acid (TIBA).

While the exact mechanism of action of these ATI remains unclear, recent studies have confirmed that the ATI inhibit auxin efflux from the cells during the cell-to-cell transport of auxin in the PAT system (Friml and Palme, 2002). Though the different types of ATI are functionally similar, considerable differences have been observed in their ability to induce undesirable phenotypic responses such as abscission, phytotoxicity and epi/hyponasty.

TIBA caused phytotoxic injuries when applied on the stem, and when applied on the leaves, the presence of TIBA shortened internodes and induced adventitious root formation (Snyder, 1949). Morphactins, on the other hand, caused structural changes to the root cap, which resulted in limited elongation of root cells, not observed with the application of phytotropins (Katekar and Geissler, 1980). Though some members of the phytotropins have been reported to cause phytotoxicity at higher concentrations, NPA is considered to be less phytotoxic (Katekar and Geissler, 1980), and has, therefore, been widely used in ATI studies related to auxin transport (Guerrero et al., 1999; Keller et al., 2004) and apical dominance/branching (Dun et al., 2006). Hence, in the current research, NPA was chosen as the ATI for application on *Zantedeschia*.

Stem application of ATI has been shown to stimulate axillary bud outgrowth by alleviating apical dominance (Snyder, 1949; Mitchell et al., 1965; Panigrahi and Audus, 1966). The endogenous auxin content in the stem was reduced below the point of application of either NPA or TIBA (Morris et al., 2005). This reduction in auxin content may stimulate axillary bud outgrowth, either via the stimulation of a second messenger, such as cytokinin (CK) (Bangerth, 1994; Nordström et al., 2004), or by facilitating the export of auxin from the axillary buds to the mainstream PAT system (Leyser, 2009). Albeit, the ability of an ATI to stimulate branching depends on the stage of growth of the plant (Morris et al., 2005), the type of ATI used (Nakajima et al., 2001) and, its mode of application (Mitchell et al., 1965).

Unlike other plant species, where the application of ATI to the stem was implemented below the apical bud (Snyder, 1949; Mitchell et al., 1965), in *Zantedeschia* the minimal internode distance between buds and their underground location on the tuber (Fig. 1.3a), deemed initial attempts to directly apply ATI below the apical bud impossible, when grown in a conventional system as potted plants. Attempts to inject a test solution into the tuber, below the apical bud, also proved futile. A subsequent pilot experiment examined the effectiveness of applying NPA (0, 0.03 and 0.3 mM) either:

- as drops in the axils of leaves,
- as a foliar spray, or
- in lanolin as a band around the petioles (1-2 mm width) of all existing leaves (Fig. 5.1).

In the pilot experiment, the treatments comprised a 3 × 3 factorial of application modes × concentration ($n = 3$). The logic behind the application of NPA as drops was that it would transport NPA directly to the tuber region as a trickle via the petiole. The foliar spray was aimed at the transport of NPA to the tuber directly via the petiole as run-off, and/or transport to the tuber via the phloem following absorption (Guerrero et al., 1999), and/or inhibition of auxin export from the corresponding leaves. The application as a band around the petioles was also aimed at inhibiting the auxin flow from the respective leaves, and/or transport of NPA to the tuber via the phloem (Guerrero et al., 1999).

Irrespective of the concentration of NPA or the mode of its application, a significant increase in branching was not observed in the pilot experiment (Data not presented). The failure to stimulate branching was attributed to one or all of the following reasons:

- Owing to the tight packing of petioles at their bases, there was no guarantee that the application of NPA as drops in the axils of leaves would have reached the physiologically intended destination i.e., the tuber region.
- The application of naphthylphthalamic compounds (NPA included) as a band around the petioles was not effective in stimulating branching in *Phaseolus vulgaris* L. (bean), compared to application directly to the stem. This was assumed to be due to the lack of transport of the ATI from the leaves to the stem (Mitchell et al., 1965).
- Though the foliar application of NPA and its application as a band around the petioles of existing leaves could have inhibited the auxin flow from the corresponding leaves to the subtending axillary buds, the axillary buds owing to their close proximity to the apical bud would still be exposed to the influence of auxin from the apical bud. This, in turn, may have been sufficient to maintain apical dominance.

- The concentrations of NPA used in the pilot experiment could have been sub-optimal compared to the concentration required to stimulate branching.
- The plants used in the pilot experiment had accumulated 2000 ± 100 degree-days at the time of treatment application. It was subsequently ascertained that this level of accumulation of heat units indicated the onset of endodormancy in *Zantedeschia* (Carrillo Cornejo et al., 2003), which may, therefore, have rendered the treatments ineffective.

So as to examine the effectiveness of NPA on *Zantedeschia* during the active growth period, and at a wider concentration range, the objectives of the current experiment were:

1. To evaluate the application of NPA during the active growth period, as a means of stimulating branching in *Zantedeschia* and,

2. To quantify any effect of these treatments on the size of the resulting tuber.

5.2 Materials and methods

5.2.1 Plant material and culture

The experimental material comprised single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' during their active growth phase of the first growth cycle, as described in Section 4.2.1. The heat-units, number of leaves, days after planting, cultural and growth conditions were similar to that described in Section 4.2.1.

5.2.2 NPA treatments and application

In other plant species, NPA has been applied at a concentration range of 0.01 to 0.1 mM (Fujita and Syōno, 1997; Guerrero et al., 1999). However, there are no studies that describe the application of NPA in aroids. Therefore, a higher concentration of NPA i.e., 0 to 0.3 mM was attempted in the pilot studies. Since one of the reasons for the failure to elicit a branching response in the pilot studies was attributed to a sub-optimal concentration of NPA (refer Section 5.1), a higher concentration of NPA was included in the current experiment. Therefore, in the current experiment, NPA was applied via each of 3 application modes (see below) at concentrations of either:

- 0 mM
- 0.03 mM
- 0.3 mM
- 3 mM

In the pilot experiment, foliar application of NPA was hypothesized to transport NPA to the tuber region, either:

- directly as run-off or,
- indirectly through absorption and subsequent transport to the tuber via the phloem (Guerrero et al., 1999).

In light of the above, the application of NPA as drops in the axils of leaves was considered redundant, and this mode of application was eliminated from the current experiment. In bean plants, the application of naphthylphthalamic compounds (NPA included) as a media drench was also reported to stimulate significant branching (Mitchell et al., 1965). As an alternative to the application of NPA as drops therefore, in the current experiment, this mode of application was included. Consequently, NPA was applied at each of the four concentrations through one of the following modes of application:

- Foliar spray (5 ml per plant) until run-off on both sides of the leaf lamina,
- As a growing medium drench (5 ml per plant) surrounding the primary shoot,
- NPA dissolved in lanolin and applied as a band (1-2 mm) around the existing petioles (Fig. 5.1).

NPA (Molecular weight = 291.3 g/mole) was a gift from Dr. Ben Van Hooijdonk, Massey University, Palmerston North, New Zealand, and was prepared as described by Hooijdonk (2009). Where NPA was applied as a foliar spray or media drench, the required weight of NPA was dissolved in water to attain the desired concentration, with Tween 20 (0.2%) added as a surfactant. When NPA was applied as a band around the petioles, the required weight of NPA was first dissolved in an adequate volume of hydrated lanolin (w/v).

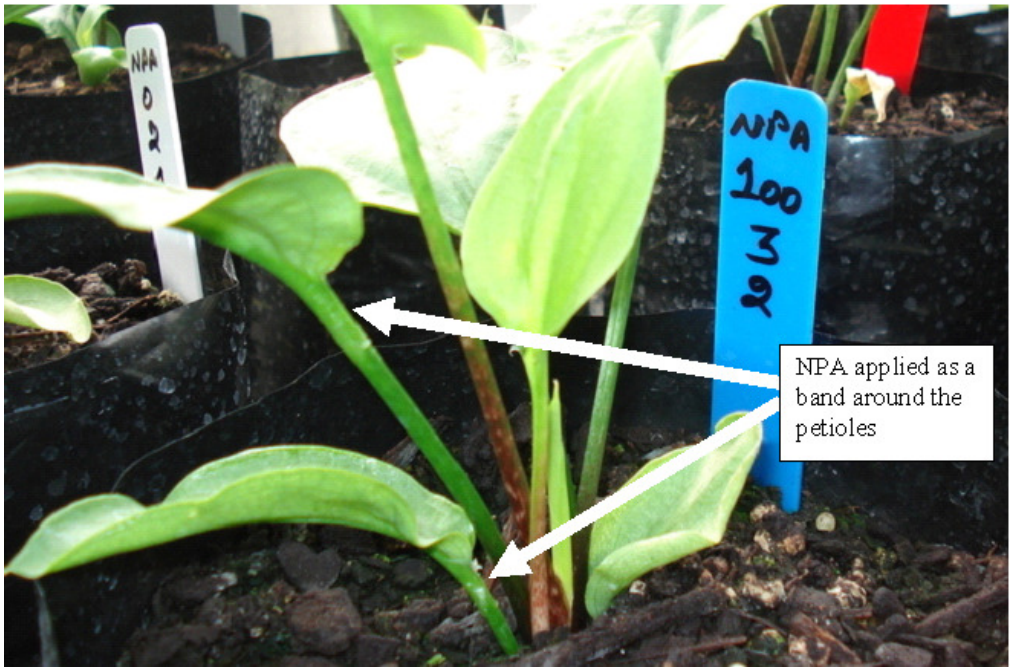


Fig. 5.1 Application of NPA as a lanolin paste around the distal end of petioles of existing leaves in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold'. Reflective surface of applied lanolin, visible on petiole.

5.2.3 Observations

As described in Section 4.2.3, the number of visible axillary shoots was recorded at fortnightly intervals, until the cessation of new leaf production. Similarly, at the end of the growth cycle, the fresh weight of lifted tubers was recorded.

5.2.4 Experimental design and statistical analysis

The concentrations of NPA and its different modes of application were arranged as a 4 × 3 factorial, with the experiment conducted as a completely randomized design. Seven single-plant replicates were allocated for each treatment combination (7 replicates × 3 modes of application × 4 concentrations of NPA = 84 planter bags). As detailed in Section 4.2.4, blocking was not adopted within the glasshouse and the planter bags were, therefore, placed at random locations.

Analysis of variance, means ± standard errors, contrasts and mean separation tests by Least Significant Difference (LSD) were carried out using PROC GLM of SAS version 9.2 (SAS Institute., USA) and MS Excel (Microsoft Corp., USA).

5.3 Results

5.3.1 Overview

At the time of treatment application, the plants were in their active growth period evident by the continuous new leaf production with a plastochron of approximately 10 days. Subsequently new leaf production ceased after the production of 7 ± 1 leaves, followed by the onset of senescence and withering of existing cataphylls and/or leaves, starting from the periphery. The advantages inherent to employing the single-shoot system for branching studies of *Zantedeschia* have been explained previously (refer Section 4.3.1). Irrespective of the concentration of NPA or its modes of application, visible phytotoxic symptoms were not observed through to the end of the growth cycle.

The presence of NPA (0.03, 0.3 or 3 mM), irrespective of the application as a foliar spray or as a band around the petiole of existing leaves, caused epinasty of the leaves (Fig. 5.2). This tendency of NPA to cause epinasty of leaves was observed only during the current experiment conducted during the active growth period of the growth cycle, and was not observed during the earlier pilot studies (refer Section 5.1). Epinastic curvature of petioles was not observed following the application of NPA as a media drench in the current experiment.

Fig. 5.2 Symptoms of epinastic curvature of leaves of *Zantedeschia* cv. 'Best Gold', following the application of NPA either as a foliar spray or as a band around the petioles

5.3.2 Branching

Two-way analysis of variance showed a significant interaction ($P < 0.06$) between the concentration of NPA and its mode of application. However, the corresponding main factors had no significant effect on branching ($P > 0.1$). The significant interaction effect was caused by a significant increase in branching ($P < 0.02$) observed with the application of NPA at 3 mM as a media drench (0.9 ± 0.3 axillary shoots), compared to its application as a foliar spray (0.1 ± 0.1 axillary shoots) or as a band around the petioles (0 axillary shoots). Foliar application of 0.03 mM NPA was as effective as a media drench of 3 mM NPA in stimulating branching (0.7 ± 0.3 axillary shoots) compared to the other modes of application (same row; Table 5.1) and the other concentrations of NPA (same column; Table 5.1).

Table 5.1 Effect of different concentrations and modes of application of NPA on the number of axillary shoots/branching ± SE in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' during their active growth period.

Concentration of NPA (mM)	Mode of application		
	Foliar spray	Media drench	Band around petioles
0	0.3 ± 0.2 a (A)	0.1 ± 0.1 a (A)	0.6 ± 0.3 a (A)
0.03	0.7 ± 0.3 a (A)	0.1 ± 0.1 a (A)	0.1 ± 0.1 a (A)
0.3	0.6 ± 0.2 a (A)	0.4 ± 0.3 a (A)	0.6 ± 0.4 a (A)
3	0.1 ± 0.1 a (B)	0.9 ± 0.3 a (A)	0.0 a (B)

Within the same column different *small letters* indicate significant differences (α = 0.05) between treatment concentrations (*n = 7*).

Within the same row different *capital letters* indicate significant differences (α = 0.05) between treatment modes (*n = 7*).

Mean separation by Least Significant Difference (LSD).

5.3.3 Tuber weight

The treatment combinations that caused an increase in branching compared to the other combinations of concentrations of NPA and modes of application i.e., media drench of 3 mM NPA and foliar application of 0.03 mM NPA (Table 5.1) also revealed a significant increase in tuber weight ($P < 0.003$; $P < 0.02$ respectively) compared to other combinations of concentration and modes of application of NPA (within the same column and row, respectively; Table 5.2). Similar to the branching response (refer Section 5.3.2), a significant interaction effect ($P < 0.0002$) of the concentration of NPA and its mode of application on the tuber weight was observed. Again, similar to the branching response, neither main factor i.e., the concentrations of NPA or their modes of application had a significant effect on the tuber weight ($P > 0.1$). NPA applied as a media drench at 3 mM increased the tuber weight by 103% and 47%, respectively, compared to foliar application and as a band around the petioles (Table 5.2). Likewise, NPA applied as a foliar spray at

0.03 mM increased the tuber weight by 54% and 34%, respectively, compared to the other modes of application (Table 5.2).

Table 5.2 Effect of different concentrations and modes of application of NPA on the fresh weight of the tuber ± SE, obtained at the end of the growth cycle of single-shoot seedlings of *Zantedeschia* cv. 'Best Gold'.

Concentration of NPA (mM)	Mode of application		
	Foliar spray	Media drench	Band around petioles
0	35.0 ± 2.5 b (A)	33.6 ± 3.0 b (A)	45.2 ± 8.4 a (A)
0.03	52.3 ± 4.1 a (A)	33.9 ± 3.9 b (B)	39.0 ± 4.6 a (B)
0.3	32.3 ± 3.2 bc (B)	37.7 ± 5.1 ab (AB)	44.9 ± 3.5 a (A)
3	25.0 ± 2.8 c (B)	50.9 ± 6.9 a (A)	34.6 ± 2.6 a (B)

Within the same column different *small letters* indicate significant differences (α = 0.05) between treatment concentrations (*n = 7*).

Within the same row different *capital letters* indicate significant differences (α = 0.05) between treatment modes (*n = 7*).

Mean separation by Least Significant Difference (LSD).

5.4 Discussion

5.4.1 Branching

In other plant systems such as legumes (Mitchell et al., 1965) and *Malus domestica* Borkh. (Apple) (Hooijdonk, 2009), the application of NPA on the stem region below the apical bud was successful in stimulating branching by inhibiting PAT (Friml and Palme, 2002). In *Zantedeschia* however, based on the results from pilot studies (refer Section 5.1), the logistical problems associated with the application of NPA in the tuber region below the apical bud after planting were discussed. In a bid to transport NPA to the tuber region therefore, alternative modes of application of NPA were attempted in the current experiment (refer Section 5.2.2). As observed in bean plants (Mitchell et al., 1965), the media drench of NPA was successful in eliciting a branching response (Table 5.1).

However, even with the highest concentration of NPA (3 mM), approximately only 1 axillary shoot was stimulated (Table 5.1). Therefore, though there is further scope to increase the degree of branching with higher concentrations of NPA applied as a media drench, as observed with the foliar application of NPA, increasing concentrations of NPA may result in a decline in branching (Table 5.1).

Though not significant, the foliar application of NPA at 0.03 mM concentration stimulated a higher degree of branching compared to the other modes of application of NPA at this concentration (same row; Table 5.1), and foliar spray at higher concentrations (same column; Table 5.1). The requirement of a higher concentration of NPA (3 mM) to stimulate branching when applied as a media drench, compared to the stimulation of branching by 0.03 mM of NPA when applied as a foliar spray, can be attributed to the possible loss of NPA in the growth medium, which would in turn affect the availability of NPA to the tuber region. Albeit, only ≤ 1 axillary shoot was stimulated by this treatment combination, with a decline in branching observed with higher concentrations of NPA (0.3 mM, 3 mM) when applied as a foliar spray (Table 5.1), thereby limiting the scope to stimulate branching further by foliar application of NPA.

A clear trend with the branching response, following the application of NPA as a band around the petioles was however not evident (Table 5.1). As suggested for the failure of the NPA treatment to stimulate branching during the pilot studies (refer Section 5.1), in the current experiment also, though the application of NPA as a band around the petioles may have hypothetically inhibited auxin flow from the corresponding leaves to the subtending axillary buds, these buds would have still been exposed to the auxin flow from the apical bud. This possibly may have resulted in the aberrant branching response observed with this mode of application (Table 5.1). Alternatively, the application of NPA as a foliar spray and media drench may have been more effectively transported to the tuber region compared to the application as a band around the petioles, which may in turn have resulted in auxin inhibition in the tuber.

Nevertheless, the results from the current experiment provided a better understanding of the effective concentration range of NPA and its preferable mode of application to conduct further studies on the stimulation of branching in *Zantedeschia*. In other studies, TIBA has

been found to be more effective in alleviating apical dominance than NPA (Nakajima et al., 2001). In future research therefore, using the results from the current experiment as a template, it would be worthwhile evaluating the efficacy of other ATI, such as TIBA or HFCA, on the branching of *Zantedeschia*.

5.4.2 Tuber weight

The importance of maintaining active leaf area and leaf area duration (LAD) on the resultant tuber weight was established in Chapter 4, where the defoliation treatment comprising the continuous removal of leaves as they emerged, failed to generate a visible tuber at the end of the growth cycle (Table 4.1). The direct relationship between an increase in leaf area and LAD, possibly caused by the stimulation of branching by the removal of the two oldest leaves, and the resultant tuber weight (Funnell et al., 2002) was also established earlier (Table 4.1). Further to these findings, in the current chapter, this relationship between an increase in branching and the resultant tuber weight was further corroborated (Table 5.2).

The treatment combination comprising the media drench of 3 mM NPA, which significantly stimulated branching (Table 5.1), also generated tubers with a significantly greater weight than the application of 3 mM NPA through other modes of application (same row; Table 5.2). Likewise, the treatment combination of a foliar spray of 0.03 mM NPA which also stimulated higher branching compared to other modes of application and concentrations of NPA (Table 5.1), generated tubers with a significantly greater weight compared to the foliar spray of increasing concentrations of NPA (same column; Table 5.2) and other modes of application of 0.03 mM NPA (same row; Table 5.2).

While the stimulation of 0.4 ± 0.2 axillary shoots following the removal of the two oldest leaves resulted in a tuber weight of 37.9 ± 2.6 g (Table 4.1), the additional 0.5 axillary shoots (0.9 ± 0.3) stimulated by the media drench of 3 mM NPA resulted in an increase in tuber weight by 35% (50.9 ± 6.9 g; Table 5.2). Put together, the results from the experiments conducted to stimulate branching via defoliation (Chapter 4) and application of ATI (Chapter 5), clearly elucidate the relationship between the stimulation of branching and the resultant tuber weight. As mentioned in Section 4.4.3, the relationship between the

increase in tuber weight and a concomitant increase in floral productivity would involve replanting the tubers and measuring floral productivity in the next (2^{nd}) growth cycle (Fig. 1.1), which was however beyond the scope of this study. Moreover, within the context of stimulation of branching by auxin inhibition (refer Section 2.4), defoliation and application of ATI were capable of stimulating only ≤ 1 axillary shoot (Table 4.1; 5.2). Therefore, the potential to stimulate branching, and thereby increase the tuber size/weight further exists in *Zantedeschia*. Consequently, the application of cytokinins (CK) and/or gibberellin (GA) was attempted at different stages of the growth cycle, the results of which have been dealt within Chapters 6 and 7.

5.4.3 Stage of growth of the seedlings

Evidence that NPA had been absorbed by the plant following its application as a foliar spray or as a band around the petioles was shown by the epinastic curvature of petioles (Fig. 5.2). Epinastic curvature of petioles has also been observed in bean plants following the application of naphthylphthalamic compounds (NPA inclusive) (Mitchell et al., 1965). It is a characteristic feature of auxin action, probably caused by the differential rates of cell elongation along the abaxial and adaxial sides of the organs (Phillips, 1975). However, this epinastic curvature was evident only during the current experiment i.e., active growth period of the growth cycle, thereby highlighting the significance of the appropriate stage of growth of seedlings to apply branch-inducing treatments.

Apart from the concentration and modes of application of NPA, the stage of growth of the seedlings is also likely to play a crucial role in determining the degree of branching response (Mitchell et al., 1965). In *Zantedeschia*, the significance of the stage of growth of the seedlings was already established through the defoliation experiment (Chapter 4). In these defoliation experiments, the efficacy of removal of the two oldest leaves in stimulating branching was evident only when the treatment was applied during the active growth period, and not after the cessation of new leaf production during which time the buds were considered to be endodormant. Also, within the current experiment involving the application of ATI, as described earlier (refer Section 5.1), a branching response whatsoever was not observed when the ATI treatments were applied after the cessation of leaf production (Data not presented). An epinastic curvature of the petiole which indicated

the absorption of NPA (Fig. 5.2), and the significant stimulation of branching, even by ≤ 1 axillary shoot occurred only following the application of ATI during the active growth period. Surely, a diagnostic tool that indicates the establishment of endodormancy in *Zantedeschia* is required to facilitate the application of branch-inducing treatments at the right stage of the growth cycle. Consequently, demarcation of the growth cycle of *Zantedeschia* based on visual landmarks, which hypothetically track the transition of buds from apical dominance to endodormancy, was proposed (Chapter 6).

5.5 Conclusions

The treatment combination of a media drench of 3 mM NPA was successful in stimulating significant branching. This increase in branching equated to a concomitant increase in tuber weight at the end of the growth cycle. While a higher concentration of NPA was required to stimulate branching when applied as a media drench, 0.03 mM NPA applied as a foliar spray stimulated higher branching compared to other modes of application. The increase in branching even by ≤ 1 axillary shoot was evident only when the treatments were applied during the active growth period, which highlights the significance of the time of application of the branch-inducing treatments. Potential to stimulate branching further exists in *Zantedeschia*.

6. Methods to stimulate branching in *Zantedeschia* – Cytokinin and/or gibberellin

6.1 Introduction

Based on the second approach to increase floral productivity in *Zantedeschia* (refer Section 1.6), the increase in branching and its consequential relevance to enhanced tuber weight were investigated in Chapters 4 and 5. While these two chapters employed the inhibition of auxin as a strategy to stimulate branching in *Zantedeschia* (refer Section 2.4), within the current chapter the strategy was based on the response to the application of cytokinin (CK) and/or gibberellin (GA) to stimulate branching. The current chapter therefore presents a series of pilot studies utilising this alternative strategy.

6.1.1 Three phases in the growth cycle of *Zantedeschia*

The series of experiments conducted within Chapters 4 and 5, where defoliation and the application of auxin transport inhibitors (ATI) were effective in stimulating branching only when applied during the active growth period of the growth cycle, led to the hypothesis that the stage of growth at the time of application of branch-inducing treatments plays a pivotal role in determining the branching response of *Zantedeschia* (refer Section 4.4.3). Towards addressing this hypothesis, before proceeding with the application of CK and/or GA to stimulate branching, the growth cycle of *Zantedeschia* was demarcated into three phases along the timeline of ontogenic development (Fig. 6.1). The rationale behind the demarcation of the growth cycle and its vindication based on the branching response stimulated by CK and/or GA formed an important component of this thesis (Chapter 7) and shall be detailed in the following sections.

As described in Section 1.3, the seasonal ontogeny of *Zantedeschia* (Fig. 1.1) is visually marked by a period of active growth where there is continuous production of new leaves, followed by the cessation of new leaf production and the formation of an atrophied leaf primordium in the axil of the last emerged leaf (Fig. 1.2). While these visual clues have previously been examined within the context of developmental morphology of the plant

(Halligan et al., 2004) and predicting the onset of endodormancy (Carrillo Cornejo et al., 2003; Halligan et al., 2004), the cessation of leaf production did not coincide with the onset of endodormancy (Halligan et al., 2004). In the current study however, these visual clues were regarded as signposts that represent progressive developmental changes in the shoot apical meristem (SAM). The SAM is a highly organized and stable structure which also maintains a large degree of flexibility to adjust its activity in response to internal and external signals (Traas and Vernoux, 2002). Therefore, the various developmental stages comprising vegetative and floral transition that the plants undergo during their ontogeny can be viewed as different episodes in the life span of the SAM (Poethig, 1990). Therefore, based on this notion that the visual markers in the ontogeny of *Zantedeschia* in fact represent underlying developmental changes in the SAM, the growth cycle of *Zantedeschia* was demarcated into three phases as (Fig. 6.1):

- Phase 1 – From the time of sowing/planting to the cessation of new leaf production
- Phase 2 – From the cessation of new leaf production to the formation of the atrophied leaf and,
- Phase 3 – From the formation of an atrophied leaf through to the end of the growth cycle

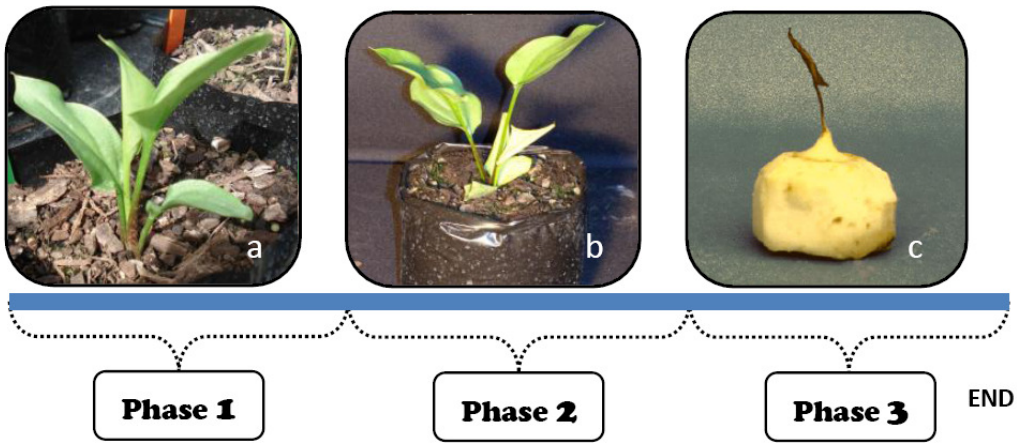


Fig. 6.1 Demarcation of the growth cycle of *Zantedeschia* into three phases based on the visual clues that represent underlying changes in the SAM as **a**) Phase 1 – from the time of seed sowing until the cessation of new leaf production, **b**) Phase 2 – from the cessation of new leaf production until the formation of an atrophied leaf and, **c**) Phase 3 - from the formation of an atrophied leaf until the end of the growth cycle.

Dormancy in plants is also a consequence of developmental changes orchestrated within the SAM (Horvath et al., 2003), which could be caused by external and/or internal factors (Lang, 1987). Though the existence of apical dominance (Clark et al., 1987; Ngamau, 2001a; Naor et al., 2005b) and endodormancy is acknowledged in *Zantedeschia* (Naor and Kigel, 2002; Carrillo Cornejo et al., 2003), the precise time of transition of buds from apical dominance to endodormancy, and the subsequent establishment of endodormancy, is unclear (Halligan et al., 2004). This ambiguity is further compounded by the potential overlapping of different types of dormancy (Lang, 1987) during the ontogeny of plants (Faust et al., 1997). The identification of the time of onset of endodormancy is crucial to the second approach of enhancing floral productivity of *Zantedeschia* via stimulating branching (refer Section 1.6), because in the pilot studies (refer Sections 4.1; 5.1) the application of branch-inducing treatments after the onset of endodormancy failed to induce a branching response.

Previous attempts to predict the onset of endodormancy based on the visual clues, have suggested that the onset of endodormancy could occur even prior to the cessation of new leaf production, i.e., before Phase 2 (Fig. 6.1; Halligan et al., 2004). The role of the

atrophied leaf in relevance to endodormancy has also been difficult to assess (refer Section 1.3). In the current study however, the visual landmarks were suggested to represent developmental changes in the SAM. Since dormancy is also a feature of developmental changes in the SAM, the demarcation of the growth cycle of *Zantedeschia* into the three phases based on these visual clues (Fig. 6.1) was believed to hypothetically track the transition of buds from apical dominance to endodormancy. As reported earlier (Chapter 4), the response of buds to branch-inducing treatments within each phase could then provide a reliable indicator of the dormancy status of the buds. Within this and the subsequent chapters (Chapters 7 and 8), the role of CKs and/or GA as the branch-inducing treatments were investigated.

6.1.2 Application of CK and/or GA

As described in Section 2.4.4, CK act antagonistically to terminally derived auxin (Nordström et al., 2004) and promote axillary bud outgrowth by alleviating apical dominance (Shimizu-Sato et al., 2009). Among other hormones such as abscisic acid (ABA) and ethylene (Chatfield et al., 2000), CK are the most prominent candidate for the second messenger hypothesis (refer Section 2.3.1.1) in the control of apical dominance (Leyser, 2005). Accordingly, CK has been shown to stimulate branching in dicots (Miller, 1961) and monocots (Hussey, 1976), including members of the *Araceae* (Imamura and Higaki, 1988).

The stimulation of branching in *Zantedeschia* with the exogenous application of CK however, depended on the group i.e., summer-flowering or winter-flowering (refer Section 1.1). While the application of BAP (6-benzylaminopurine) was successful in the stimulation of branching of summer-flowering cultivars (Naor et al., 2005b), a significant increase in branching was not observed with the application of BAP in winter-flowering *Z. aethiopica* (Underwood, 1993). Within the summer-flowering group, considerable difference in the inherent degree of branching has been observed between different species (Funnell, 1993) and cultivars (Naor et al., 2005a). This difference in the inherent capacity to branching may also influence the degree of branching response to the application of CKs. Moreover, the age of the plant (Shimizu-Sato et al., 2009), and the stage of growth (refer Section 6.1.1) at the time of application of CK, could also influence the branching

response. *Zantedeschia* cv. 'Best Gold' used throughout this thesis is naturally less prone to branching (D'Arth et al., 2007). The single-shoot seedlings in their first growth cycle (Fig. 1.1a-d) used for branching studies in this thesis have also been suggested to possess stronger apical dominance (refer Chapter 3) compared to the flowering-sized tubers obtained at the end of the second-growth cycle (Fig. 1.1d-h) used in other studies (Naor et al., 2005b). To date, the role of CK in the regulation of branching of single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' have not been investigated and, therefore is promising in the context of evaluating the role of CK at different phases of the growth cycle.

The role of GA reported until date in the regulation of branching in *Zantedeschia* has been consistent. Exogenous application of GA_3 alone failed to stimulate branching from flowering-sized tubers of different cultivars (Funnell et al., 1992; Naor et al., 2005b). This, as described above, could be due to the type of experimental material used and/or inherent branching capabilities of the cultivar. The role of GA alone in the regulation of branching in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' has not been explored before and is, therefore, worthy of further investigation at different phases of the growth cycle.

With regards the control of apical dominance, the interaction between GA and other hormones such as auxin and CK was reviewed earlier in Section 2.3.1.3. While the application of CK alone was sufficient to stimulate branching during the early stages of growth in seedlings of *Glycine max* L. (Soybean), a sequential application of GA was required to stimulate branching at later stages (Ali and Fletcher, 1970). Also, a sequential application of GA was found to be more effective in stimulating branching compared to the simultaneous application (Ali and Fletcher, 1970) which, in other plants, has also resulted in complete arrest of bud outgrowth (Woolley and Wareing, 1972). Simultaneous application of the CK 6-benzylaminopurine (BAP) and GA as Promalin^{TM} (Valent BioSciences, USA) did not promote significant axillary bud outgrowth of *Zantedeschia* compared to the application of BAP alone (Funnell and MacKay, 1988a). Therefore, the stage of growth of the plants at the time of application of CK and/or GA, and the order of application of CK and GA, could determine the degree of branching response.

While previous studies with *Zantedeschia* have successfully stimulated branching with the application of BAP during the active growth period, i.e., Phase 1 of the growth cycle (Fig.

6.1; Naor et al., 2005b), their role in the regulation of branching along the progressive developmental changes in the SAM, i.e., Phases 1, 2 and 3 (Fig. 6.1), have not been reported previously. Determination of the branching response along the three phases (Fig. 6.1), may aid in the identification of the ideal time of application of branch-inducing treatments to induce maximum response. As reported in soy bean (Ali and Fletcher, 1970), the implications of a sequential application of CK followed by GA, depending on the stage of growth along the three phases of growth, may also provide a better understanding of the manipulation of branching in *Zantedeschia*. Moreover, given that the three phases of the growth cycle also hypothetically track the transition of buds from apical dominance to endodormancy (refer Section 6.1.1), the branching response induced within these phases following the application of CK and/or GA, may indicate the dormancy status of the buds.

Hence, within the context of this thesis, following the development of techniques to demarcate three phases in the growth cycle of *Zantedeschia*, i.e., phases 1, 2 and 3, the current series of pilot studies were conducted to:

1. Determine if the application of CK and/or GA can stimulate branching in single-shoot seedlings of *Zantedeschia* 'Best Gold' during their first growth cycle and, if so,
2. Quantify the degree of branching stimulated by the application of CK and/or GA along the three phases of growth,
3. Quantify the effect of these treatments on the size of the resulting tuber, and thereby,
4. Determine if an increase in the size of the tuber equates to an increase in floral productivity in the next growth cycle.

As explored later in this chapter (Section 6.2), the treatments applied in the current series of pilot studies reflect a progressive evolution in understanding the potential role of CK and GA with progressive changes in phase of growth. Hence not all pilot studies utilised the same set of treatment combinations.

6.2 Materials and Methods

6.2.1 Plant material and culture

Single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' (Fig. 4.1a) were used in the pilot studies conducted in each of the three phases of the growth cycle. For the study conducted in phase 1 i.e., from the time of sowing/planting to the cessation of new leaf production, seedlings comprised 4 ± 1 leaves on a single primary shoot (~ 80 days after seed sowing; ~ 1500 heat-units). As described in Section 4.2.1, under similar growing conditions, the seedlings would be expected to produce 7 ± 1 leaves before the cessation of new leaf production. Therefore, the seedlings selected for phase 1 were considered to be in their active growth period. For the study in phase 2 i.e., from the cessation of new leaf production to the formation of the atrophied leaf, seedlings comprised 7 ± 1 leaves on a single primary shoot (~ 135 days after seed sowing; ~ 2331 heat-units). At this time, the seedlings had passed through the time interval required for at least one plastochron after the cessation of new leaf production without any new leaves, and it was also confirmed that the atrophied leaf was not evident in a sample of dissected buds. For the study in phase 3 i.e., from the formation of an atrophied leaf through to the end of the growth cycle, seedlings were selected with 2 ± 1 leaves remaining on a single primary shoot (~ 250 days after seed sowing; ~ 4000 heat-units). The rest of the expanded leaves on the shoot had undergone natural senescence and had withered. Dissection of a sample of buds, prior to the commencement of the pilot study during phase 3, revealed an atrophied leaf primordium in the axil of the last-emerged leaf.

To measure floral productivity in the second growth cycle, tubers obtained at the end of the first growth cycle following treatment applications in phase 1 (refer Section 6.2.2.1) were lifted and cured for two weeks (Funnell, 1993). To alleviate endodormancy (refer Section 2.3.3), the tubers were subsequently transferred to a cool store maintained at 7 ± 2 °C and 70 ± 10% relative humidity (~ 90 days). On removal from the cool store, tubers were planted and grown in 2.8 l polythene bags in a commercial bark-based medium (Appendix 11.1) with a slow-release fertilizer and automated irrigation, as described in Chapter 3 (Appendix 11.2). The temperature and irrigation conditions in the glasshouse were also as described in Chapter 3.

6.2.2 Treatment applications

Since BAP is capable of acropetal and basipetal transport (Black and Osborne, 1965) and successful stimulation of branching was achieved with the foliar spray of BAP in *Zantedeschia* (Naor et al., 2005b), the foliar application of all treatments was utilized in the current pilot studies. Two formulations of BAP were used as follows, with the objective of selecting the better based on the branching response and the minimal extent of undesirable effects caused:

- BAP (Molecular weight = 225.25 g/mole; OlChemIm Ltd., Czech Republic) dissolved in 15 ml of 1N NaOH, the final volume made up with distilled water and Tween 20 (Sigma CAS No. 9005-64-5) added as a surfactant.
- Cylex™ (Valent BioSciences, USA), a commercial preparation of BAP (20 g l^{-1}) dissolved in tetrahydrofurfuryl alcohol, with a surfactant included.

Where applied, GA_3 (OlChemIm Ltd., Czech Republic) was dissolved in 10 ml of 95% ethanol, and the final volume was made up with distilled water. Tween 20 was added as a surfactant. The control treatment (0 mM) comprised Tween 20 and distilled water only.

6.2.2.1 Treatment application in phase 1

For single-shoot seedlings in phase 1, the following concentrations of each form of BAP i.e., BAP in 1N NaOH or Cylex, was applied as a foliar spray (5 ml per plant) until run-off on both sides of the leaves

- 0 mM (control)
- 0.4 mM (100 mg l^{-1})
- 2.2 mM (500 mg l^{-1})
- 4.4 mM (1000 mg l^{-1})
- 6.6 mM (1500 mg l^{-1})

All treatments were applied twice. The first foliar spray was at ~ 80 days after seed sowing, and the second application 10 days after the first.

Prior to replanting for the second growth cycle, the tubers treated with BAP (0 mM, 0.4 mM, 2.2 mM, 4.4 mM or 6.6 mM) in their first growth cycle were soaked in a solution of GA_3 at 0.3 mM concentration (in accordance with commercial recommendations), as described in Section 3.2.3. The control treatment comprised tubers without the application of either BAP or GA_3 during the first or second growth cycles, respectively. In summary, the treatment combinations for the measurement of floral productivity in the second growth cycle were:

- Control – Intact plants in their second growth cycle without the application of BAP in the first growth or GA_3 in the second growth cycle
- GA_3 alone (0.3 mM) - BAP in 1N NaOH/Cylex™ (0 mM) in the first growth cycle followed by GA_3 (0.3 mM) in the second growth cycle
- BAP in 1N NaOH/Cylex™ (0.4 mM) in the first growth cycle followed by GA_3 (0.3 mM) in the second growth cycle
- BAP in 1N NaOH/Cylex™ (2.2 mM) in the first growth cycle followed by GA_3 (0.3 mM) in the second growth cycle
- BAP in 1N NaOH/Cylex™ (4.4 mM) in the first growth cycle followed by GA_3 (0.3 mM) in the second growth cycle
- BAP in 1N NaOH/Cylex™ (6.6 mM) in the first growth cycle followed by GA_3 (0.3 mM) in the second growth cycle

6.2.2.2 Treatment application in phase 2

For seedlings in phase 2, a sequential application of BAP as Cylex™ at 0 mM, 2.2 mM or 6.6 mM concentrations, followed by GA_3 at 0.9 mM (300 mg l^{-1}), were applied as foliar sprays (5 ml per plant each) on both sides of the leaves until run-off. Two sprays of each concentration of Cylex were undertaken at ~ 135 days after seed sowing, and again 10 days after the first application. A single foliar application of GA_3 was performed a further 10 days after the second application of Cylex. The control treatment comprised the application of 0 mM concentration of Cylex alone at the respective times of application of the different treatments. In summary, the treatment combinations in phase 2 were:

- Control – Cylex^{TM} (0 mM)
- Cylex^{TM} (0 mM) followed by GA_3 (0.9 mM)
- Cylex^{TM} (2.2 mM) followed by GA_3 (0.9 mM)
- Cylex^{TM} (6.6 mM) followed by GA_3 (0.9 mM)

6.2.2.3 Treatment application in phase 3

For seedlings in phase 3, BAP and GA_3 were applied in two sequential orders, where BAP as Cylex was applied first at 0 mM, 2.2 mM or 6.6 mM followed by GA_3 at 0 mM or 1.4 mM (500 mg l^{-1}) (BAP → GA_3) or vice-versa, where GA_3 was applied first at 1.4 mM, followed by BAP at any one of the concentrations stated above (GA_3 → BAP). In either sequential order, the component applied first (BAP or GA_3) was applied thrice, and the component applied second (GA_3 or BAP) applied twice, by spraying on both sides of the leaves until run-off. In contrast to that applied in phases 1 and 2, a higher concentration of GA_3 and the additional number of applications, were accommodated because the buds in phase 3 were hypothesized to be under deeper endodormancy (refer Section 6.1.1). The reverse order of application (GA_3 → BAP) was included in phase 3, so as to determine the significance of the sequential orders of application. The first spray of BAP or GA_3 was performed at ~ 250 days after seed sowing, with subsequent treatment applications at 10 day intervals. In summary, the following treatment combinations were applied in phase 3:

- Control – Cylex^{TM} (0 mM) followed by GA_3 (0 mM)
- Cylex^{TM} (2.2 mM) applied alone
- Cylex^{TM} (6.6 mM) applied alone
- GA_3 (1.4 mM) applied alone
- Cylex^{TM} (2.2 mM) followed by GA_3 (1.4 mM)
- Cylex^{TM} (6.6 mM) followed by GA_3 (1.4 mM)
- GA_3 (1.4 mM) followed by Cylex^{TM} (2.2 mM)
- GA_3 (1.4 mM) followed by Cylex^{TM} (6.6 mM)

6.2.3 Observations

Within each pilot study the number of visible axillary shoots, i.e. branch number, was counted every 10 days, commencing from the date of the last foliar application until the end of the respective three phases i.e., until the cessation of new leaf production for phase 1, until the formation of the atrophied leaf for phase 2, and until the end of the growth cycle for phase 3. As utilised previously (refer Section 4.2.3), any visible axillary shoot with a height > 1 cm was counted.

For each treatment application within each phase, as a measure of the size of the tuber (Naor et al., 2005a) the fresh weight of the tubers, where applicable, was measured at the end of the growth cycle, following the withdrawal of irrigation and drying down of the foliage.

As a measure of the floral productivity in the second growth cycle, the total number of flowers per tuber i.e., flower number comprising primary, secondary and tertiary flowers (Fig. 1.3b) was recorded for the plants treated with BAP in both forms (in 1N NaOH or as Cylex^{TM}) during phase 1. Any structure containing a spadix was counted as a flower. Flowers were counted as and when they emerged until at least 120 days after planting, after which flowering was not observed.

6.2.4 Experimental design and statistical analysis

Three single-plant replicates were allocated to each treatment application in phases 1, 2 and 3, in a completely randomized design. As described previously, blocking was not adopted in this experiment (refer Section 4.2.4).

Within each phase (1, 2 or 3), analysis of variance, means ± standard errors, mean separation tests and contrasts for the number of axillary shoots and fresh weight of tubers, were carried out using PROC GLM of SAS version 9.2 (SAS Institute., USA) and MS Excel (Microsoft Corp., USA).

6.3 Results

6.3.1 Overview

In phase 1, visible axillary shoots were observed from 10 days after the second foliar application of BAP, either as Cylex or in 1N NaOH, for up to 1 month after the first observation. After this time, the cessation of new leaf production was noticed in control plants, which indicated the commencement of phase 2. While BAP in 1N NaOH and Cylex were both capable of stimulating a branching response in Best Gold, the application of higher concentrations of Cylex in phase 1 (4.4 mM and 6.6 mM) resulted in scorching of leaves, not observed with the application of BAP in 1N NaOH.

Flowering in the second growth cycle, from the tubers obtained following the application of BAP in 1N NaOH or Cylex^{TM} in phase 1 of the first growth cycle, always commenced in the SAM i.e., dominant bud 1 (Fig. 1.3a), followed by primary flowers from the remaining dominant buds (Fig. 1.3a), and subsequently secondary and tertiary flowers from the respective shoots. The first flower was noticed at 75 ± 10 days after planting and continued until 100 ± 10 days.

Visible symptoms of active growth were observed in the control plants in phase 3, which produced 1-2 axillary shoots in contrast to phases 1 and 2, where the control plants comprised only the primary shoot. These axillary shoots also possessed leaves that were notably darker green, thicker and maculated (Fig. 6.2a), compared to the leaves in phases 1 or 2 (Fig. 6.2b). From experience, these features were inherent to plants in their second and third growth cycles (Fig. 1.1d-h).

Fig. 6.2 Phenotypic difference in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' in, **a**) phase 3 depicting dark-green, thicker leaves with maculation, compared to leaves without these features in, **b**) phase 1.

6.3.2 Phase 1

6.3.2.1 Branching in phase 1

Both forms of BAP i.e., BAP in 1N NaOH and Cylex, at all concentrations above 0 mM, were effective in stimulating branching significantly ($P < 0.0005$) higher than the control (Fig. 6.3). Though a significant difference in the degree of branching ($P > 0.1$) was not observed between 2.2 mM, 4.4 mM and 6.6 mM concentrations of both BAP in 1N NaOH and Cylex (Fig. 6.3), these concentrations produced at least 1-2 additional axillary shoots than the 2.3 ± 0.3 axillary shoots stimulated by 0.4 mM. Overall, amongst all the concentrations of BAP tested, 6.6 mM of BAP in 1N NaOH (Fig. 6.4a) stimulated the highest degree ($P < 0.0005$) of branching (4.7 ± 0.3) than the control (no axillary shoots) (Fig. 6.4b).

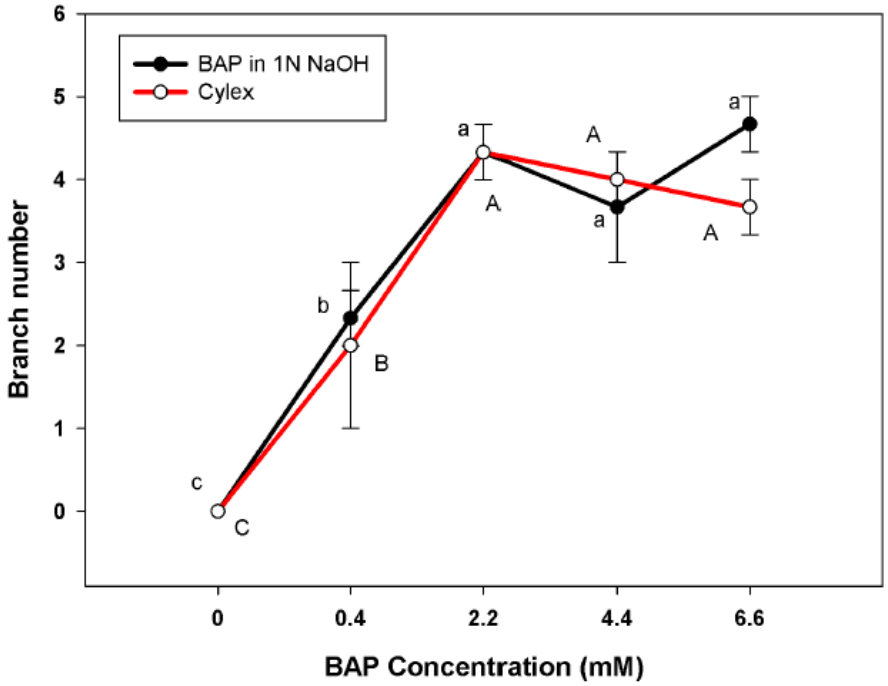


Fig. 6.3 Number of axillary shoots/branch number per plant ± standard error stimulated by the application of increasing concentrations of BAP either as BAP in 1N NaOH or Cylex (Valent BioSciences, USA) during phase 1 of the growth cycle in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold'. Different *small letters* indicate significant differences (α = 0.05) between the number of axillary shoots stimulated by different concentrations of BAP in 1N NaOH, and different *capital letters* indicate significant differences (α = 0.05) between the number of axillary shoots stimulated by different concentrations of Cylex™. Mean separation by Least Significant Difference (LSD) (*n = 3*).

Fig. 6.4 Single-shoot seedling of *Zantedeschia* cv. 'Best Gold' showing, **a**) 4 axillary shoots obtained following the application of BAP in 1N NaOH at 6.6 mM concentration in phase 1, compared to, **b**) control with only the primary shoot.

6.3.2.2 Tuber weight in phase 1

Though the difference was not significant (Fig. 6.5; $P > 0.1$), the lowest concentration of BAP in 1N NaOH (0.4 mM) produced tubers with a greater weight (an increase of 6.4 g) than the control (31.7 ± 1.7 g). Further increases in the concentration of BAP also generated tubers with greater weight than the control (Fig. 6.5). Visually, the size of the tubers (Fig. 6.6a) was also consistent with this pattern where the application of BAP at higher concentrations (4.4 mM and 6.6 mM) resulted in bigger-sized tubers than the control. Accordingly, the highest concentration of BAP in 1N NaOH (6.6 mM), which stimulated the highest degree of branching (4.7 ± 0.3 axillary shoots; Fig. 6.3) produced tubers with the largest tuber weight (48.0 ± 11.1 g; Fig. 6.5) and visual size (Fig. 6.6a) compared to the control (31.7 ± 1.7 g).

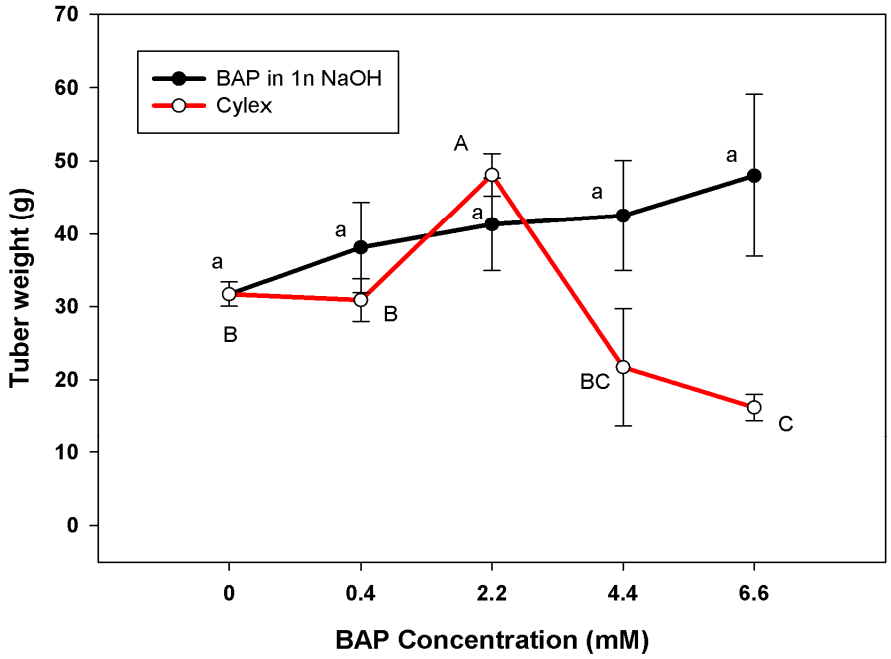


Fig. 6.5 Tuber weight ± standard error obtained following the application of increasing concentrations of BAP either as BAP in 1N NaOH or Cylex (Valent BioSciences, USA) during phase 1 of the growth cycle in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold'. Different *small letters* indicate significant differences (α = 0.05) between the tuber weight obtained following the application of different concentrations of BAP in 1N NaOH, and different *capital letters* indicate significant differences (α = 0.05) between the tuber weight obtained following the application of different concentrations of Cylex. Mean separation by Least Significant Difference (LSD) (*n* = *3*).

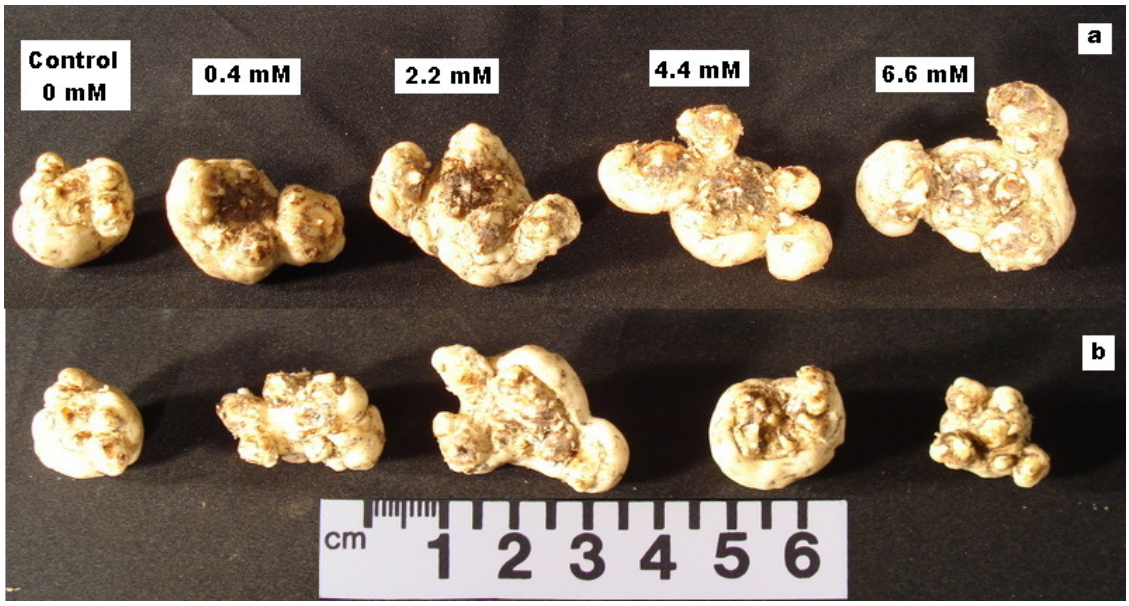


Fig. 6.6 Size of the tubers obtained at the end of the growth cycle following the application of increasing concentrations of BAP as, **a**) BAP in 1N NaOH or, **b**) Cylex (Valent BioScinces, USA) during phase 1 of the growth cycle of single-shoot seedlings of *Zantedeschia* cv. 'Best Gold'.

Concomitant with the branching response (Fig. 6.3), the application of 2.2 mM of Cylex produced tubers with a greater weight than the control ($P < 0.003$; Fig. 6.5). This was also evident in the resultant visual size of the tuber generated by this concentration (Fig. 6.6b). However, at higher concentrations of Cylex (4.4 mM and 6.6 mM), the weight of the tuber diminished significantly ($P < 0.003$) compared to 2.2 mM (Fig. 6.5), which was also apparent with the visual size of the tuber at these concentrations, respectively (Fig. 6.6b).

6.3.2.3 Floral productivity in the second growth cycle

Compared to tubers from the control treatment, tubers obtained following the application of BAP in 1N NaOH in the first growth cycle (except 4.4 mM) showed a significant increase ($P < 0.06$) in floral productivity in their second growth cycle (Fig. 6.7). Compared to the existing commercial recommendation comprising the application of GA_3 alone, these treatments also caused an increase of 66.5% (0.4 mM), 33.5% (2.2 mM) and 66.5% (6.6 mM) of the floral productivity, though the difference was not significant (Fig. 6.7; $P > 0.1$). Following the application of Cylex^{TM} in the first growth cycle, an increase in the floral

productivity (P = 0.1) was observed only between the 0.4 mM concentration and the control. Higher concentrations of Cylex^{TM} (4.4 mM and 6.6 mM) resulted in floral productivity equal to or less than that of the control (1.0 ± 0.6 flowers per tuber). Within this assessment following replanting, as observed before (Figs. 6.3; 6.5) the highest concentration of BAP in 1N NaOH (6.6 mM) also produced the highest number of flowers per plant (3.3 ± 0.7), compared to the control (1.0 ± 0.6) and the commercial recommendation comprising the application of GA_3 alone (2.0 ± 0.0) (Fig. 6.7).

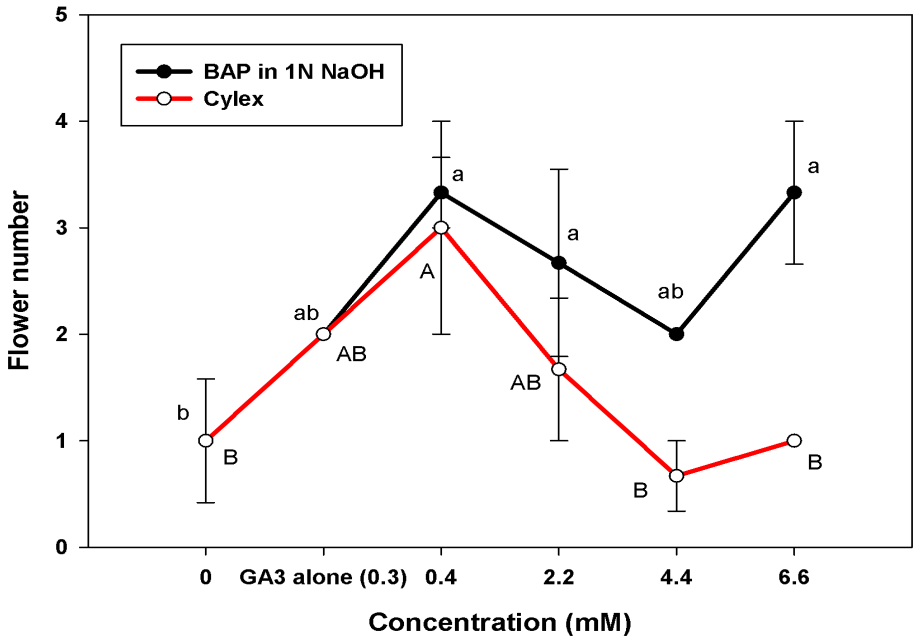


Fig. 6.7 Flower number, i.e. total number of flowers (primary, secondary and tertiary), per tuber ± SE obtained following the replanting of tubers treated with BAP in 1N NaOH or Cylex^{TM} in their first growth cycle and GA_3 (0.3 mM) prior to replanting in their second growth cycle as described in Section 6.2.2.1. Different *small letters* indicate significant differences (α = 0.05) between the number of flowers obtained following the application of different concentrations of BAP in 1N NaOH or GA_3 alone (0.3 mM), and different *capital letters* indicate significant differences (α = 0.05) between the number of flowers obtained following the application of different concentrations of Cylex^{TM} or GA_3 alone (0.3 mM). Mean separation by Least Significant Difference (LSD) (n = *3*).

6.3.3 Phase 2

6.3.3.1 Branching in Phase 2

In contrast to that observed in phase 1 (refer Section 6.3.1), visible emergence of axillary shoots was not noticed 10 days after the second foliar spray of Cylex at any concentration (Fig. 6.8). Following the sequential application of GA_3 at 0.9 mM however, the number of branches increased significantly ($P < 0.0001$), with all concentrations of Cylex above 0 mM. A minimum of 7 axillary shoots were stimulated by this sequential order of application (BAP → GA_3), compared to the control (no axillary shoots). The application of GA_3 alone also failed to stimulate branching compared to the control ($P > 0.1$; Fig. 6.8).

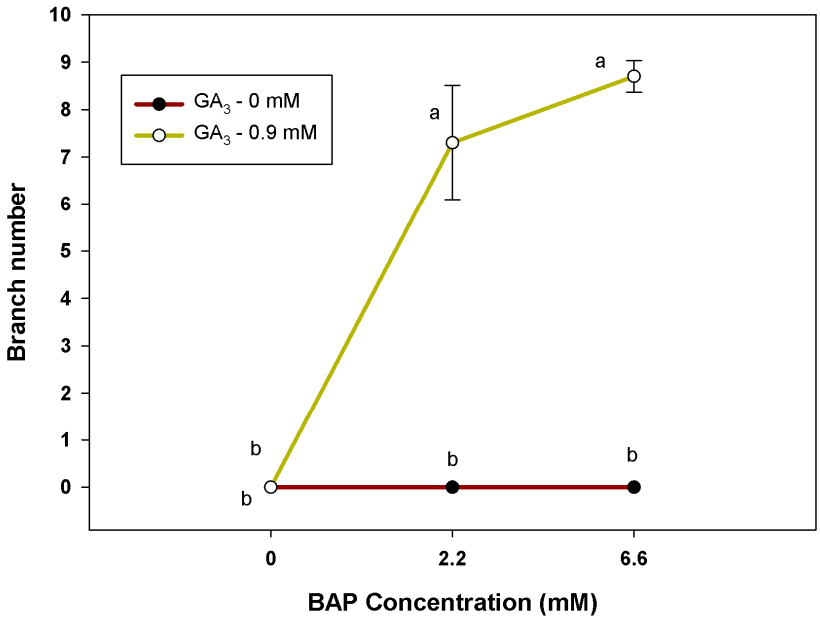


Fig. 6.8 Number of axillary shoots/branch number per plant ± standard error stimulated by the application of increasing concentrations of BAP applied as Cylex (Valent BioSciences, USA) applied either alone (GA_3 – 0 mM) or followed by a sequential application of GA_3 at 0.9 mM, during phase 2 of the growth cycle in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold'. Different *small letters* indicate significant differences ($\alpha = 0.05$) between the number of axillary shoots stimulated by different concentrations of Cylex alone (GA_3 – 0 mM) or followed by a sequential application of GA_3 at 0.9 mM. Mean separation by Least Significant Difference (LSD) ($n = 3$).

6.3.3.2 Tuber weight in phase 2

Concomitant with the degree of branching (Fig. 6.8), the sequential application of Cylex at 6.6 mM followed by GA_3 at 0.9 mM, generated tubers with the largest weight (42.3 ± 12.9 g), followed by the treatment comprising Cylex at 2.2 mM followed by GA_3 at 0.9 mM (28.7 ± 3.0 g), compared to the control (24.7 ± 1.4 g). As observed in phase 1 however, treatment differences were not significant ($P > 0.1$).

6.3.4 Phase 3

In contrast to phases 1 and 2, where the control plants failed to produce any axillary shoots (Figs. 6.3, 6.8), in phase 3, plants within the control treatment produced 1.7 ± 0.3 axillary shoots. As described in section 6.3.1, the plants in this phase also displayed characteristic features inherent to plants in their second growth cycle (Fig. 6.2). Though the sequential application of Cylex at 2.2 mM followed by GA_3 at 1.4 mM (BAP → GA_3), and the reverse order (GA_3 → BAP) at the respective concentrations induced 4.7 ± 0.9 and 3.7 ± 1.2 axillary shoots, respectively, the increase was not significantly higher ($P > 0.1$) than the control (1.7 ± 0.3 axillary shoots).

Since, at this time, the plants were assumed to have commenced their second growth cycle (refer Section 6.3.1), the measurement of tuber weight at the end of this cycle would not truly represent the treatment effects, and therefore was not measured at the end of phase 3 in the current experiment.

6.4 Discussion

6.4.1 The three phases in the growth cycle of *Zantedeschia*

The branching response from the pilot studies conducted during phases 1 and 2 of the growth cycle (Fig. 6.1) corroborated the demarcation of the growth cycle into these phases. While the application of BAP alone was sufficient to stimulate branching in phase 1 (Fig. 6.3), BAP alone was not capable of stimulating branching in phase 2 (Fig. 6.8), during which time the dynamics of the SAM was potentially altered (i.e., cessation of leaf production). These results, as described in Chapters 4 and 5, and hypothesized at the beginning of the experiment (refer Section 6.1.1), reiterated the significance of the time of

application of branch-inducing treatments in eliciting a branching response. The identification of the ideal time of application of branch-inducing treatments within the growth cycle has now been facilitated by employing the visual clues in the current experiment. Future studies aiming to stimulate branching in *Zantedeschia* can utilize these visual clues as a roadmap to manipulate the branching response accordingly. While the branching response following the application of BAP in phase 3, i.e., from the formation of an atrophied leaf through to the end of the growth cycle (Fig. 6.1), could have added to the existing knowledge on the significance of the visual clues in the ontogeny of *Zantedeschia*, the possible commencement of the second growth cycle in these plants failed to permit a rational interpretation of the branching response (refer Section 6.3.4). In the subsequent main experiment (Chapter 7) therefore, for phase 3 the treatments were planned to be applied earlier than that utilized in the current pilot study.

That the SAM had undergone developmental changes between phases 1 and 2, represented visually by the cessation of leaf production, was further supported by the branching response stimulated in phase 2, only following the sequential application of GA_3 (BAP → GA_3; Fig. 6.8). These results were similar to those reported by Ali and Fletcher (1970; 1971) in seedlings of soy bean, where a sequential application of GA following CK was required to elicit a branching after cell division had ceased. Likewise, for seedlings of 'Best Gold' in phase 2, the application of BAP alone may have rekindled cell division (Miller et al., 1955), which probably required the sequential application of GA_3 to stimulate shoot elongation (Stowe and Yamaki, 1959). Without the induction of cell division in phase 2, in the absence of BAP, the application of GA_3 alone failed to stimulate any branching response (Fig. 6.8). In the current pilot studies, a sequential application of GA_3 was included with the aim of stimulating branching in phase 2. Therefore, a sequential application of GA_3 following BAP (BAP → GA_3) or the application of GA_3 alone was not attempted in phase 1. Also, the effect of the reverse order of application (GA_3 → BAP) on the degree of branching was not evaluated in both phases 1 and 2. While treatments comprising the reverse order were included for plants in phase 3, as stated above, since these plants were believed to have commenced their second growth cycle, the resultant branching response was considered to be unrepresentative of the treatment effects. To analyze the effect of BAP and GA_3, applied alone and/or in sequential combination on the

degree of branching, a common set of treatments was planned along the three phases of the growth cycle in the subsequent main experiment (Chapter 7).

Decapitation can be used as a diagnostic tool to determine the dormancy status of the buds. While decapitation of the apical bud stimulated the outgrowth of axillary buds under apical dominance (Thimann and Skoog, 1933), decapitation after the onset of endodormancy would fail to stimulate bud outgrowth. Likewise, the dormancy status of the buds can be determined by the branching response stimulated by the application of CKs. Based on this logic, the demarcation of the growth cycle of *Zantedeschia* into the three phases (Fig. 6.1) was hypothetically suggested to track the transition of buds from apical dominance to endodormancy (refer Section 6.1.1). While the application of BAP was capable of stimulating branching when applied in phase 1 (Fig. 6.3), it failed to elicit a branching response in phase 2 (Fig. 6.8), thereby suggesting that the buds in phase 1 were under apical dominance, whereas those in phase 2 were possibly endodormant. Given that dormancy is also a feature of developmental changes in the SAM (Horvath et al., 2003) and in *Zantedeschia*, the onset of endodormancy is autonomously controlled (Naor and Kigel, 2002), it is more likely that intrinsic factors such as cell division determine the dynamics of the SAM (Robinson and Wareing, 1969). The branching response following the application of BAP in phase 3 would have further corroborated the establishment of endodormancy concomitant with the three phases, which was however thwarted due to the possible commencement of the second growth cycle in these seedlings. Therefore, the transition of buds from apical dominance to endodormancy and the parallel establishment of endodormancy along the three phases of growth was proposed to be vindicated through the subsequent main experiment (Chapter 7).

6.4.2 Phase 1

6.4.2.1 Branching

Foliar application of CK in *Zantedeschia* as BAP in 1N NaOH or Cylex was capable of stimulating visible outgrowth of axillary buds present on a subterranean tuber (Fig. 6.3), thereby suggesting that these buds were controlled by apical dominance. Among other possible mechanisms involving hormonal cross-talk (Ross et al., 2001; Ferguson and Beveridge, 2009), BAP (Black and Osborne, 1965) or its metabolites (refer Section 2.3.1.2.1), may have been transported from the point of application (leaves) to the point of action (subterranean buds), culminating in a branching response. As examined in Chapter 8, further studies involving the tracking of [8-^{14}C] BAP would elucidate the possible mechanisms of uptake, transport and metabolism of BAP in *Zantedeschia*.

Though both BAP in 1N NaOH and Cylex^{TM} were equally effective in stimulating a branching response in *Zantedeschia* (Fig. 6.3), the application of Cylex^{TM} at higher concentrations caused scorching of leaves, not observed with the application of BAP in 1N NaOH. Since the leaf area and LAD bear a direct relationship to the size of the tuber (Funnell et al., 2002), it was not surprising that the scorching of leaves resulted in tubers with a smaller weight and size than those produced by lower concentrations (Fig. 6.5 and Fig. 6.6b). Therefore, in further studies involving the application of BAP in *Zantedeschia* (e.g. Chapter 7), BAP dissolved in 1N NaOH was proposed to be used.

6.4.2.2 Tuber weight

Stimulation of 0.4 ± 0.2 axillary shoots by defoliation resulted in a tuber weight of 37.9 ± 2.6 g at the end of the growth cycle (Table 4.1). Subsequently, the stimulation of an additional 0.5 axillary shoots (0.9 ± 0.3) by the media drench of ATI resulted in an increase in tuber weight by 13 g (50.9 ± 6.9 g) compared to that after defoliation (Table 5.2). Therefore, given the direct relationship between the degree of branching and the resultant tuber weight, the highest concentration of BAP in 1N NaOH (6.6 mM), which stimulated the highest degree of branching (4.7 ± 0.3 axillary shoots; Fig. 6.3) would be expected to produce tubers with a weight of approximately 140 g at the end of the growth cycle. However, this treatment produced tubers with a weight of 48.0 ± 11.1 g (Fig. 6.5). This

anomaly can be explained by differences in source-sink partitioning of assimilates. In *Zantedeschia*, leaf canopy development can be viewed as a competing sink for assimilates (Funnell et al., 2002). Therefore, the increase in leaf area and LAD by the stimulation of branching by the application of BAP may have potentially altered the source-sink relationship, ultimately resulting in a decline in the tuber weight than expected. An alteration in the source-sink relationship concomitant with increased branching was further corroborated by the branching response and the resultant tuber weight in phase 2 (refer Section 6.3.3.2). In this phase, approximately 7 axillary shoots were stimulated to growth by the sequential application of BAP followed by GA_3 (Fig. 6.8). However, the weight of tubers obtained at the end of the growth cycle was 42.3 ± 12.9 g, which was less than that obtained in phase 1 (4.7 ± 0.3 axillary shoots; 48.0 ± 11.1 g). Based on these observations, it is clear that the dynamics of source-sink partitioning between the leaf area and LAD and the resultant tuber weight needs to be revisited in the context of the regulation of branching. Nevertheless, within the context of this thesis, and the commercial objective stated at the beginning of this study (refer Section 1.6), the increase in tuber weight obtained following the application of BAP in phase 1, compared to the control (Fig. 6.5), equated to an increase in floral productivity in the next growth cycle, compared to the existing standard (refer Section 6.4.2.3 below).

6.4.2.3 Floral productivity

Given that the tuber size/weight bears a direct relationship to the floral productivity of *Zantedeschia* (Naor et al., 2005a), it was not surprising that the larger sized tubers obtained following the application of BAP in 1N NaOH during their first growth cycle (Fig. 6.6a) resulted in an increase in floral productivity compared to the control, which had not been treated with BAP in the first growth and/or GA_3 prior to replanting in the second growth cycle (Fig. 6.7). However, the 30-65% increase in floral productivity caused by these treatments compared to the existing commercial recommendation provided sufficient motivation to recommend pursuit of this strategy on a larger commercial scale (Data not presented). This finding that equated the stimulation of branching in *Zantedeschia* (Fig. 6.3) to an increase in the resultant tuber weight (Fig. 6.5) and ultimately the floral productivity of these tubers in the next growth cycle fulfilled the commercial objective

framed at the beginning of this thesis (refer Section 1.6). The significance of this equation was further established by the decline in floral productivity observed (Fig. 6.7) following the application of Cylex ^{TM} at the highest concentrations (4.4 mM and 6.6 mM) during the first growth cycle. These treatments had generated visually smaller sized tubers (Fig. 6.6b) caused presumably by the scorching of leaves. Therefore, it was evident that the formulation of BAP rather than its mere presence was also a critical factor in determining the branching response. Moreover, the branching response (or the lack of it) obtained following the application of BAP alone in phase 2, also highlighted the significance of the right time of application of the branch-inducing treatments. To obtain the desired flowering response therefore, the application of branch-inducing treatments should be targeted during phase 1 of the growth cycle.

Due to the lack of common treatment applications within each the three phases of growth, the effect of the sequential application of GA_3 following BAP (BAP $\rightarrow$ GA_3), as implemented in phases 2 and 3 was not undertaken in phase 1. In the subsequent main experiment (Chapter 7) therefore, the effect of BAP and/or GA_3 on branching was planned to be evaluated. Since GA catalyses floral transition in *Zantedeschia* (Naor et al., 2005a), the effect of these components on the flowering response was also targeted to be measured.

6.4.3 Phase 2

Between phases 1 and 2, further developmental changes had occurred in the SAM, evident by the cessation of leaf production in phase 2 (Fig. 6.1b), and the failure of BAP alone to evoke a visible branching response in phase 2 of the growth cycle (Fig. 6.8). This failure of BAP to evoke a branching response in phase 2 could signal the onset of endodormancy in these buds compared to those in phase 1, which were predominantly governed by apical dominance (refer Section 6.4.1). The transition of buds from apical dominance (phase 1) to endodormancy (phase 2) therefore, could involve a decline in the sensitivity of the buds (refer Section 2.2) to exogenous BAP. A comparison of dose-response curves measuring the branching response across a range of BAP concentrations between phases 1 and 2 may, therefore, provide an estimate of the sensitivity changes in the bud to CKs (Weyers et al., 1995), and the factors that contribute to these changes (Firn, 1986). However, due to the limited number of replications used in the current pilot studies (n = 3), a valid inference on

the sensitivity changes could not be derived. The dose-response curves obtained following the application of BAP along the three phases of growth was, therefore, planned to be investigated in the subsequent main experiment (Chapter 7).

While BAP alone was not sufficient to stimulate significant axillary branching in phase 2 (Fig. 6.8), a sequential application of GA_3 provoked a significant increase in the branching response. At later stages of growth of soybean seedlings, marked by the cessation of cell division, a sequential application of GA following an initial application of CK was required to stimulate branching (Ali and Fletcher, 1970). A role for CK in the induction of cell division, and GA in the subsequent shoot elongation was proposed (Ali and Fletcher, 1970). Though the measurement of cell division was not undertaken in the current pilot studies, and was beyond the scope of the stated objectives, the application of BAP in phase 2 was assumed to stimulate axillary bud outgrowth to some extent, which was however, not visible. In performing its characteristic role of shoot elongation (Stowe and Yamaki, 1959), the sequential application of GA_3 (BAP → GA_3) may have provided the stimulus to promote visible outgrowth of the axillary buds. The efficacy of GA_3 in provoking a branching response in phase 2 however, was evident only when it was applied in sequential combination to BAP. GA_3 alone had no effect on the branching response (Fig. 6.8). Surely, the role of GA either alone or in sequential combination with BAP, in forward (BAP → GA_3) and reverse (GA_3 → BAP) orders, requires further investigation. While the pilot studies provided an initial direction towards this cross-talk, the implications of this cross-talk between CK and GA in the transition of buds from apical dominance to endodormancy was planned to be corroborated in the subsequent main experiment (Chapter 7). Tracking the foliar application of [8-^{14}C] BAP with/without a sequential application of GA_3 in phase 2 was, also planned to provide a better understanding of the changes in the transport and metabolism of BAP in phase 2 compared to that in phase 1 (Chapter 8).

Concomitant with the degree of branching (Fig. 6.8), the sequential application of BAP followed by GA_3 (BAP → GA_3) also produced tubers with the largest weight compared to the control (refer Section 6.3.3.2), though the difference was not significant. Since the direct relationship between branching, tuber weight, and floral productivity, was already established during phase 1 (Section 6.3.2), replanting of the tubers obtained in phase 2 was

not attempted. However, the measurement of floral productivity was reserved for the subsequent main experiment (Chapter 7).

6.4.4 Phase 3

The intention of demarcating phase 3 i.e., from the formation of an atrophied leaf through to the end of the growth cycle (Fig. 6.1c), was to evaluate the branching response following the application of BAP and/or GA_3 at this stage and thereby corroborate the establishment of deeper endodormancy, compared to the buds in phase 2. The measurement of this response would further vindicate the demarcation of the growth cycle based on visual clues. However, the plants in phase 3 exhibited characteristic features of the second growth cycle (Fig. 6.2), thereby leading to the assumption that these plants had actually commenced phase 1 of the second growth cycle (Fig. 1.1). It was therefore not surprising that during phase 3 both the forward and reverse orders of application of BAP and GA_3 failed to elicit a significant branching response compared to the control (refer Section 6.3.4). Since a comparison of the branching response was not within the physiologically intended context of phase 3 during the pilot studies, the evaluation of the true branching response of BAP and GA_3 alone, and their corresponding cross-talk in both forward and reverse orders in phase 3 was planned to be executed earlier than that attempted in the current pilot studies i.e., ~ 250 days after seed sowing; ~ 4000 heat-units (Chapter 7).

For the same reasons stated in this section, i.e., the natural progression of the plants in phase 3 to their next growth cycle, the tuber weight obtained at the end of the growth cycle following the application of BAP and/or GA_3 was not measured. Also, the floral productivity of the plants in phase 3 following the application of BAP and/or GA_3 was planned to be measured in the subsequent main experiment (Chapter 7).

6.5 Conclusions

Depending upon the timing of application i.e., the phase of the growth cycle, the exogenous application of BAP on single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' resulted in a significant increase in the branching response. The increase in branching, which equated to an increase in the size/weight of the resultant tuber, and led to a subsequent increase in floral productivity in the next growth cycle, infers that the commercial objective framed at the beginning of this thesis (refer Section 1.6) can be achieved through this strategy. In contrast to phase 1, the decline in the efficacy of BAP alone to stimulate branching in phase 2 and the need for a sequential application of GA_3 in this phase, not only vindicated the demarcation of the growth cycle into different phases, but also highlighted the need for a sequential application of GA_3 to stimulate branching at later stages of the annual growth cycle. By applying the same set of treatment combinations within each phase, and conducting phase 3 at an earlier time, the results from the current pilot studies were planned to be substantiated and extended, through a subsequent main experiment (Chapter 7).

The current pilot studies supported the hypothesis that axillary buds in phase 1 were under apical dominance, whereas the buds in phase 2 were probably endodormant. The suggested decline in the sensitivity of the buds to BAP associated with the transition of buds from apical dominance to endodormancy, was proposed to be tested in future experiments by using a dose-response curve to depict the sensitivity changes (Chapter 7). Since the sequential application of GA_3 following BAP was found to stimulate branching in phase 2 and GA is already known to catalyse floral transition in *Zantedeschia*, the floral productivity following the application of BAP and/or GA_3 was also planned to be measured with the aim of contributing to the recent studies drawing common mechanisms between dormancy and flowering in plants. Overall, the pilot studies described in this chapter laid the foundation for conducting a full fledged experiment that aims to elucidate the hormonal control of dormancy and flowering in *Zantedeschia*, elaborated in Chapter 7.

7. Hormonal control of branching and flowering in *Zantedeschia*

7.1 Introduction

By stimulating branching (refer Section 1.6), floral productivity of *Zantedeschia* was proposed to be enhanced via a subsequent increase in tuber weight and/or by triggering the sympodial flowering cascade in the branched plant (refer Section 4.1). Following the demarcation of the growth cycle of *Zantedeschia* into phases 1, 2 and 3 (Fig. 6.1a-c), the successful stimulation of branching in phase 1 by the application of the cytokinin (CK) 6-benzylaminopurine (BAP) alone (Fig. 6.3), the concomitant increase in the tuber weight/size (Fig. 6.5; 6.6) and the resultant increase in the floral productivity of these plants in the second growth cycle (Fig. 6.7) corroborated the first mechanism of increasing floral productivity by stimulating branching (Chapter 6). The current experiment was therefore devised to address, among other issues such as vindication of the three phases of the growth cycle, the second mechanism of increasing floral productivity by triggering the sympodial flowering cascade in a branched plant.

With regards to branching, the failure of BAP alone to induce a branching response, and the need for a sequential application of gibberellin (GA) (BAP → GA_3) to stimulate branching in phase 2, with the parallel establishment of endodormancy was also highlighted (Fig. 6.8). Given that BAP alone and GA_3 alone had no significant effect on branching in phase 2, and branching induction occurred only following the sequential application of BAP and GA_3 (Fig. 6.8), this topic of cross-talk between CK and GA in the regulation of branching during the transition of buds from apical dominance to endodormancy i.e., phase 1 through to phase 3, demanded further investigation. Consequently, since GA catalyses floral transition in *Zantedeschia* irrespective of meristem size and age (Naor et al., 2005a), an analysis of the influence of the sequential application of BAP→ GA_3 and vice-versa on the flowering response could elucidate the impact of CK-GA cross-talk on flowering. Put together, cross-talk between CK and GA was assumed to regulate branching and flowering in *Zantedeschia*.

Though the pilot studies described in Chapter 6 indicated a role for CK-GA cross-talk in the control of branching and flowering in *Zantedeschia*, the progressive evolution in understanding the potential role of CK and GA parallel to changes in the phases of growth, did not permit a comparison of the same set of treatment combinations across the three phases of growth. Moreover, since the plants in phase 3 exhibited characteristic features of the second growth cycle, the rationale behind the demarcation of the growth cycle into three phases, which were suggested to hypothetically track the transition of buds from apical dominance to endodormancy, could not be vindicated. These lacunae inherent to pilot studies, combined with the lack of sufficient plant replicates (n = 3; Chapter 6), underlined the need to address these issues through a main experiment.

The main experiment described in the current chapter was therefore conducted in a bid to address these aspects of the hormonal control of branching and flowering in *Zantedeschia* that arose as a result of the pilot studies.

7.2 Dormancy and flowering are regulated by the reciprocal interaction between CK and GA in Zantedeschia

Refer to the attached paper (With kind permission from Springer Science + Business media: Journal of Plant Growth Regulation, Dormancy and flowering are regulated by the reciprocal interaction between cytokinin and gibberellin in *Zantedeschia*, Volume 29 (4), 2010, Pages 487-499, Arvind Kumar Subbaraj, Keith Allen Funnell, David John Woolley).

7.3 Discussion

7.3.1 Relationship between branching and flowering in *Zantedeschia*

The sequential application of BAP followed by GA_3 (BAP → GA_3) was capable of stimulating significant branching compared to the reverse order of application (GA_3 → BAP) (Table 7.1). As hypothesized (refer Section 4.1), increasing floral productivity of *Zantedeschia* via triggering the sympodial flowering cascade of a branched plant, means that this order of application (BAP → GA_3), should in turn stimulate a higher degree of flowering. However, in contrast, the sequential application of GA_3 followed by BAP (GA_3 → BAP), which stimulated a significantly lesser degree of branching in phases 2 and 3, consistently produced a significantly higher degree of flowering compared to BAP → GA_3 (Table 7.1). This increase in floral productivity was contributed by a significant increase in the capacity of this sequential order of application i.e., GA_3 → BAP, to initiate floral transition in several primary shoots (Table 7.2B). On the other hand, BAP → GA_3 acted by triggering the flowering cascade in a few primary shoots, evident by the increase in axillary flowering induced by this order of application (Table 7.2B). Albeit, the results from the current experiment indicated that an increase in branching does not necessarily equate to an increase in floral productivity. This notion was stated earlier in Chapter 3, where the significant increase in the number of shoots after 6 months of tuber storage (Table 3.1) did not equate to a concomitant increase in the number of flowers per tuber (Table 3.2) compared to that after 9 months of storage. Therefore, this approach of increasing floral productivity of *Zantedeschia* by triggering the flowering cascade in a branched plant needs further evaluation. While Naor et al. (2005a) have reported that GA catalyses floral transition in *Zantedeschia* regardless of meristem size and age, in the same study, they reported a basipetal decline in the flowering potential of the buds on the tuber. Though GA triggered floral transition in plantlets *in vitro*, subsequent inflorescence development ceased at later stages of growth (Naor et al., 2004). Therefore, though GA mediates floral transition in *Zantedeschia*, additional factors are required to convert this transition to visual manifestation of flowering. In the current study also, histological examination of the buds revealed that the presence of GA_3 mediated floral transition, irrespective of the order of application (Table 7.3). However, the order comprising GA_3 → BAP produced more

number of flowers per plant than the reverse order i.e., BAP → GA_3 (Table 7.1). The sequential application of BAP following GA_3, probably enhanced organogenesis of different floral organs, resulting in visual manifestation of flowering (refer Section 2.5.2.2). In the presence of GA, BAP was also shown to increase differentiation of floral organs in plantlets *in vitro* (Naor et al., 2004). This topic of hormonal cross-talk between CK and GA in the regulation of flowering is definitely worth pursuing. However, determination of the differential capacity of the shoots to flower is fundamental to interpreting flowering data.

Nevertheless, towards addressing the commercial objective stated at the beginning of this study (refer Section 1.6), in addition to achieving an increase in floral productivity via an increase in tuber size (Chapter 6), the sequential application of GA_3 → BAP can be recommended. This order of application was capable of increasing the floral productivity by at least 2-3 additional flowers, compared to the existing commercial recommendation of the application of GA_3 alone at 0.3 mM concentration (Fig. 7.5d).

7.3.2 Possible mechanisms of branching control by CK and/or GA

The dose-response curves obtained in the three phases of growth, following the application of BAP alone and in sequential application with GA_3 i.e., BAP → GA_3 (Fig. 7.4a-c), indicated a concomitant change in the sensitivity of the buds to CK and/or GA with the establishment of endodormancy. While BAP and GA_3 alone had minimal/no effect on branching in phase 3 (Fig. 7.4c), a significant increase in branching was observed following the application of BAP → GA_3. The importance of this order of application was further corroborated by the minimal branching stimulated by the reverse order of application i.e., GA_3 → BAP, in phase 3 (Fig. 7.4f). This difference in the sensitivity of the buds to CK and/or GA can be caused by one or all of the following mechanisms:

- As observed in soybean (Ali and Fletcher, 1971), a mitotic role for CK and a shoot elongation role for GA can be suggested. In the absence of mitotic activity stimulated by CK, the bud may fail to respond to the GA stimulus. Likewise, in the absence of shoot elongation by GA, the mitotic activity induced by CK may fail to manifest as visible bud outgrowth. This mechanism of control partly explains the results obtained from the current experiment, where BAP alone and GA_3 alone had

no effect on branching in phase 3 (Fig. 7.4c; f). Though not measured in the current experiment, it is likely that BAP induced mitosis in the endodormant buds in phase 3, which enabled the buds to respond to the shoot elongation stimulus of the sequential application of GA_3.

- Triple interaction between abscisic acid (ABA), CK and GA may enable a permissive role for CK in allowing GA to mediate the release of endodormancy (Khan, 1975). As widely reported in the context of seed dormancy, CK antagonizes dormancy induction by ABA, thereby permitting GA to perform its role in dormancy release (Khan, 1975). Though this mechanism of control has also been reported in the context of bud dormancy (Khan, 1975), recent studies involving molecular/genetic approaches have not been conducted to establish this interaction in bud dormancy. Albeit, based on the results from the current study, it is possible that CK neutralizes ABA action, thereby allowing GA to release endodormancy. Alternatively, given the well known antagonistic relationship between ABA and GA in the control of endodormancy (Tanino, 2004), GA may neutralize ABA inhibition, thereby allowing CK to perform its characteristic role of stimulating axillary bud outgrowth.

- Cross-talk between CK and GA is also evident at the biosynthesis level, where CK inhibits GA biosynthesis and vice-versa (Weiss and Ori, 2007). This reciprocal interaction between CK and GA has been shown to regulate meristem function at different stages of growth (Greenboim-Wainberg et al., 2005; Jasinski et al., 2005). Such a mechanism of control cannot be ruled out in *Zantedeschia*, where exogenously applied CK may regulate GA biosynthesis and vice-versa, depending on the dormancy status of the buds.

- Finally, as suggested by Firn (1986; refer Section 2.2), sensitivity changes of the buds to CK and/or GA can be caused by changes in

    a) Receptivity – number of receptors
    b) Affinity of the receptors
    c) Response capacity of the signal transduction chain

d) Endogenous hormone concentration
e) Uptake efficiency
f) Metabolism
g) Transport *en route* to the site of action

Among the possible mechanisms of control described above, to begin with, changes in the uptake, availability and metabolism of BAP as influenced by the three phases of growth and a sequential application of GA_3 was explored subsequently (Chapter 8). [8-^{14}C] BAP chased with unlabelled BAP and GA_3 was used towards this purpose.

7.4 Conclusions

Compared to the sequential order of application of BAP followed by GA_3 i.e., BAP → GA_3, which stimulated a higher degree of branching, the reverse order of application i.e., GA_3 → BAP stimulated higher floral productivity. An increase in branching therefore, does not always equate to an increase in floral productivity. Further studies are required to evaluate the differential flowering potential of the shoots in the context of branching. The sensitivity of the buds to CK and/or GA changes with the transition of buds from apical dominance to endodormancy i.e., phases 1 through to 3. Possible mechanisms of this control were suggested.

8. Factors that contribute to sensitivity changes between phases 1, 2 and 3 – Uptake, availability and metabolism of [8-^{14}C] BAP

8.1 Introduction

In Chapter 7, it was shown that, while 6-benzylaminopurine (BAP) alone was sufficient to stimulate branching in the buds under apical dominance i.e., phase 1, a sequential application of gibberellin (GA) (BAP → GA_3) was required to stimulate axillary bud outgrowth/branching in predominantly endodormant buds i.e., phase 3 (Fig. 7.4a-c). Based on the dose-response curves obtained from the three phases of growth (Fig. 7.4a-c), it was assumed that, among other factors, a change in the sensitivity of the buds to cytokinin (CK) and/or GA was associated with the transition of buds from apical dominance to endodormancy, which was manifested as a differential branching response. Therefore, among several factors that contributes to sensitivity changes of the responding tissue/organ (Firn, 1986; refer Section 2.2.1), the uptake, availability and metabolism of [8-^{14}C] BAP as influenced by the three phases of growth, simultaneous application of unlabelled BAP (8.9 mM), and a sequential application of unlabelled GA_3 (1.4 mM) were investigated within the current chapter.

8.1.1 Hormonal control of dormancy

In the control of endodormancy, the antagonistic relationship between abscisic acid (ABA) and GA is widely implicated (Wareing and Saunders, 1971; Tanino, 2004). Exogenous application of ABA induced dormancy in woody perennials (El-Antably et al., 1967), and soaking of dormant potato (*Solanum tuberosum* L.) tubers in a GA solution, resulted in the breaking of dormancy (Brian et al., 1955). In *Zantedeschia* however, the onset of endodormancy, which is autonomously controlled (Naor and Kigel, 2002), did not coincide with an increase in endogenous ABA levels, and the endogenous GA content remained relatively low or constant throughout the growth period (Naor et al., 2008). The exogenous application of ABA and GA also failed to impose and release buds from endodormancy, respectively (Naor et al., 2008), thereby raising scepticism about the applicability of the ABA/GA paradigm for endodormancy control in *Zantedeschia*.

CK are a group of biologically versatile (Mok and Mok, 2003; Sakakibara, 2006) and mobile (Hirose et al., 2008) hormones, that are well-known in the control of apical dominance (refer Section 2.3.1.2). However, CK have been largely neglected from the paradigm of endodormancy control (Turnbull and Hanke, 1985a; Horvath, 2009). In a few plant species such as potato (Turnbull and Hanke, 1985a) and *Spirodela polyrrhiza* (Duckweed) (Chaloupková and Smart, 1994), CK have been shown to release buds from endodormancy. In potato, the release of endodormancy coincided with an increase in endogenous CK content (Turnbull and Hanke, 1985b; Suttle, 1998) and in duckweed, CK alleviated ABA-induced growth inhibition (Chaloupková and Smart, 1994). The role of CK and its interaction with ABA, GA and auxin are relatively well established in the context of seed dormancy in comparison to bud dormancy (Khan, 1975; Kucera et al., 2005).

Given the scepticism about the applicability of the ABA/GA paradigm for endodormancy control in *Zantedeschia*, and the successful stimulation of branching by the sequential order of application of BAP followed by GA_3 (BAP → GA_3) in phase 3 (Fig. 7.4c), it was not unreasonable to hypothesize a role for CK in the control of endodormancy in *Zantedeschia*. While the dose-response curves within the three phases of growth (Fig. 7.4a-c) demonstrated a concomitant change in the sensitivity of the buds to CK and/or GA, the exact mechanism behind this regulation is still dubious (refer Section 7.3.2). CK could regulate GA biosynthesis (Jasinski et al., 2005) or vice-versa (Greenboim-Wainberg et al., 2005), and/or as proposed by Ali and Fletcher (1970b), the sequential application of CK followed by GA may stimulate mitosis/cell division and shoot elongation respectively, and/or drawing a parallel mechanism from seed dormancy, the permissive role of CK to GA or vice-versa (Khan, 1975) may coordinate the sensitivity of the buds to CK and/or GA, which was manifested as the differential branching response (Fig. 7.4a-c) within the three phases of growth. Nevertheless, differences in the uptake, availability and metabolism of CK between the three phases of growth ± GA may influence one or more of these underlying mechanisms.

Based on antibodies used exclusively for the detection of the isoprenoid group of CK (ISCK) (refer Section 2.3.1.2), D'Arth et al. (2007) identified a 10 fold increase in the endogenous ISCK concentration during the release of endodormancy, compared to the endodormant buds of *Zantedeschia*. However, a significant difference in the ISCK concentration was not observed between a branched and non-branched (control) plant

within the same cultivar (D'Arth et al., 2007). Given that BAP, which belongs to the aromatic group of CK (ARCK) (refer Section 2.3.1.2), was successful in stimulating branching during phase 1 of the growth cycle of *Zantedeschia* and the ability of BAP to stimulate branching declined with the establishment of endodormancy (Fig. 7.3a), it was hypothesized that the ARCK group could play a pivotal role in the control of dormancy in *Zantedeschia*. Moreover, the existence of topolins (refer Section 2.3.1.2.1) naturally in *Zantedeschia* (das Neves and Pais, 1980a) provided further impetus to explore the ARCK group within the context of dormancy.

8.1.2 Sensitivity as a controlling factor

While the concentration of hormones was considered to be a key determinant of plant developmental processes (Cleland, 1983), Trewavas (1983) put forward the extreme view that sensitivity of the responding tissue/organ was the primary controlling factor. Subsequently, and to date, the consensus supports the existence of a combined control, where both the concentration of a hormone and the sensitivity of the responding tissue/organ are considered to regulate plant processes in unison (Weyers and Paterson, 2001). However, sensitivity changes can be contributed by several factors (refer Section 2.2) and there is no unifying principle that defines the sensitivity of the responding tissue (Firn, 1986). Under these circumstances, sensitivity changes are best demonstrated by dose-response curves and, therefore, several modelling approaches have been developed based on these curves (Firn, 1986; Weyers et al., 1995).

Ontogenetic sensitivity comprises changes in sensitivity due to developmental changes, and is indicated by spatial and temporal differences in responses (Trewavas, 1986). The bud transition hypothesis (refer Section 2.3.1.4) for the control of branching highlights the temporal changes in the sensitivity of the buds, where the buds in the transition stage are more sensitive to growth-promoting signals than the buds in the dormant stage. From the transition stage, the buds can proceed to a stage of sustained growth or revert back to the dormant stage (Dun et al., 2006). Sensitivity changes are confined to the responding tissue rather than the whole plant (Weyers and Paterson, 2001), and spatial changes in sensitivity to different hormones have been noted in different plant parts (Davies, 1995). Therefore, for branching related studies, a measure of the branching response of the buds to CK i.e., dose-response curves, would ideally depict sensitivity changes in contrast to studies that have employed a root-length bioassay to

determine changes in the sensitivity of the buds of *Zantedeschia* to CK (D'Arth et al., 2002).

The decline in the branching response to CK within the three phases of growth, and the simultaneous increase in the need for a sequential application of GA to stimulate branching, combined with the inability of GA alone to stimulate branching (Fig. 7.4), clearly depicted concomitant changes in the ontogenetic sensitivity of the buds to CK and/or GA during the transition of buds from apical dominance to endodormancy (Firn, 1986; Trewavas, 1986). Conventionally, CK has been applied directly to the suppressed buds to stimulate branching (Sachs and Thimann, 1967; Ali and Fletcher, 1970; Sakakibara, 2006). In geophytes such as *Zantedeschia*, the direct application of CK to the subterranean buds is impossible after planting. Since the leaves constitute the sole conduit of aerial signals to the underground buds, a foliar application of BAP and GA_3 was employed to stimulate branching in the subterranean buds (refer Chapter 7). The change in the ontogenetic sensitivity of the buds to CK and/or GA, evident during the transition of buds from apical dominance to endodormancy (Fig. 7.4), could therefore constitute spatial and/or temporal changes in CK and/or GA from the point of application i.e., leaves *en route* to the buds. These changes are, among other factors, represented by changes in the uptake, availability and metabolism of hormones. Uptake efficiency, especially in studies involving exogenous application of hormones, is a key determinant of sensitivity changes in the responding tissue (Firn, 1986), and in turn could affect the availability of hormones to different plant parts. Availability, denoting the amount of hormone present in different plant parts, is governed by the transport of hormone from the point of application to different plant parts (Davies, 1995), and the sink strength of these parts (Abo-Hamed et al., 1984). In addition to the uptake and availability of the hormone, metabolism also determines physiological response because metabolites differ in their transport (Auer et al., 1992) and biological capabilities (Jameson, 1994; Strnad, 1997).

Therefore, as a first step, among several factors that contribute to sensitivity changes (Firn, 1986; refer Section 2.2), changes in the uptake, availability and metabolism of BAP as influenced by the three phases of growth and the sequential application of GA_3 was planned to be investigated in the current study. Albeit, the role of other factors that contribute to sensitivity changes, such as changes in the affinity, receptivity and response capacity of the signal transduction pathway (Firn, 1986), in addition to

concomitant changes in the endogenous concentrations of CK and GA during the transition of buds from apical dominance to endodormancy, cannot be ruled out.

In a bid to identify the factors that contribute to the changes in the ontogenetic sensitivity of the buds to CK and/or GA during the transition of buds from apical dominance to endodormancy, which was manifested as the differential branching response between phases 1, 2 and 3, following the sequential application of BAP → GA_3 (Fig. 7.4a-c), the objective of the current experiment was to measure and thereby compare the uptake, availability and metabolism of [8-^{14}C] BAP as influenced by:

1. The three phases of growth i.e., phases 1, 2 and 3,
2. The simultaneous application of unlabelled BAP and,
3. The sequential application of unlabelled GA_3.

8.2 Materials and methods

8.2.1 Plant selection and culture

Seedlings of *Zantedeschia* cv. 'Best Gold' used in the current experiment comprised a single-shoot during their first growth cycle (single-shoot system). Plants for experimentation within phases 1, 2 and 3 were selected based on the criteria described earlier (refer Chapter 7). Growth and culture conditions were also as described in Chapter 7, except that in order to avoid extraneous diffusion of radioactivity, the plants were isolated by using individual plastic trays, with irrigation provided as a drench (50-60 ml per plant per day) to the growing medium until sampled.

For identification of key metabolites within the metabolic profile of [8-^{14}C] BAP using mass spectrometry (MS) (described later in Section 8.2.7), an additional population of plants was maintained in phase 1 as described in Chapter 7.

8.2.2 Treatment application

[8-^{14}C] BAP (Specific activity: 45-60 mCi/mmol; Moravek Biochemicals Inc., California, USA) was dissolved in 95% ethanol and made up to the required volume with distilled water. To enhance the absorption of [8-^{14}C] BAP, Tween 20 (Sigma CAS no. 9005-64-5; 0.2%) was added to the treatment solution (Zhu and Matsumoto, 1987). Approximately 6,226,000 disintegrations per minute (DPM) of [8-^{14}C] BAP per leaf was applied using a paint brush, on the abaxial and adaxial surfaces of the distal-half of

the youngest (leaf 1) and the penultimate youngest (leaf 2) leaves of plants in all three phases i.e., phases 1, 2 and 3 (Fig. 8.1). To measure and compare the uptake, availability and metabolism of [8-^{14}C] BAP in the three phases as influenced by unlabelled BAP, the highest concentration of BAP employed in the main experiment (8.9 mM) was prepared as described previously (refer Chapter 7), and sprayed on the distal-half of leaves 1 and 2 after the application of [8-^{14}C] BAP was visibly dry (5-6 hours). Likewise, to measure and compare the uptake, availability and metabolism of [8-^{14}C] BAP along the three phases as influenced by unlabelled GA_3, the highest concentration of GA_3 employed in the main experiment (1.4 mM) was prepared as described previously (refer Chapter 7), and sprayed on the distal-half of leaves 1 and 2, 10 days after the application of [8-^{14}C] BAP. In summary, the treatment applications in the current experiment comprised:

- [8-^{14}C] BAP + simultaneous application of unlabelled BAP (8.9 mM)
- [8-^{14}C] BAP + unlabelled GA_3 (1.4 mM) applied sequentially 10 days after the application of [8-^{14}C] BAP

To retain [8-^{14}C] BAP, unlabelled BAP and GA_3 at the area of application, and to prevent diffusion of [8-^{14}C] BAP to the remaining plant parts, the treated leaves were held at a horizontal position by fastening them to a tripod stand, until the treatment solutions were visibly dry.

For the identification of key metabolites within the metabolic profile of [8-^{14}C] BAP using mass spectrometry (MS) (described later in Section 8.2.7), unlabelled BAP at 8.9 mM was prepared and applied as a foliar spray (Chapter 7) on the additional population of plants maintained exclusively for this purpose in phase 1 (refer Section 8.2.1).

8.2.3 Plant parts

Radioactivity was measured in 14 different plant parts (Fig. 8.1), representing the conduit of transport from the area of application to all other major parts of the plant, 10 days after the application of [8-^{14}C] BAP + unlabelled BAP (8.9 mM), and 10 days after the application of unlabelled GA_3. These comprised the distal-halves of leaf lamina (area of application), proximal-halves of leaf lamina, petioles, and the corresponding axillary buds of the two treated leaves (1 and 2), other axillary buds (i.e., those in the axils of the remaining leaves), apical bud, roots and tuber. Magnetic resonance imaging

(MRI) of the *Zantedeschia* tuber has revealed a random network of strands of small parenchyma cells comprising xylem tissues in the region of the tuber below the apical bud, large intracellular spaces in the middle region, and large parenchyma cells in the lower region of the tuber (Robinson et al., 2000). Due to this inherent variation in the cellular structure of the tuber, and since the buds i.e., responding tissue/organ, reside on the upper region of the tuber in *Zantedeschia* (Fig. 1.3a), measurement of radioactivity in the tuber was further classified as that in the upper, middle and lower regions of the tuber (Fig. 8.1).

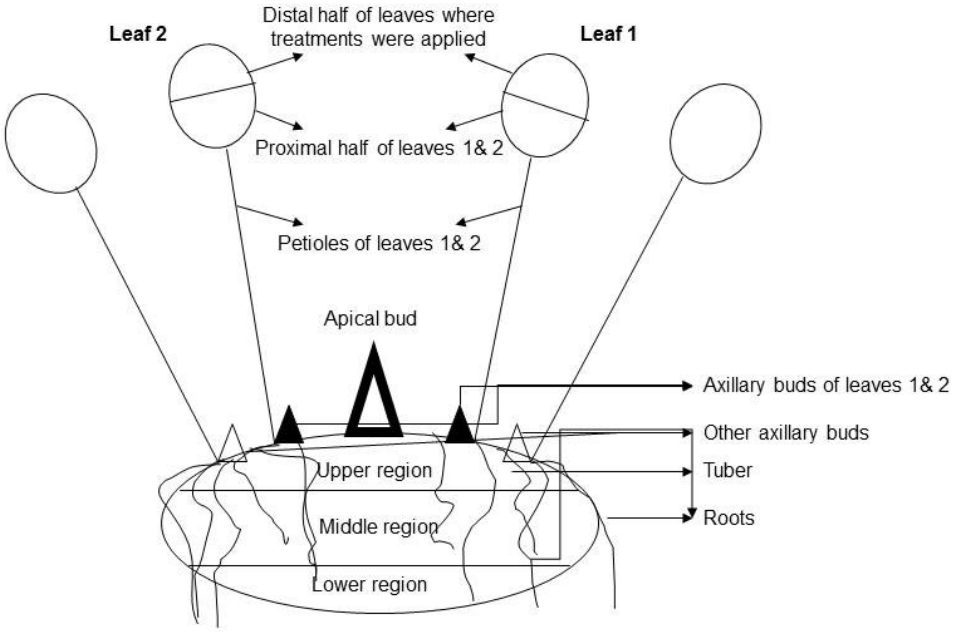


Fig. 8.1 Schematic representation of a single-shoot seedling of *Zantedeschia* cv. 'Best Gold' depicting the 14 different plant parts harvested to study the uptake, availability and metabolism of [8-^{14}C] BAP

8.2.4 Cytokinin extraction and purification

The respective plant parts (Fig. 8.1) were excised and their fresh weight (FW) was measured. For plant parts exceeding 1 g FW, only 1 g of excised tissue was used. Plant parts weighing less than 1 g FW were used *in toto*. CK extraction was performed according to the method of Hoyerova et al. (2006). Briefly, the plant parts were homogenized to a powder using liquid nitrogen, and 10 ml of modified Bieleski's solvent (MeOH-HCOOH-H_2O; 15/1/4, v/v/v) at 4°C, was immediately added. After

overnight extraction at -20°C, the samples were centrifuged, re-extracted with 5 ml of the same solvent, vortexed for 30 seconds, and allowed to stand for 1 hour at -20°C. The pooled extracts were passed through a 28 mm syringe filter containing a 0.2 µm glass fibre/cellulose acetate membrane (Phenomenex, California, USA), and a Sep-Pak Plus short C_{18} cartridge (Waters Corp., Massachusetts, USA) attached in tandem and the final extract was stored at -20°C, until further analysis.

Following the foliar application of unlabelled BAP (8.9 mM) for the identification of key metabolites within the metabolic profile of [8-^{14}C] BAP using mass spectrometry (MS) (described later in Section 8.2.7), CKs were extracted from 5g FW of leaves as described above.

8.2.5 Chromatographic separation

The purified extracts of different plant parts were evaporated to dryness under vacuum at 40°C, in a miVAC Quattro concentrator (Genevac Ltd., UK). Separation of metabolic products was accomplished by a reverse-phase HPLC system, with a Spheri-5, 220 × 4.6 mm octadecyl silica (ODS) column and 3 × 4.6 mm ODS guard cartridge (PerkinElmer, USA), attached in tandem. The solvents, HPLC grade acetonitrile and 40 mM acetic acid (adjusted to pH=3.38 with triethylamine), were filtered through 0.2 µm Teflon and cellulose nitrate membrane filters respectively, and degassed by sonicating for 30 minutes. A solvent flow rate of 1 ml per minute was delivered to the column via Waters 510 and 501 pumps (Millipore Corp., USA) for acetonitrile, and 40 mM acetic acid, respectively. A Waters automated gradient controller (Millipore Corp., USA) was used to maintain a linear gradient starting with 6% acetonitrile, and increasing to 20%, 30% and 95% acetonitrile at 5, 10 and 30 minutes, respectively. Between 30 and 40 minutes, the gradient was brought back to the starting conditions.

The retention times of a mixture of authentic standards of CK (refer Section 8.2.7.1 below) was determined using a programmable Waters UV detector (Millipore Corp., USA), at 268 nm. Subsequently, the dried extract of each plant part was dissolved in 6% acetonitrile (200 µl) and injected through a Rheodyne (IDEX Corp., USA) injector port with a loop capacity of 1 ml. Fractions were collected using an ISCO model 1200 pup (ISCO Inc., USA) fraction collector, at 0.5 minute intervals from 0-30 minutes. The same procedure as described above, was also followed for the chromatographic separation of CKs extracted from leaves (5g FW) exclusively for the identification of

key metabolites within the metabolic profile of [8-^{14}C] BAP using MS (refer Section 8.2.7).

8.2.6 Radioactivity measurements

Both prior to chromatographic separation, and in the fractions of the corresponding plant parts collected after chromatographic separation, radioactivity in the different plant parts was determined using a 2 ml polypropylene microcentrifuge tube (LabServ, New Zealand) containing 0.1 ml of the purified extract (prior to drying) and 0.5 ml of the collected fraction, respectively. The remaining volume was made up with a scintillant (Ultima Gold; PerkinElmer, USA), and the mixture vortexed for 10 seconds.

After background and quenching corrections, radioactivity was measured in a Tri-Carb 2900 TR scintillation counter (PerkinElmer, USA), and was finally expressed as DPM per gram FW.

8.2.7 Identification of key metabolites

The radioactive peaks of interest within the metabolic profile of [8-^{14}C] BAP were identified based on a comparison with the retention times of a mixture of authentic standards of CK and the Multiple Reaction Monitoring (MRM) method of MS.

8.2.7.1 Authentic standards

Authentic standards of adenosine monophosphate (AMP), adenine (Ade), adenosine (Ado), *para*-topolin riboside (pTR), *ortho*-topolin (oT), *ortho*-topolin riboside (oTR), *meta*-topolin (mT), *meta*-topolin riboside (mTR), BAP and 6-benzylaminopurine riboside (BAR) (OlChemim Ltd., Czech Republic) were each dissolved in a mixture of 3% (v/v) acetonitrile and 3% (v/v) methanol and the final volume made up with nanopure water. The retention time of each standard (1000 ng per injection) was then determined using the HPLC system as described above (refer Section 8.2.5). Subsequently, a mixture comprising 1 ml each of the above standard solutions was prepared, and the retention times of the standards in mixture was determined using the same HPLC system (refer Section 8.2.5).

8.2.7.2 Mass spectrometry

Following CK extraction from the leaves treated with unlabelled BAP (8.9 mM) (refer Section 8.2.4), fractions corresponding to the retention times of the radioactive metabolic peaks were collected after chromatographic separation (refer Section 8.2.5). These fractions were purified and vacuum evaporated as described earlier (refer Section 8.2.5). Subsequently, these fractions were sent to Dr. Colin Turnbull, Imperial College, London (UK) for LC-MS analyses.

The LC-MS/MRM analyses were performed as described by Foo et al. (2007). Essentially, the LC-MS system comprised a gradient of acetonitrile in 10 mM ammonium acetate (pH 3.4), starting with 5% for 4 min, rising to 14% at 20 min and 32% at 25 min, using a flow rate of 200 µL min^{-1}. The solvent was passed through a Phenomenex 3-µm C_{18} Luna 100- × 2-mm column on an Agilent 1100 Binary LC system, coupled to an Applied Biosystems Q-Trap hybrid mass spectrometer fitted with a TurboIonspray (electrospray) source operating in positive ion multiple reaction monitoring mode. The dwell time for each MS-MS ion pair was 30 min.

The retention times of a mixture of authentic ARCK standards comprising mT, oT, mTR, [$^{2}H_{7}$] BAP, BAP, 6-benzylaminopurine 9-glucoside (BA9G), methoxy oT (oMeoT), oTR, [$^{2}H_{7}$] BAR, BAR and methoxy oTR (oMeoTR) was determined using the LC-MS system described above. Subsequently, identification of the compounds in the fractions corresponding to the radioactive metabolic peaks of [8-^{14}C] BAP was performed by measuring the peak area of these compounds using two runs (equivalent to 1g FW of leaves each) in the LC-MS/MRM method described above.

8.2.8 Experimental design and statistical analysis

To determine the uptake of [8-^{14}C] BAP and availability of radioactivity as influenced by unlabelled BAP (8.9 mM) or GA_3 (1.4 mM) within each phase i.e., phases 1, 2 and 3, four single-plant replicates were allocated to each treatment application (refer Section 8.2.2) in a completely randomized design (3 phases × 2 treatment applications × 4 single-plant replicates per treatment = 24 plants). Radioactivity in each plant part of all four replicates (refer Section 8.2.6) prior to chromatographic separation, was determined from three sub-replicates of 0.1 ml each. The metabolic profile of [8-^{14}C] BAP in each of the 14 different plant parts following the application of BAP or GA_3 in

phases 1, 2 and 3 was determined from CK extracts of one plant replicate after chromatographic separation.

Natural log transformation of raw radioactivity data, analysis of variance, means ± standard errors, and mean separation tests for differences in the uptake and availability of [8-^{14}C] BAP following the application of BAP or GA_3 between the three phases, and between BAP and GA_3 within each phase was determined using PROC GLM of SAS version 9.2 (SAS Institute, Cary, NC, USA) and MS Excel (Microsoft Corp., USA).

8.3 Results

8.3.1 Overview

The plastochron of the plants within phase 1, as observed previously (Chapter 7), was 10 days. As previously diagnosed for the plants within phase 2 (Chapter 7), at least two plastochrons had passed without the production of new leaves. Visual symptoms of withering of existing leaves in phase 3 had just begun at the time of removal of plants from the glasshouse for CK extraction, 10 days after the application of unlabelled GA_3 (1.4 mM) (refer Section 8.2.2). The manual method of irrigation adopted for the current experiment did not impose any visual symptoms of stress in the plants along the three phases of growth.

8.3.2 Uptake of [8-^{14}C] BAP

Radioactivity (DPM) remaining in the distal half of the two leaves, where the treatments were applied (refer Section 8.2.2), was counted after the respective durations of application (refer Section 8.2.3). The difference between the radioactivity (DPM) applied (6,226,000 DPM of [8-^{14}C] BAP) and radioactivity remaining in these parts, was expressed as the percentage uptake of [8-^{14}C] BAP.

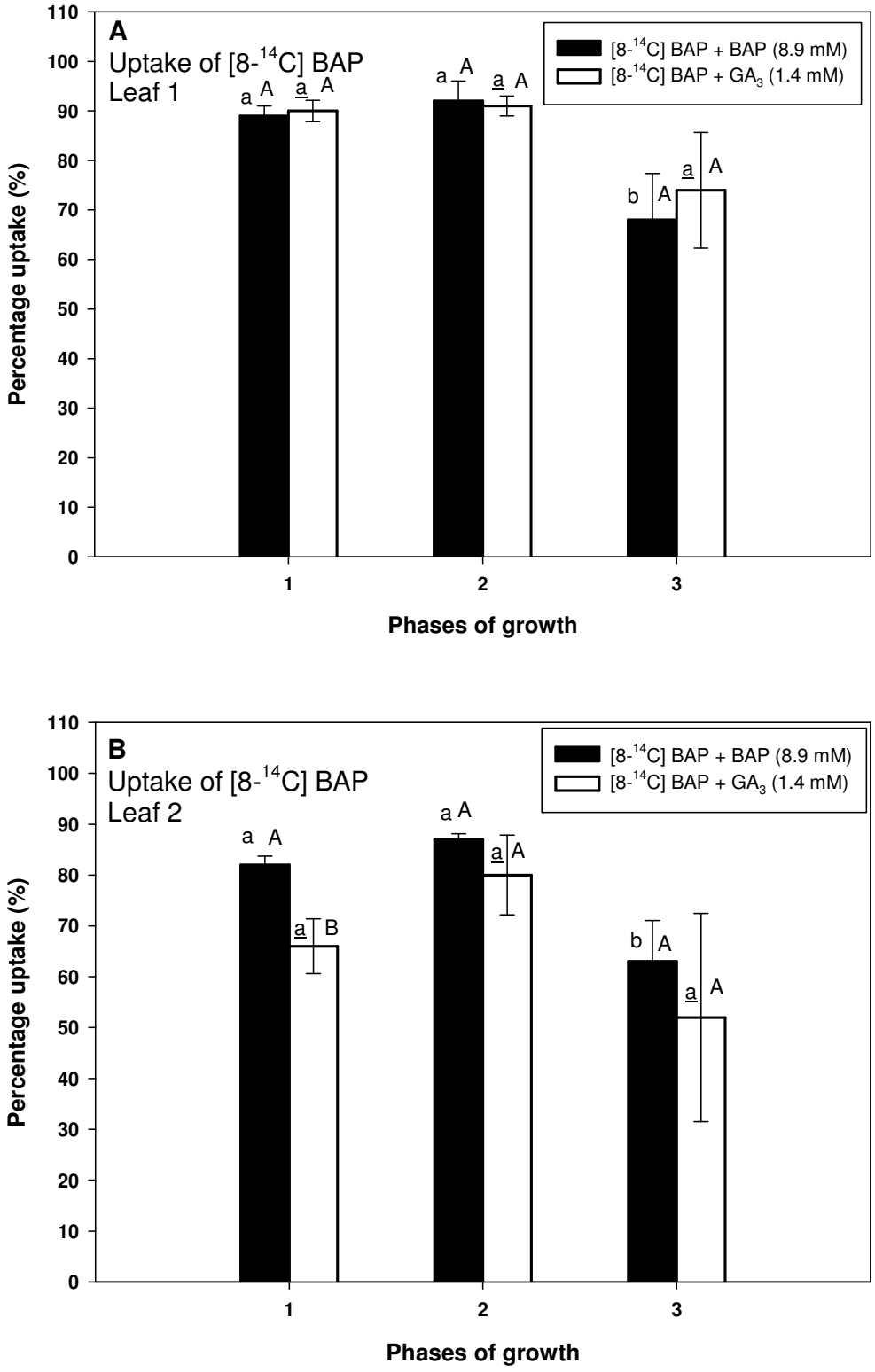


Fig. 8.2 Percentage uptake of [8-^{14}C] BAP (± SE) in single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' measured along the three phases of growth i.e., phases 1, 2 and 3, at 10 days after the simultaneous application of [8-^{14}C] BAP + BAP (8.9 mM) and sequential application of [8-^{14}C] BAP + GA_3 (1.4 mM) from **A** leaf 1 and **B** leaf 2.

For each leaf, different *small regular* (not underlined) *letters* indicate significant differences ($\alpha = 0.05$) in the percentage of uptake of [8-^{14}C] BAP between the three phases of growth following the simultaneous application of BAP.

Different *small underlined letters* indicate significant differences ($\alpha = 0.05$) in the percentage of uptake of [8-^{14}C] BAP between the three phases of growth following the sequential application of GA_3.

Different *capital letters* indicate significant differences in the percentage of uptake of [8-^{14}C] BAP between BAP and GA_3 within each phase of growth.

Mean separation by Least Significant Difference (LSD) ($n = 4$)

8.3.2.1 Uptake of [8-^{14}C] BAP as influenced by the three phases

Following the application of BAP (8.9 mM), a significantly higher uptake of [8-^{14}C] BAP was observed in phases 1 and 2, from both leaves i.e., leaf 1 ($P < 0.04$; Fig. 8.2A) and leaf 2 ($P < 0.02$; Fig. 8.2B), compared to that in phase 3. A significant difference was however not noticed between phases 1 and 2 ($P > 0.1$). The uptake of [8-^{14}C] BAP declined by at least 20% in phase 3 to 68.0 ± 9.3% and 63.0 ± 8.0% for leaves 1 and 2 respectively, compared to that in phases 1 and 2 (Fig. 8.2A; B).

In contrast, following the application of GA_3 (1.4 mM), a significant difference in the uptake of [8-^{14}C] BAP was not observed between the three phases of growth ($P > 0.1$; Fig. 8.2A; B). For leaf 1 (Fig. 8.2A), while 90.0 ± 2.2% and 91.0 ± 2.0% uptake was observed in phases 1 and 2 respectively, 74.0 ± 11.7% uptake was observed in phase 3.

8.3.2.2 Uptake of [8-^{14}C] BAP as influenced by the treatment applications within each phase

Within each of the three phases of growth, a significant difference in the uptake of [8-^{14}C] BAP was not observed ($P > 0.1$) between the application of BAP (8.9 mM) and GA_3 (1.4 mM) for leaf 1 (Fig. 8.2A).

For leaf 2 (Fig. 8.2B), while there was no significant difference in the uptake between BAP and GA_3 in phases 2 and 3, in phase 1, the uptake was significantly higher after the application of BAP ($P < 0.03$; 82.0 ± 1.7%) compared to that after GA_3 (66.0 ± 5.4%).

8.3.3 Availability of radioactivity

The availability of [8-^{14}C] BAP and/or its metabolites as influenced by the three phases of growth and different treatment applications (refer Section 8.2.1) was measured by the availability of radioactivity in 14 different plant parts (refer Section 8.2.3). Availability of radioactivity was denoted by the amount of radioactivity present in each plant part at the respective times of observation (refer Section 8.2.3). Radioactivity (DPM) in each plant part was finally expressed per gram of fresh weight of the corresponding parts.

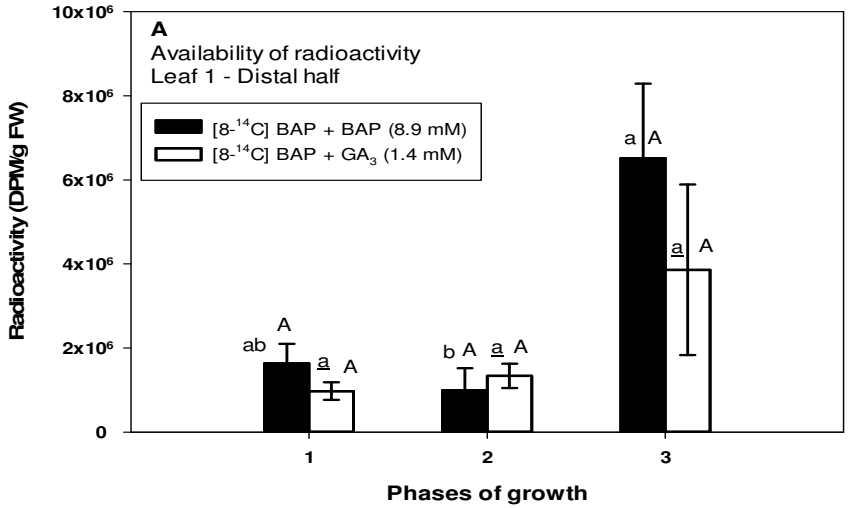
8.3.3.1 Availability of radioactivity as influenced by the three phases

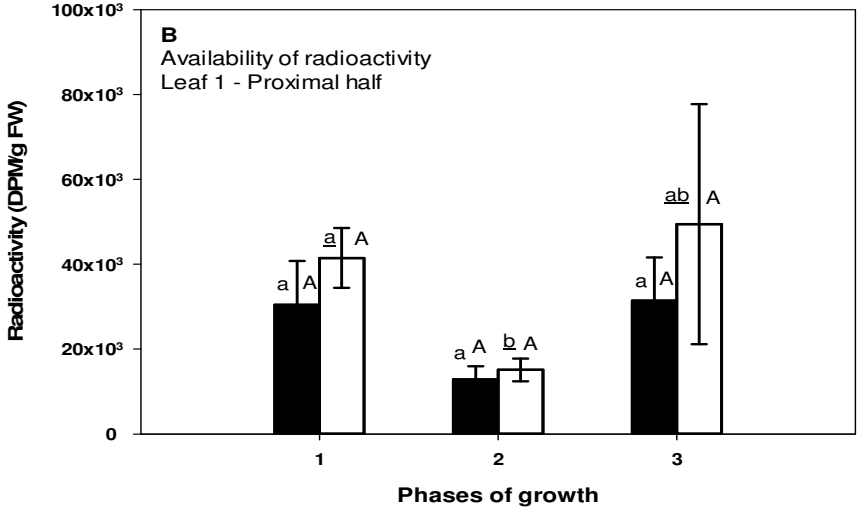
Concomitant with the decline in the uptake of [8-^{14}C] BAP observed in phase 3 following the application of BAP (8.9 mM) in both the leaves (Fig. 8.2A; B), a 2-4 fold higher amount of radioactivity was retained in the distal half of both the leaves (Fig. 8.3A; E) in phase 3, compared to that in phases 1 and 2 (Table 8.3). For both the leaves i.e., leaf 1 ($P < 0.03$; Fig. 8.3A) and leaf 2 ($P < 0.004$; Fig. 8.3E), the difference was significant only between phases 2 and 3. Again, similar to the uptake (Fig. 8.2A; B), a significant difference in radioactivity was not observed between the three phases of growth ($P > 0.1$) in either leaf (Fig. 8.3A; E), following the application of GA_3 (1.4 mM).

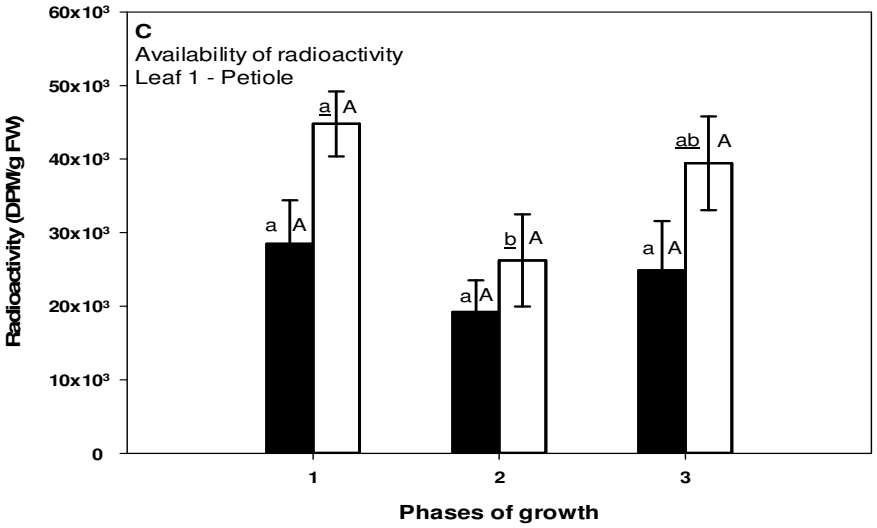
There was no difference in radioactivity ($P > 0.1$) in the proximal half of leaves 1 and 2 across the three phases after application of BAP and GA_3, though a marginal decline ($P = 0.08$) in radioactivity was alone observed in the proximal half of leaf 1 (Fig. 8.3B) between phases 1 and 2 following the application of GA_3. In plants treated with BAP, there was no significant difference in the available radioactivity ($P > 0.1$) in the petioles of leaf 1 (Fig. 8.3C) and leaf 2 (Fig. 8.3G) between the three phases. However, when treated with GA_3, a marginal increase in radioactivity was present in the petiole of leaf 1 ($P = 0.08$; Fig. 8.3C) in phase 1 compared to that in phase 2. In the petiole of leaf 2 also (Fig. 8.3G), following the application of GA_3, a significantly higher amount of radioactivity ($P < 0.02$) was noticed in phases 1 and 3, compared to those in phase 2.

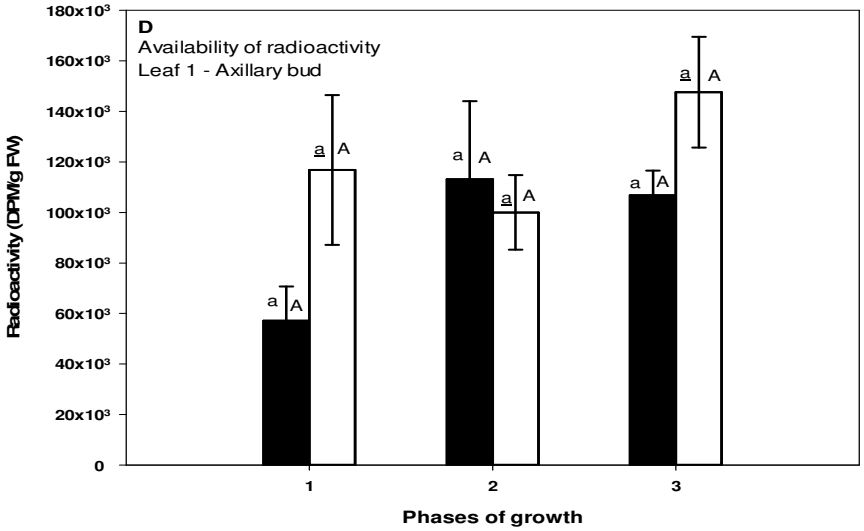
Though not significant ($P > 0.1$), at least 50-80% higher radioactivity was present in the corresponding axillary buds of leaf 1 (Fig. 8.3D) and 2 (Fig. 8.3H) in phase 3 than in phase 1, following the application of BAP (Table 8.3). Likewise, 20-65% higher

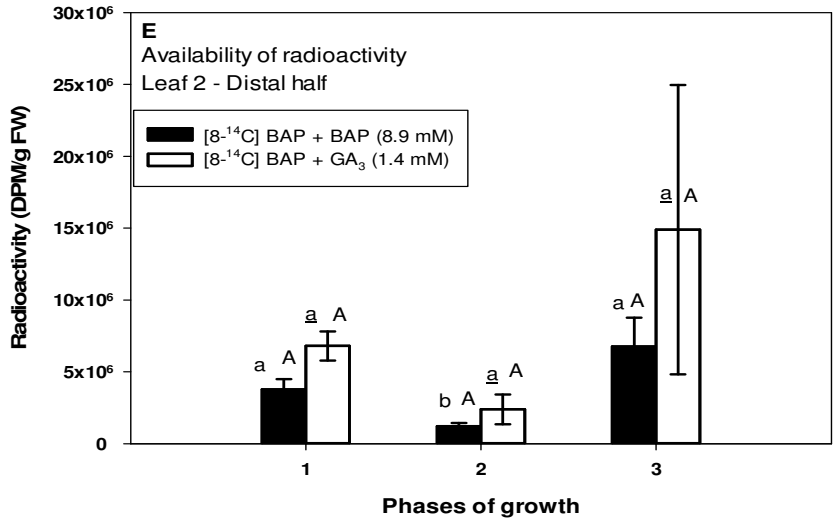
radioactivity was also found in these buds in phase 3 ($P > 0.1$; Table 8.3), following the application of GA_3, compared to that in phase 1 (Fig. 8.3D; H).

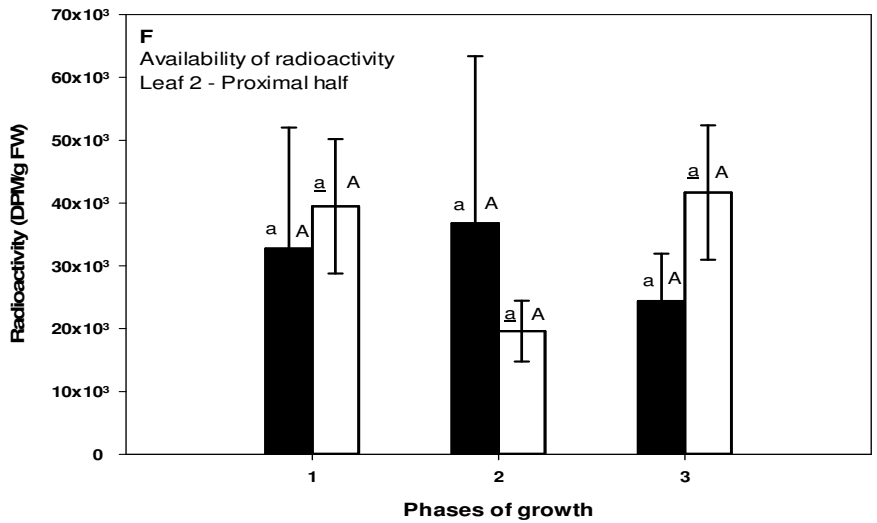

A
Availability of radioactivity
Leaf 1 - Distal half
[8-14C] BAP + BAP (8.9 mM)
[8-14C] BAP + GA3 (1.4 mM)
Radioactivity (DPM/g FW)
10x10⁶
8x10⁶
6x10⁶
4x10⁶
2x10⁶
0
ab A
a A
b A
a A
a A
a A
1
2
3
Phases of growth

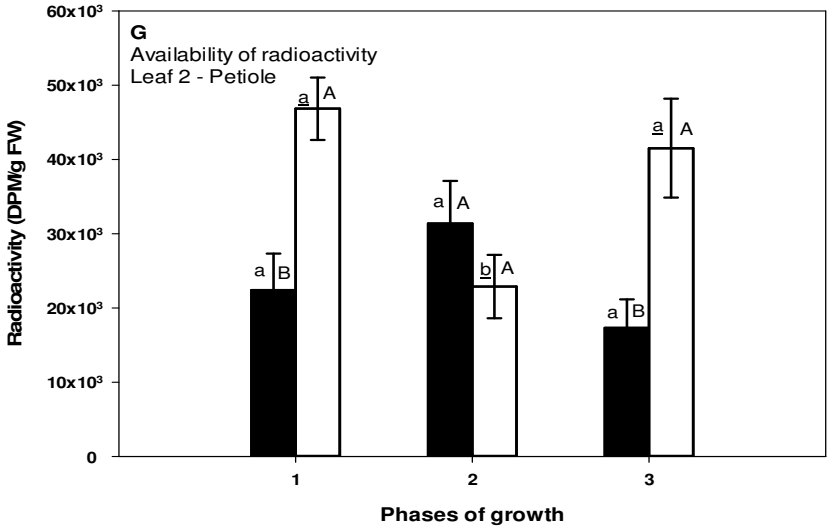

B
Availability of radioactivity
Leaf 1 - Proximal half
Radioactivity (DPM/g FW)
100x10³
80x10³
60x10³
40x10³
20x10³
0
a A
a A
a A
b A
a A
ab A
1
2
3
Phases of growth

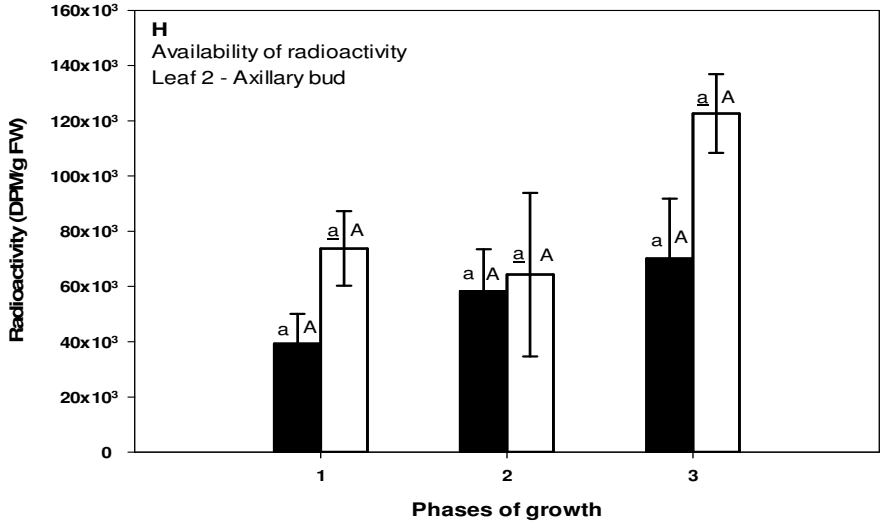

C
Availability of radioactivity
Leaf 1 - Petiole
Radioactivity (DPM/g FW)
60x10³
50x10³
40x10³
30x10³
20x10³
10x10³
0
a A
a A
a A
b A
a A
ab A
1
2
3
Phases of growth

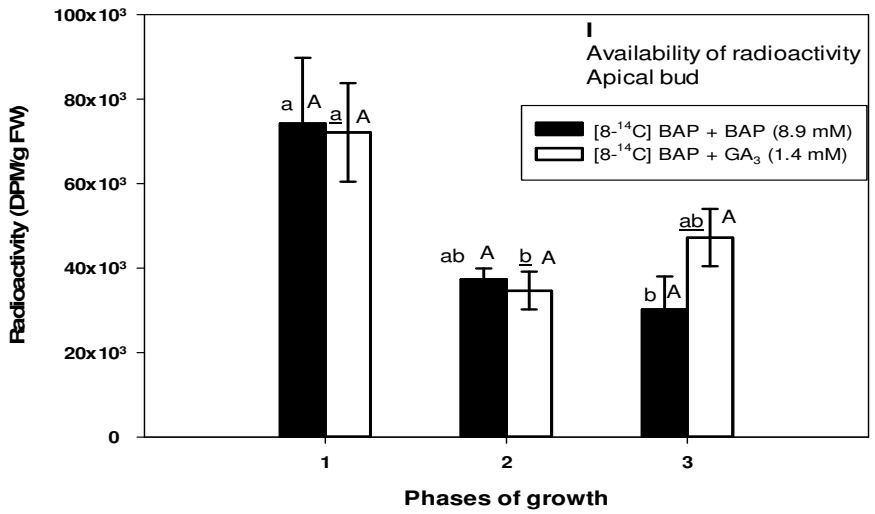

D
Availability of radioactivity
Leaf 1 - Axillary bud
Radioactivity (DPM/g FW)
180x10³
160x10³
140x10³
120x10³
100x10³
80x10³
60x10³
40x10³
20x10³
0
a A
a A
a A
a A
a A
a A
1
2
3
Phases of growth

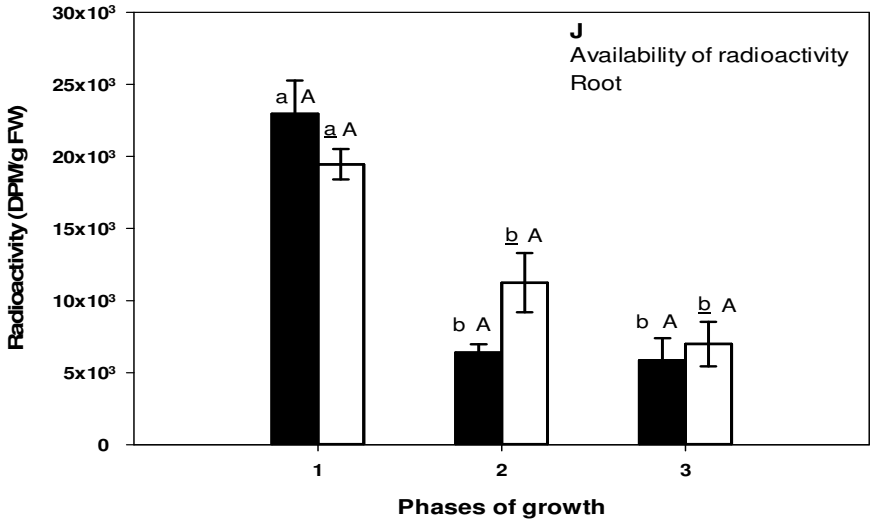

E
Availability of radioactivity
Leaf 2 - Distal half
[8-14C] BAP + BAP (8.9 mM)
[8-14C] BAP + GA3 (1.4 mM)
Radioactivity (DPM/g FW)
Phases of growth

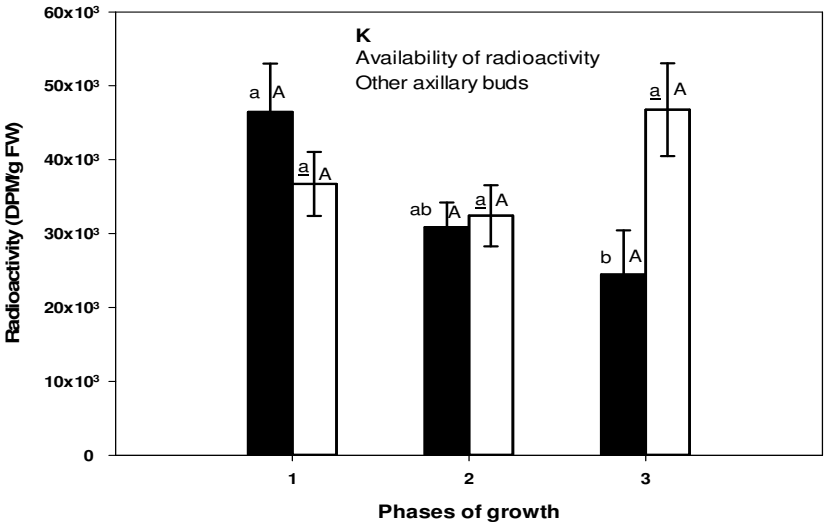

F
Availability of radioactivity
Leaf 2 - Proximal half
Radioactivity (DPM/g FW)
Phases of growth

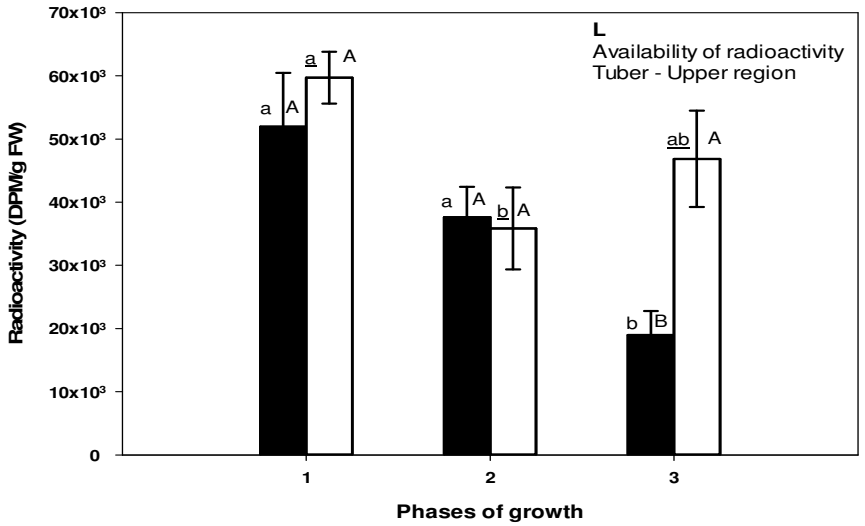

G
Availability of radioactivity
Leaf 2 - Petiole
Radioactivity (DPM/g FW)
Phases of growth


H
Availability of radioactivity
Leaf 2 - Axillary bud
Radioactivity (DPM/g FW)
Phases of growth


I
Availability of radioactivity
Apical bud
[8-14C] BAP + BAP (8.9 mM)
[8-14C] BAP + GA3 (1.4 mM)
Radioactivity (DPM/g FW)
100x10³
80x10³
60x10³
40x10³
20x10³
0
a A a A
ab A b A
b A ab A
1
2
3
Phases of growth


J
Availability of radioactivity
Root
Radioactivity (DPM/g FW)
30x10³
25x10³
20x10³
15x10³
10x10³
5x10³
0
a A a A
b A b A
b A b A
1
2
3
Phases of growth


K
Availability of radioactivity
Other axillary buds
Radioactivity (DPM/g FW)
60x10³
50x10³
40x10³
30x10³
20x10³
10x10³
0
a A a A
ab A a A
b A a A
1
2
3
Phases of growth


L
Availability of radioactivity
Tuber - Upper region
Radioactivity (DPM/g FW)
70x10³
60x10³
50x10³
40x10³
30x10³
20x10³
10x10³
0
a A a A
a A b A
b B ab A
1
2
3
Phases of growth

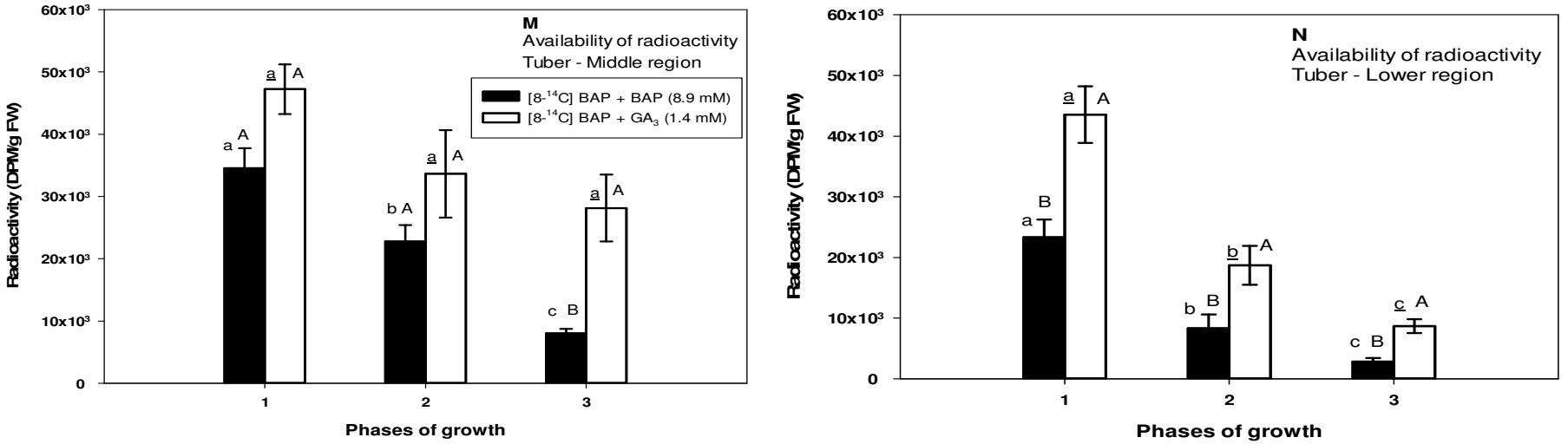


Fig. 8.3 Availability of radioactivity (DPM/ g FW ± SE) in 14 different plant parts of single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' measured along the three phases of growth i.e., phases 1, 2 and 3, at 10 days after the simultaneous application of [8-^{14}C] BAP + BAP (8.9 mM) and the sequential application [8-^{14}C] BAP + GA_3 (1.4 mM) in **A-D** the distal-half, proximal-half, petiole and corresponding axillary bud of leaf 1, **E-H** the distal-half, proximal-half, petiole and corresponding axillary bud of leaf 2, **I** the apical bud, **J** roots, **K** other axillary buds and **L-N** the upper, middle and lower regions of the tuber, respectively. Analysis of variance (ANOVA) was conducted on log transformed data.

For each plant part, different *small regular* (not underlined) *letters* indicate significant differences ($\alpha = 0.05$) in the availability of radioactivity (log transformed) between the three phases of growth following the simultaneous application of BAP.

Different *small underlined letters* indicate significant differences ($\alpha = 0.05$) in the availability of radioactivity (log transformed) between the three phases of growth following the sequential application of GA_3.

Different *capital letters* indicate significant differences ($\alpha = 0.05$) in the availability of radioactivity (log transformed) between BAP and GA_3 within each phase of growth.

Mean separation by Least Significant Difference (LSD) ($n = 4$)

In the apical bud however, located in close proximity to the corresponding axillary buds of leaves 1 and 2 (Fig. 8.1), a significantly higher amount of radioactivity ($P < 0.03$) was found in phase 1 following the application of BAP, than that in phase 3 (Fig. 8.3I). This increase was at least 50% higher in phase 1 than in phases 2 and 3 (Table 8.3). A significantly higher amount of radioactivity was also found in the apical bud in phase 1 ($P < 0.02$), following the application of GA_3 (1.4 mM), compared to that in phase 2 (Fig. 8.3I). However, between phases 2 and 3, 25% higher radioactivity was found in phase 3 than in phase 2 (Table 8.3).

The application of both BAP ($P < 0.0006$) and GA_3 ($P < 0.006$) resulted in a significant increase in the availability of radioactivity in the roots in phase 1, compared to that in phases 2 and 3 (Fig. 8.3J). This increase was at least 2-4 folds higher in phase 1 than in phases 2 and 3 (Table 8.3).

Significantly higher radioactivity was also found in the other axillary buds i.e., axillary buds not in the axils of leaves 1 and 2 (Fig. 8.1), in phase 1 ($P < 0.08$) following the application of BAP (8.9 mM) (Fig. 8.3K). This radioactivity declined by 34% between phases 1 and 2 and by 20% between phases 2 and 3, respectively (Table 8.3). Though not significant ($P > 0.1$; Fig. 8.3K), following the application of GA_3, at least 20% higher radioactivity was found in phase 3 than the buds in phases 1 and 2 (Table 8.3).

Irrespective of the application of BAP or GA_3 and the three phases of growth, a comparison of radioactivity present in different regions of the tuber i.e., upper, middle and lower regions (Fig. 8.1), revealed that the upper region of the tuber, where the buds reside, possessed the highest degree of radioactivity (Table 8.3). This amount of radioactivity declined concomitantly within the middle and lower regions of the tuber (Table 8.3). A similar trend as observed with the other axillary buds (Fig. 8.3K) following the application of BAP was also observed in the upper region of the tuber (Fig. 8.3L). Significantly higher radioactivity ($P < 0.008$) was present in phase 1 (Fig. 8.3L), with the available radioactivity declining gradually with the progression of phases 2 and 3 (Fig. 8.3L). A significant decline in radioactivity was observed between phases 1 and 2 ($P < 0.07$) following the application of GA_3 (1.4 mM). However, between phases 2 and 3, though not significant ($P > 0.1$; Fig. 8.3L), the amount of radioactivity increased by at least 20% in phase 3, and was similar to that present in phase 1 (Table 8.3). In the middle ($P < 0.0001$; Fig. 8.3M) and lower ($P < 0.0002$; Fig.

8.3N) regions of the tuber, as observed with the upper region following the application of BAP, a significant decline in the amount of radioactivity was observed as the buds progressed from phase 1 through to phase 3. Though a similar trend was also noticed in the lower region of the tuber (Fig. 8.3N) following the application of GA_3, where a significant decline in the amount of radioactivity was found ($P < 0.0001$) as the buds progressed from phase 1 through to phase 3, there was no significant difference ($P > 0.1$; Fig. 8.3M) in the amount of radioactivity with the natural progression of the growth cycle in the middle region of the tuber.

Overall, following the simultaneous application of [8-^{14}C] BAP + BAP, the distal halves of leaves 1 (Fig. 8.3A) and 2 (Fig. 8.3E), where the treatments were applied, retained higher radioactivity in phase 3, compared to that in phases 1 and 2. The corresponding axillary buds i.e., in the axils of leaves 1 (Fig. 8.3D) and 2 (Fig. 8.3H) also possessed higher radioactivity in phase 3, compared to those in phase 1. In contrast all the other subterranean parts of the plant (Fig. 8.3I-N), possessed higher radioactivity in phase 1 compared to that in phase 3. Amongst these parts examined in phase 1 (Table 8.3), the apical bud had the highest radioactivity (74283 ± 15500 DPM/g FW), followed by the upper region of the tuber (51972 ± 8485 DPM/g FW) and the other axillary buds (46460 ± 6533 DPM/g FW). The lowest radioactivity of all the subterranean plant parts in phase 1 was present in the roots (22947 ± 2305 DPM/g FW).

8.3.3.2 Availability of radioactivity as influenced by the treatment applications within each phase

Similar to the uptake of [8-^{14}C] BAP observed in leaf 1 (Fig. 8.2A), the amount of radioactivity retained in the distal half of leaf 1 (where the treatments were applied) was not significantly different ($P > 0.1$) between the application of BAP (8.9 mM) and GA_3 (1.4 mM) within each of the three phases of growth (Fig. 8.3A). In the distal half of leaf 2 however, concomitant with the decline in uptake of [8-^{14}C] BAP observed between BAP and GA_3 in all the three phases (Fig. 8.2B), GA_3 retained higher radioactivity in all the three phases compared to BAP (Fig. 8.3E), though the differences were not significant ($P > 0.1$; Fig. 8.3E).

The amount of radioactivity available in the proximal halves of leaf 1 (Fig. 8.3B) and leaf 2 (Fig. 8.3F) was not significantly influenced between the application of BAP and GA_3 within the three phases of growth. Differences in the availability of radioactivity

was also not noticed between the treatment applications ($P > 0.1$) in the petioles of leaf 1 (Fig. 8.3C) across the three phases of growth. However, in phases 1 ($P < 0.02$) and 3 ($P < 0.02$), GA_3 significantly increased the availability of radioactivity in the petioles of leaf 2 (Fig. 8.3G), compared to the application of BAP.

Within each of the three phases, significant differences in the available radioactivity was not noticed ($P > 0.1$) in the corresponding axillary buds of leaf 1 (Fig 8.3D) and leaf 2 (Fig. 8.3H), between the treatment applications comprising BAP and GA_3. The application of GA_3 compared to the application of BAP, did not significantly influence ($P > 0.1$) the radioactivity in the apical bud in phases 1 and 2 (Fig. 8.3I). However in phase 3, though not significant ($P > 0.1$), GA_3 resulted in a 56% increase in the availability of radioactivity in the apical bud, compared to the application of BAP (Table 8.3).

In the roots, a significant difference in the available radioactivity was not noticed between the treatment applications, within each of the three phases ($P > 0.1$; Fig. 8.3J). However, 75% and 20% higher radioactivity was present in phases 2 and 3 respectively, following the application of GA_3 compared to that of BAP (Table 8.3).

The application of GA_3 compared to BAP, resulted in 20% lesser or equal availability of radioactivity in the other axillary buds, within phases 1 and 2 respectively (Fig. 8.3K; Table 8.3). In phase 3 however, though not significant ($P > 0.1$), GA_3 resulted in 90% increase in radioactivity in these buds compared to the application of BAP (Table 8.3).

The same trend was observed in the upper region of the tuber (Fig. 8.3L), where a significant difference in the available radioactivity was not detected between the treatment applications in phases 1 and 2 ($P > 0.1$). However in phase 3, GA_3 resulted in a significant ($P < 0.02$; by at least 145%) increase in available radioactivity, compared to the application of BAP (Fig. 8.3K; Table 8.3). While the same trend as the upper region of the tuber was also observed in the middle region (Fig. 8.3M), in the lower region of the tuber (Fig. 8.3N), the application of GA_3 significantly enhanced the availability of radioactivity within all three phases of growth, compared to the application of BAP ($P < 0.04$).

Overall, within each of the three phases of growth, the availability of radioactivity in most of the aerial components of leaves 1 and 2 i.e., the distal half, proximal half and petioles, and the corresponding axillary buds was not significantly different between the treatment applications comprising BAP (8.9 mM) and GA_3 (1.4 mM). However, in the key subterranean plant parts comprising the apical bud, other axillary buds and the upper region of the tuber, GA_3 increased the availability of radioactivity in phase 3 compared to the application of BAP, whereas in phases 1 and 2, the difference between these treatment applications was not prominent.

8.3.4 Metabolism of [8-^{14}C] BAP

While the distribution/availability of radioactivity in 14 different plant parts (Fig. 8.1) as influenced by the three phases of growth (refer Section 8.3.3.1) and the presence of unlabelled BAP (8.9 mM) or GA_3 (1.4 mM) (refer Section 8.3.3.2) was reported earlier (refer Section 8.3.3 above), in the current section the influence of these factors on the metabolism of [8-^{14}C] BAP in the 14 different plant parts was measured and expressed as radioactivity (DPM) per gram of fresh weight of each part.

8.3.4.1 Identification of the key metabolites of [8-^{14}C] BAP

The retention times of a mixture of authentic standards of CK derivatives (refer Section 8.2.7.1), mainly comprising topolins was identified as follows (Fig. 8.4) through chromatographic separation in a HPLC system (refer Section 8.2.5). In this system, the retention times of the topolin free bases i.e., mT (13.5-14 mins) and oT (16.5-17 minutes) was earlier than that of BAP (19.5-20 mins), and the ribosides of mT, oT and BAP i.e., mTR, oTR and BAR respectively, emerged earlier than their corresponding free bases (Fig. 8.4).

The retention times of a mixture of authentic standards, exclusively prepared for the identification of metabolites using the LC-MS/MRM method (refer Section 8.2.7.2) was identified as follows (Fig. 8.5). In this system also, the retention times of the topolin bases mT (16.6 mins) and oT (21.5 mins) was earlier than that of BAP (22.3 mins). However, in this system, the respective ribosides emerged later than their corresponding bases (Fig. 8.5).

Among the metabolic profiles of [8-^{14}C] BAP constructed from the 14 different plant parts in all the three phases of growth, following the simultaneous application of BAP (8.9 mM) or sequential application of GA_3 (1.4 mM), six prominent radioactive peaks were identified (Fig. 8.6). Based on the retention times of authentic standards in the HPLC system at Massey University, Palmerston North (Fig. 8.4) these peaks, representing the key metabolites of [8-^{14}C] BAP were identified as follows (Table 8.1). The peak areas of the respective metabolites obtained through the LC-MS/MRM method (Table 8.2) provided further confirmation of the existence of the key metabolites in the corresponding fractions.

Table 8.1 Identification of the six key metabolites of [8-^{14}C] BAP obtained following the simultaneous application of BAP (8.9 mM) or sequential application of GA_3 (1.4 mM) on single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' in phases 1, 2 and 3 of the growth cycle, based on comparison with retention times of authentic standards of cytokinins (Fig. 8.4) obtained through the HPLC system at Massey University, Palmerston North

Radioactive peak	Retention time (minutes)	Metabolites
1	2.8-3.2	AMP
2	5.5-6	Ado
3	10-10.5	Unknown
4	11-11.5	Unknown
5	13.5-14	mT
6	15.5-16	BAR

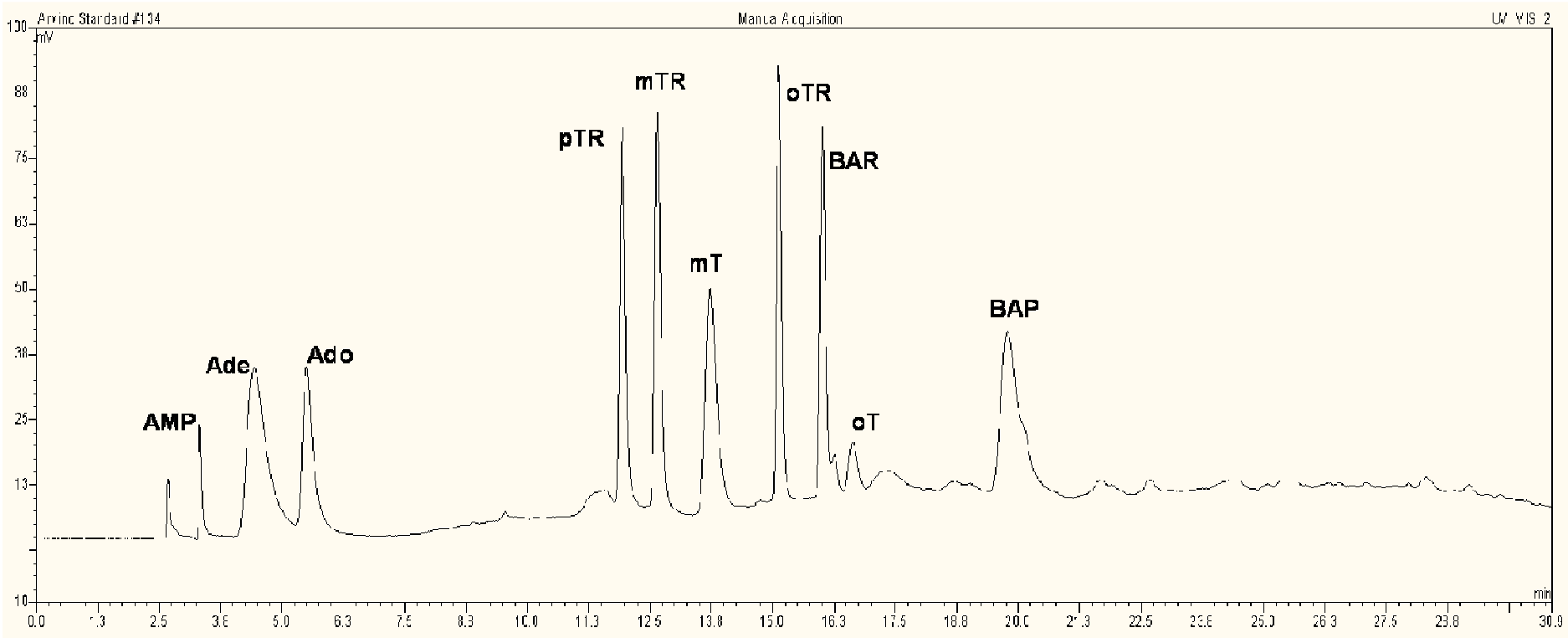


Fig. 8.4 Retention times of a mixture of authentic standards of CK comprising Adenosine monophosphate (AMP), Adenine (Ade), Adenosine (Ado), *para*-Topolin riboside (pTR), *meta*-Topolin riboside (mTR), *meta*-Topolin (mT), *ortho*-Topolin riboside (oTR), 6-benzylaminopurine riboside (BAR), *ortho*-Topolin (oT) and 6-benzylaminopurine (BAP) obtained through the HPLC system at Massey University, Palmerston North

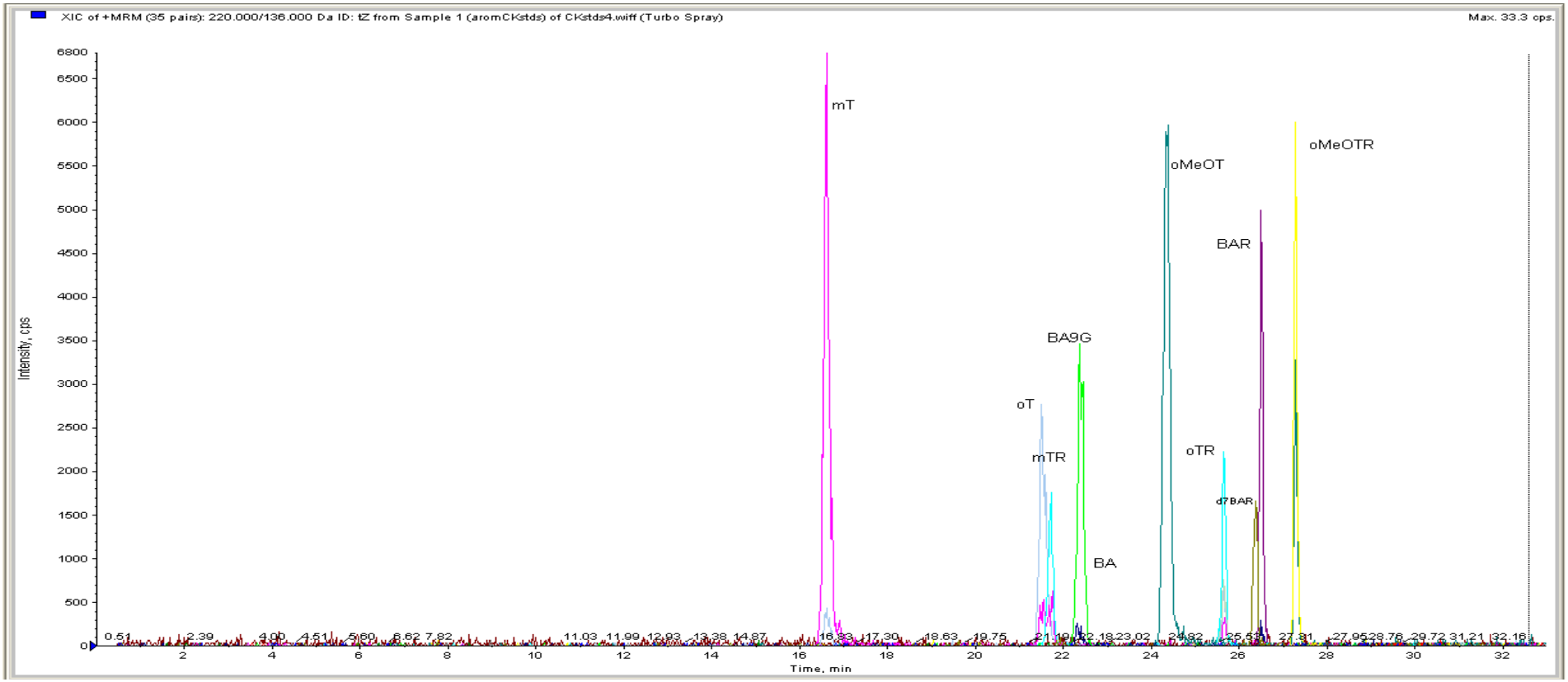


Fig. 8.5 Retention times of a mixture of authentic standards of CK comprising *meta*-Topolin (mT), *ortho*-Topolin (oT), *meta*-Topolin riboside (mTR), 6-benzylaminopurine 9-glucoside (BA9G), 6-benzylaminopurine (BA), Methoxy *ortho*-Topolin (oMeoT), *ortho*-Topolin riboside (oTR), [$^{2}H_{7}$] 6-benzylaminopurine riboside (d7 BAR), 6-benzylaminopurine riboside (BAR) and Methoxy *ortho*-Topolin riboside (oMeoTR) obtained through the HPLC system at Imperial College, London (Courtesy: Dr. Colin Turnbull)

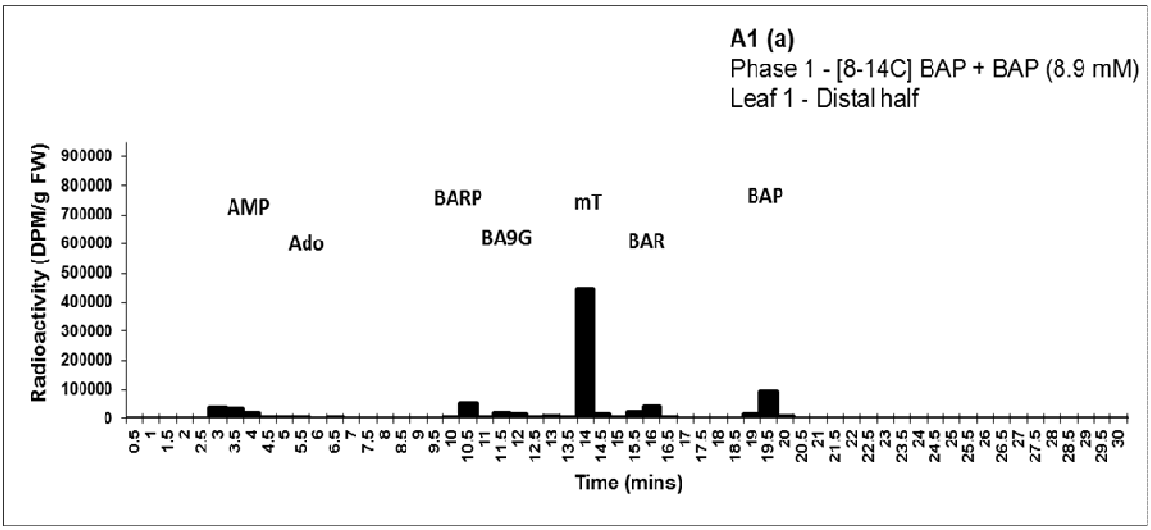
Table 8.2 Identification of metabolites of [8-^{14}C] BAP in the fractions corresponding to the retention times of the six metabolic radioactive peaks (Table 8.1) obtained following the simultaneous application of [8-^{14}C] BAP + BAP (8.9 mM) or the sequential application of [8-^{14}C] BAP + GA_3 (1.4 mM) on single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' in phases 1, 2 and 3 of the growth cycle, based on the peak area of the respective compounds obtained from the LC-MS/MRM system at Imperial College, London (Courtesy: Dr. Colin Turnbull)

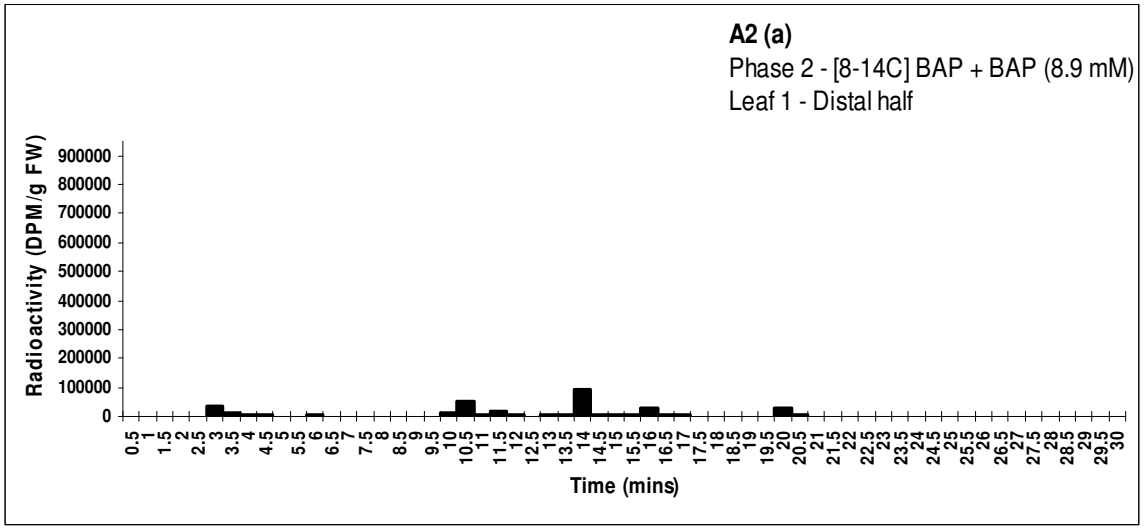
Fraction corresponding to radioactive peak	Peak area*		Metabolite
	1st run	2nd run	
1	3753	3567	AMP
2	180000	3517500	Ado
3	121100	106581	BARP (Tentative)
4	75494	80568	BA9G (Tentative)
5	5746	6046	mT
6	295300	151460	BAR

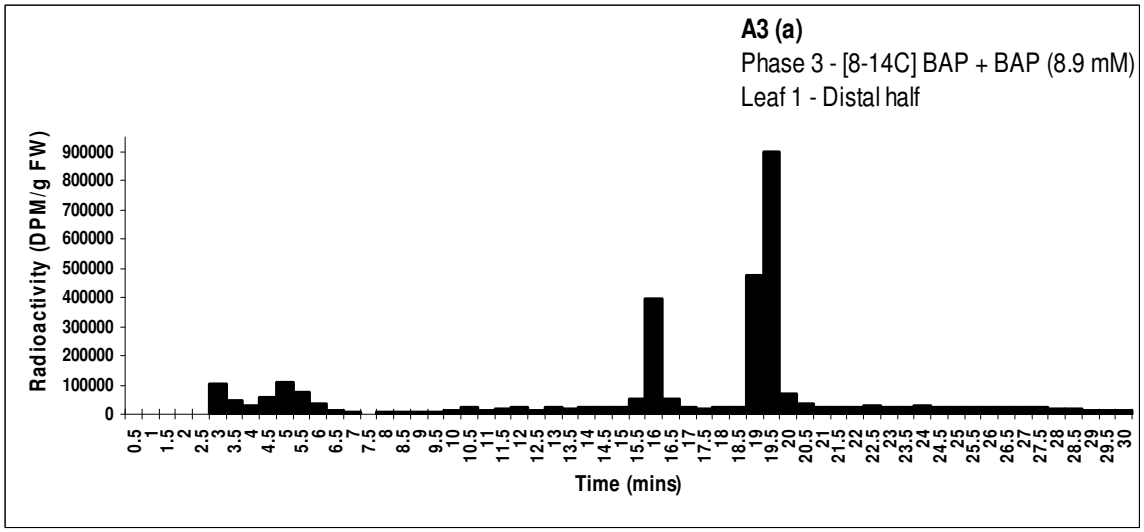
* An area of 200 is considered the absolute minimum ($\alpha = 0.05$)

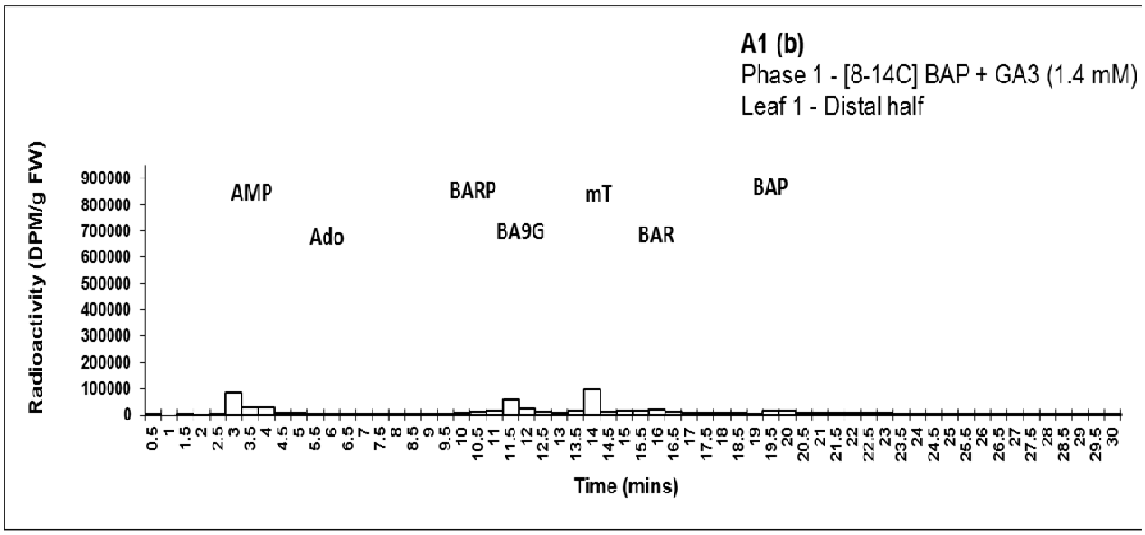
8.3.4.1 Metabolism of [8-^{14}C] BAP as influenced by the three phases

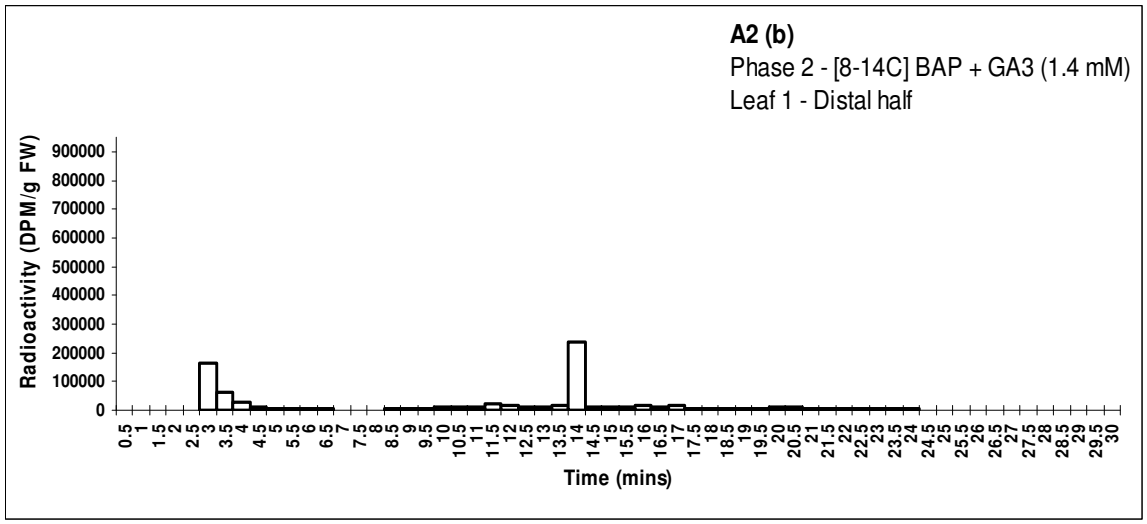
The high amount of radioactivity observed in the distal halves of leaf 1 (Fig. 8.3A) and leaf 2 (Fig. 8.3E) in phase 3 following the application of BAP, was due to unmetabolized [8-^{14}C] BAP (Fig. 8.6A3 (a); 8.6E3 (a)). In the corresponding halves of leaf 1 in phases 1 (Fig. 8.6A1 (a)) and 2 (Fig. 8.6A2 (a)), [8-^{14}C] BAP was scarcely present, whereas mT was the prominent metabolite. In leaf 1, this mT fraction was at least 19 times higher in phase 1 than that in phase 3 (Table 8.3). In the corresponding halves of leaf 2 in phases 1 (Fig. 8.6E1 (a)) and 2 (Fig. 8.6E2 (a)) however, the mT fraction was at least 5 times less than that present in leaf 1 (Fig. 8.6A1 (a); Table 8.3). In contrast to mT, BAR was the primary metabolite identified in these parts of leaves 1 (Fig. 8.6A3 (a)) and 2 (Fig. 8.6E3 (a)) in phase 3. The BAR content of leaf 1 in phase 3 (Fig. 8.6A3 (a)) was at least 10 times higher than that in phase 1 (Fig. 8.6A1 (a); Table 8.3).

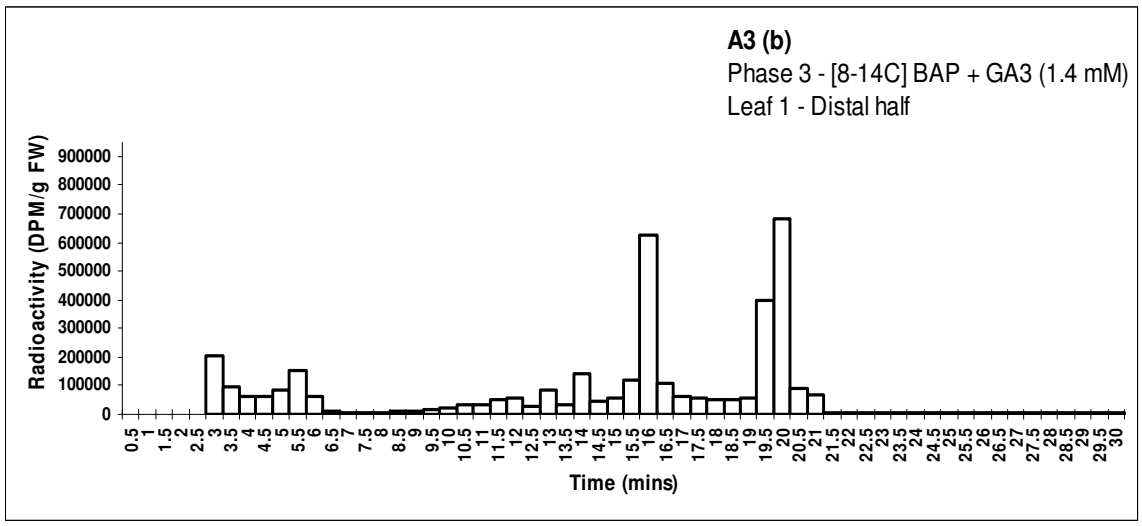

A1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Distal half
Radioactivity (DPM/g FW)
Time (mins)
AMP
Ado
BARP
BA9G
mT
BAR
BAP

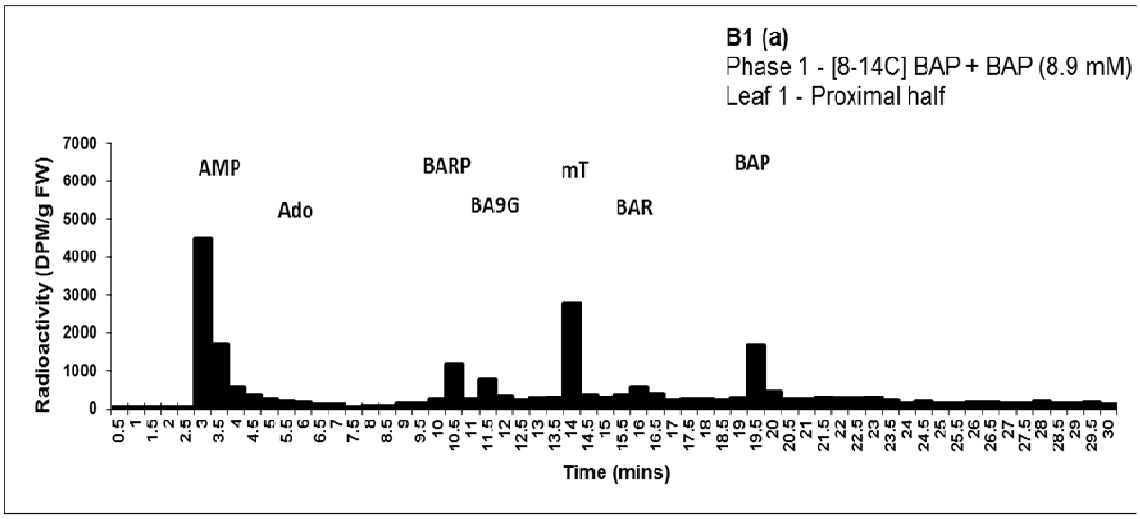

A2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

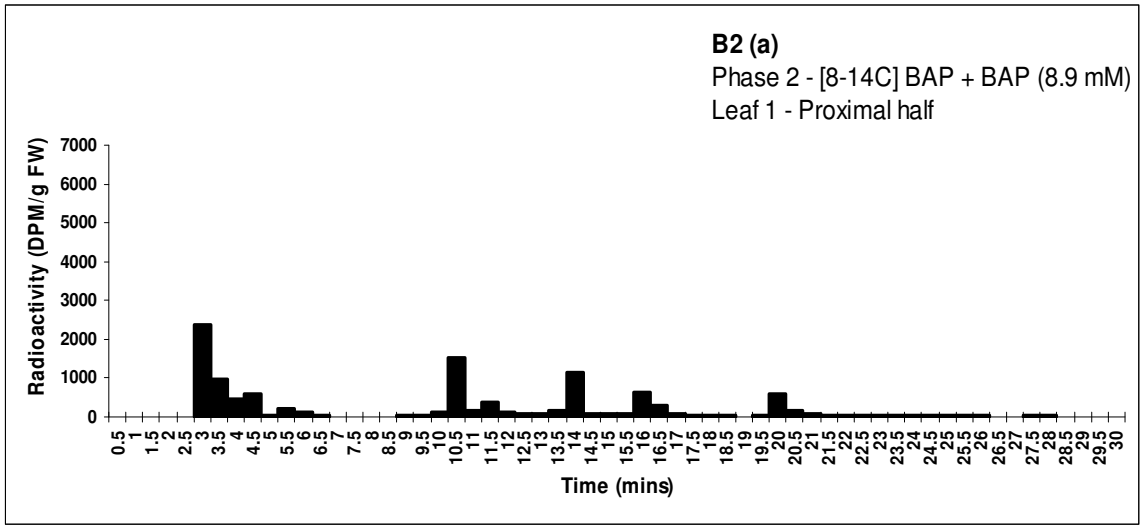

A3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

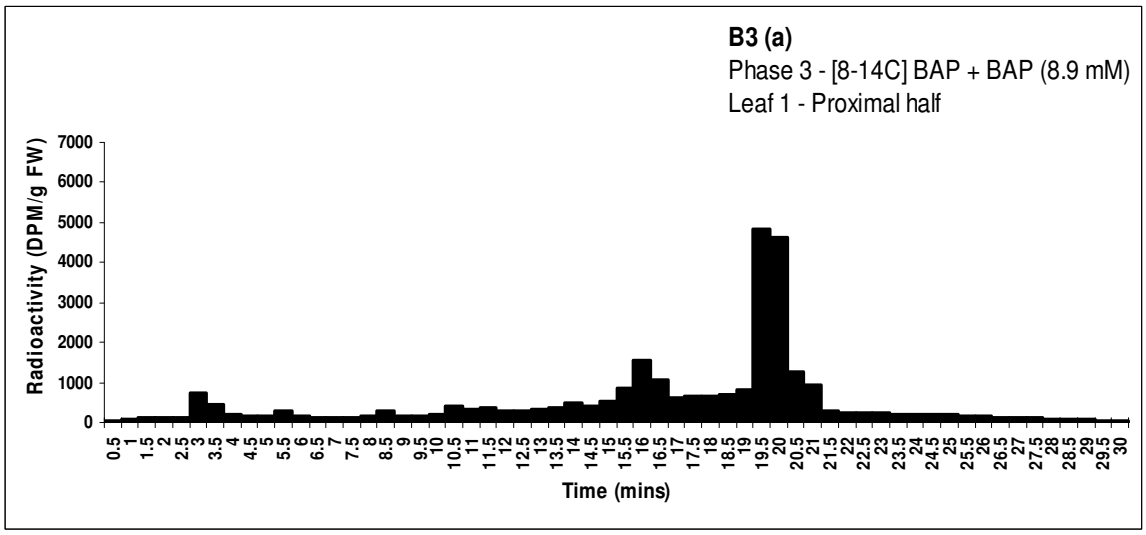

A1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Distal half
Radioactivity (DPM/g FW)
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Time (mins)

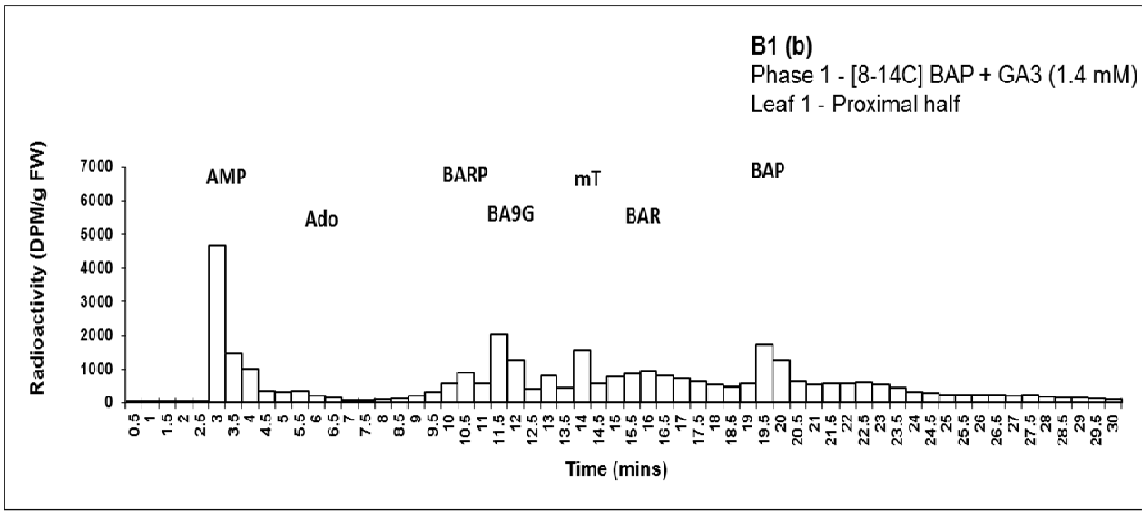

A2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

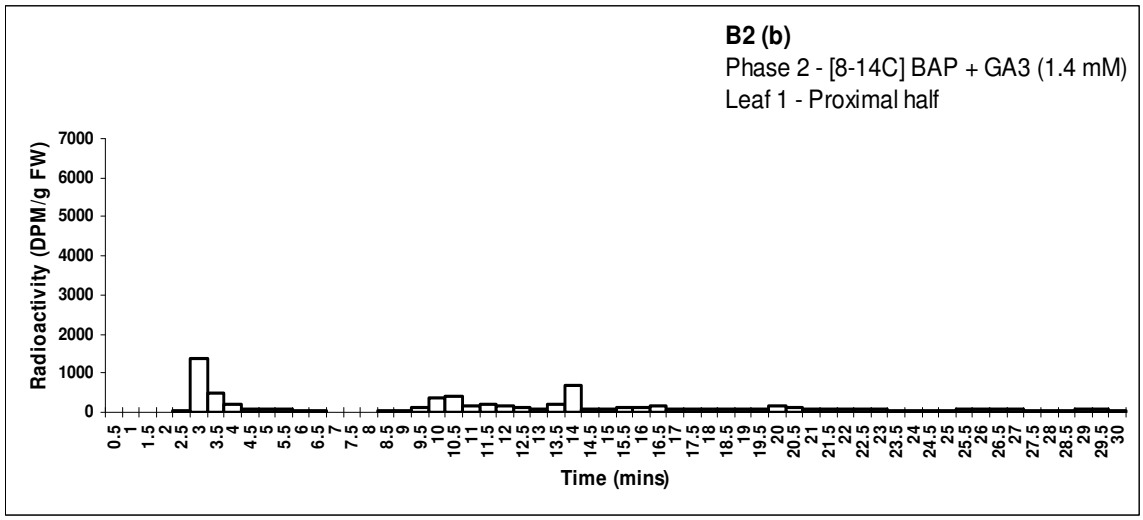

A3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

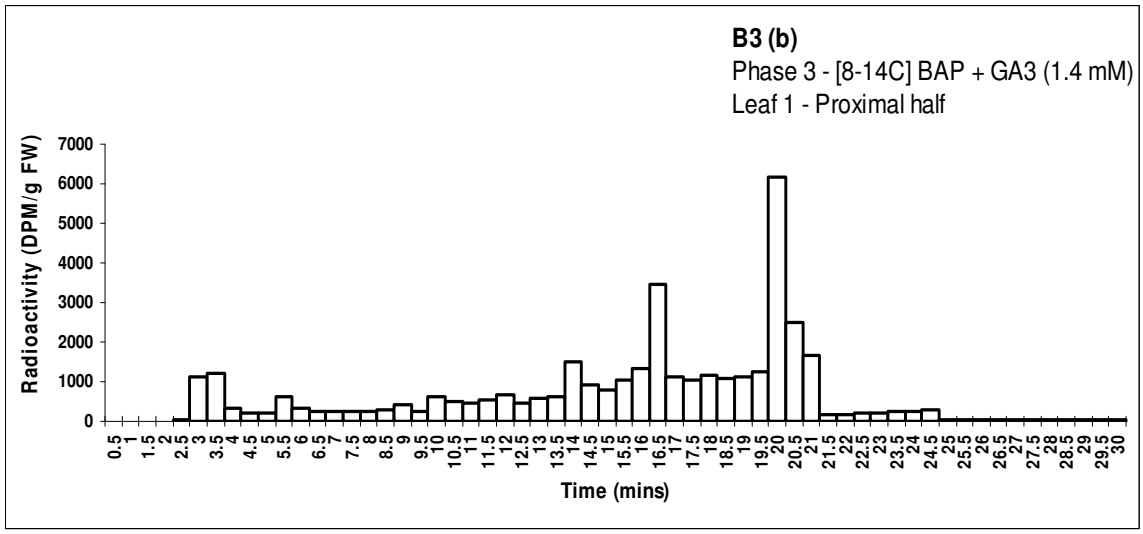

B1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Proximal half
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

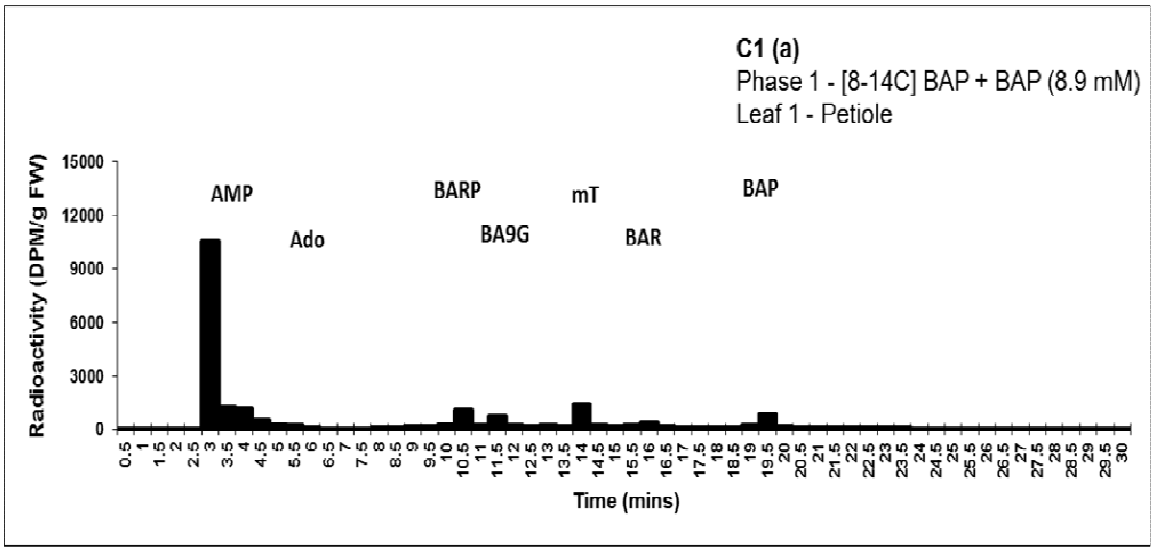

B2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

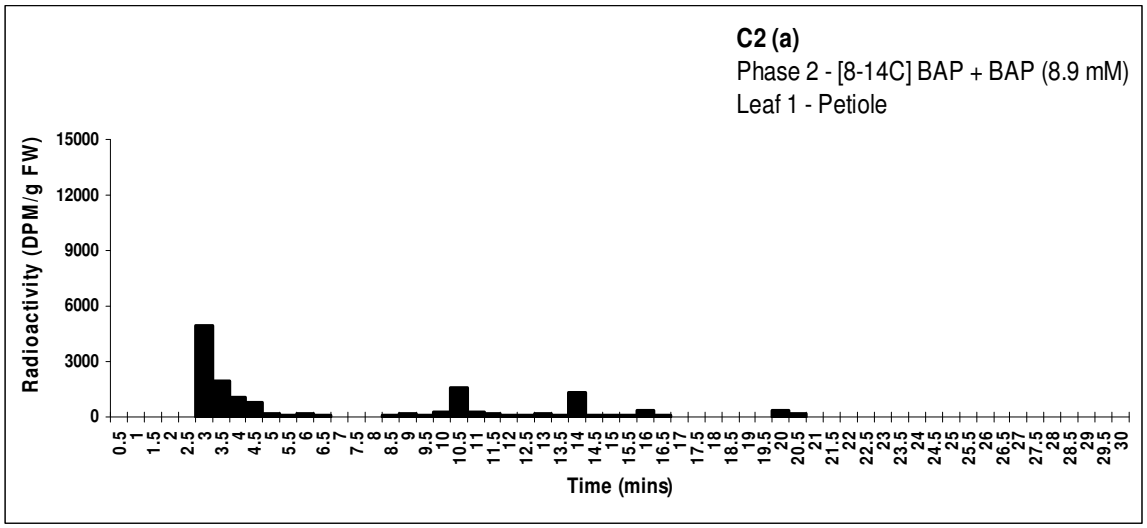

B3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

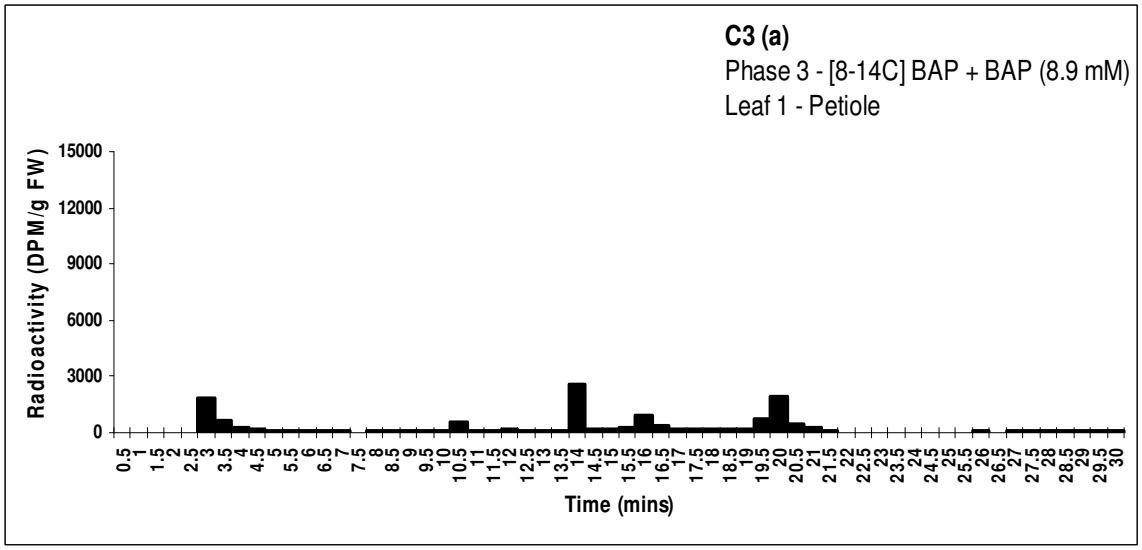

B1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Proximal half
Radioactivity (DPM/g FW)
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Time (mins)

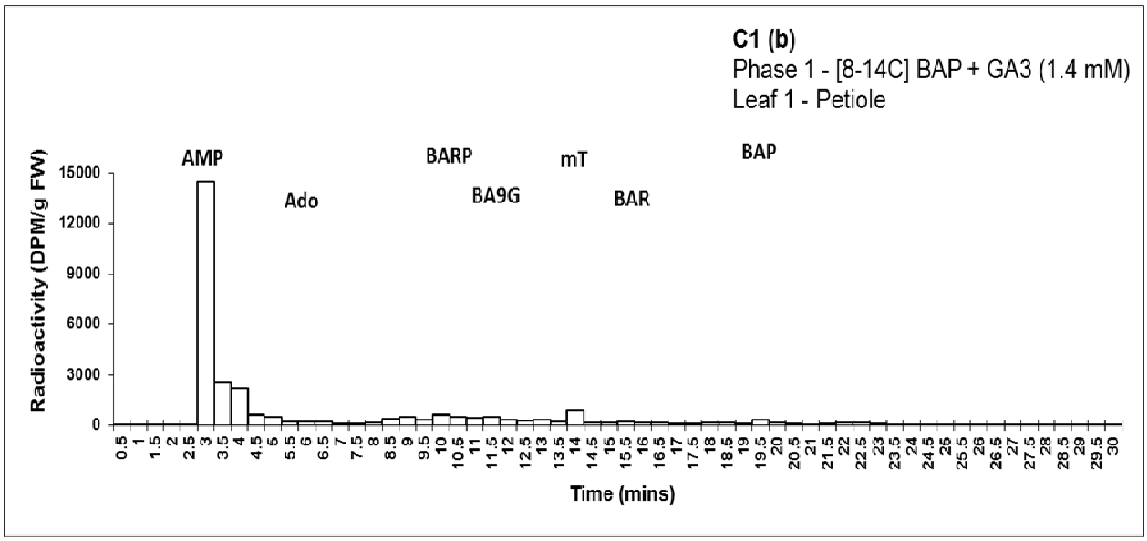

B2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

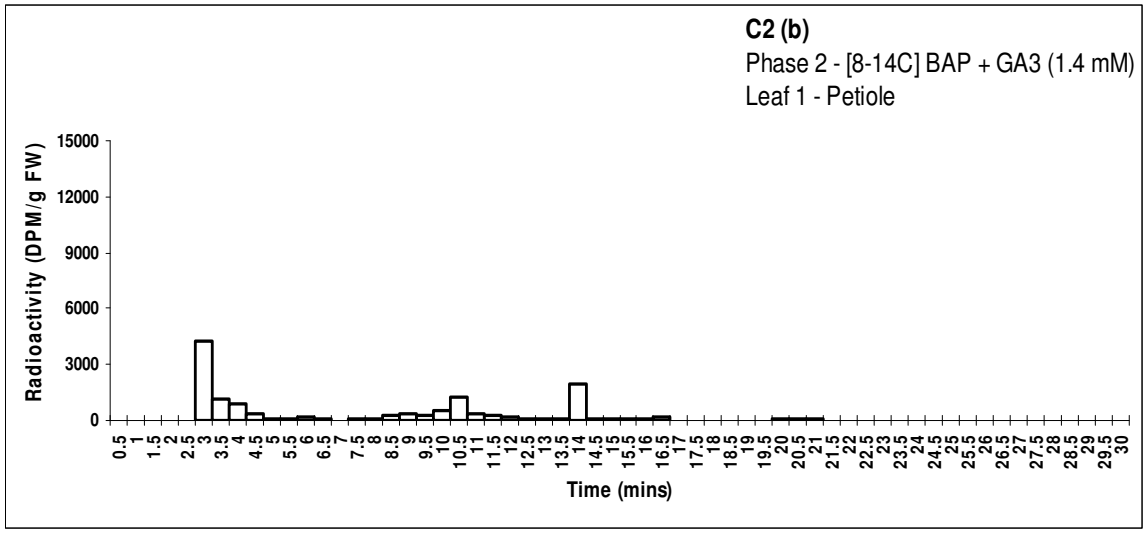

B3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

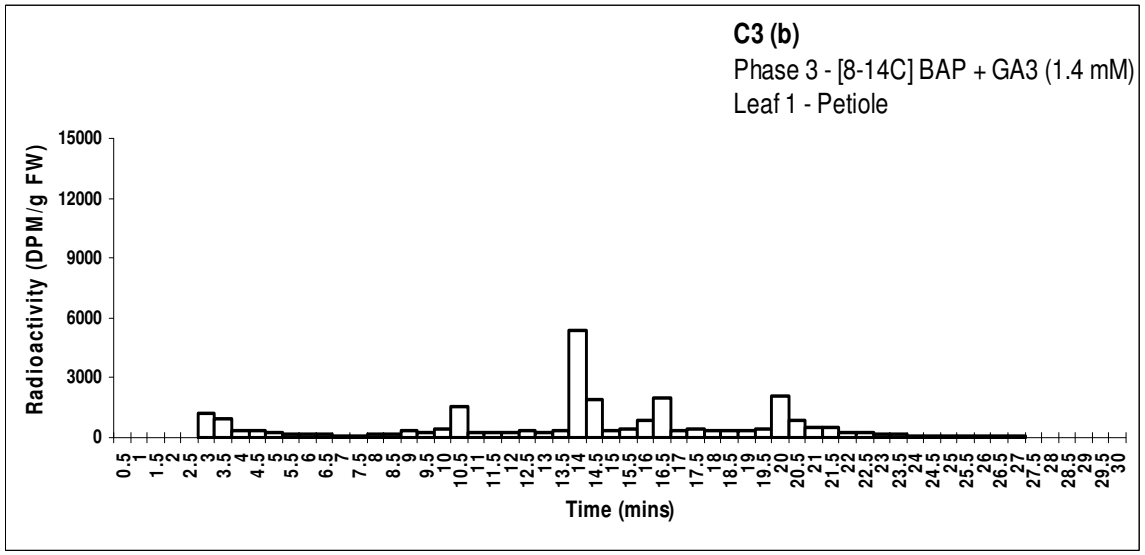

C1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Petiole
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

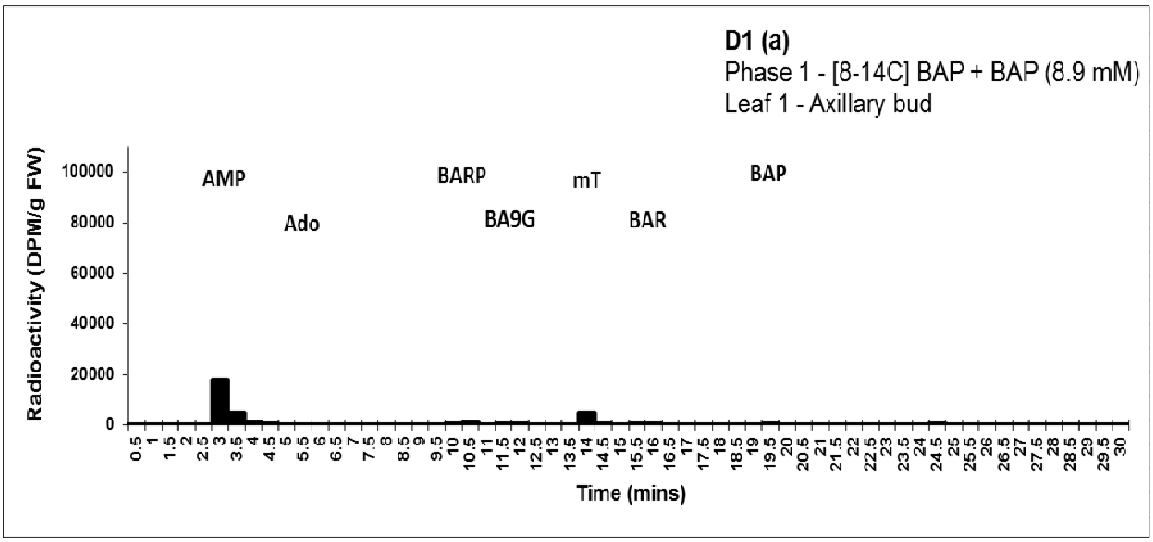

C2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

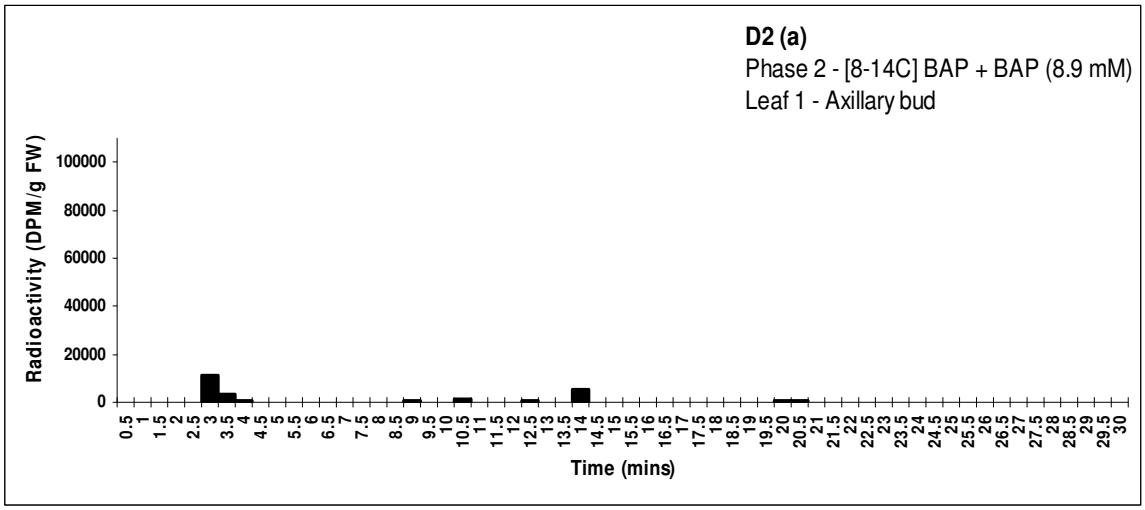

C3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

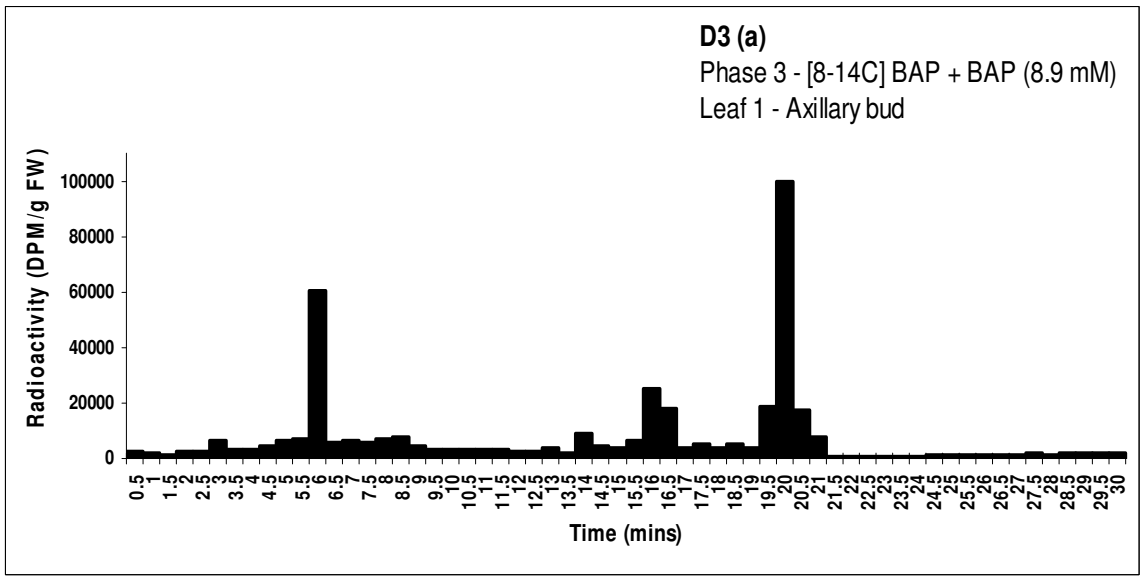

C1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Petiole
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

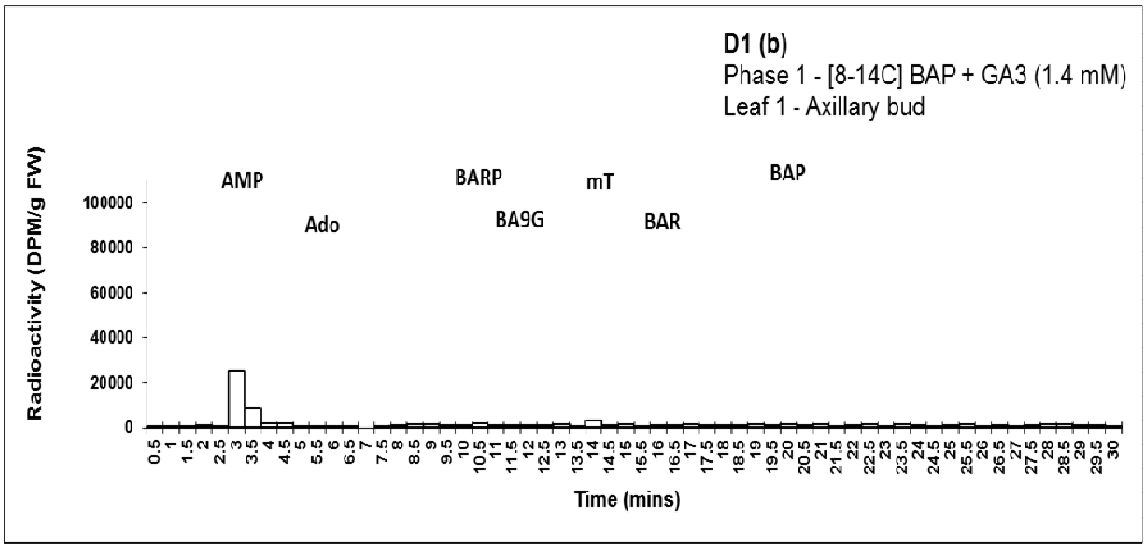

C2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

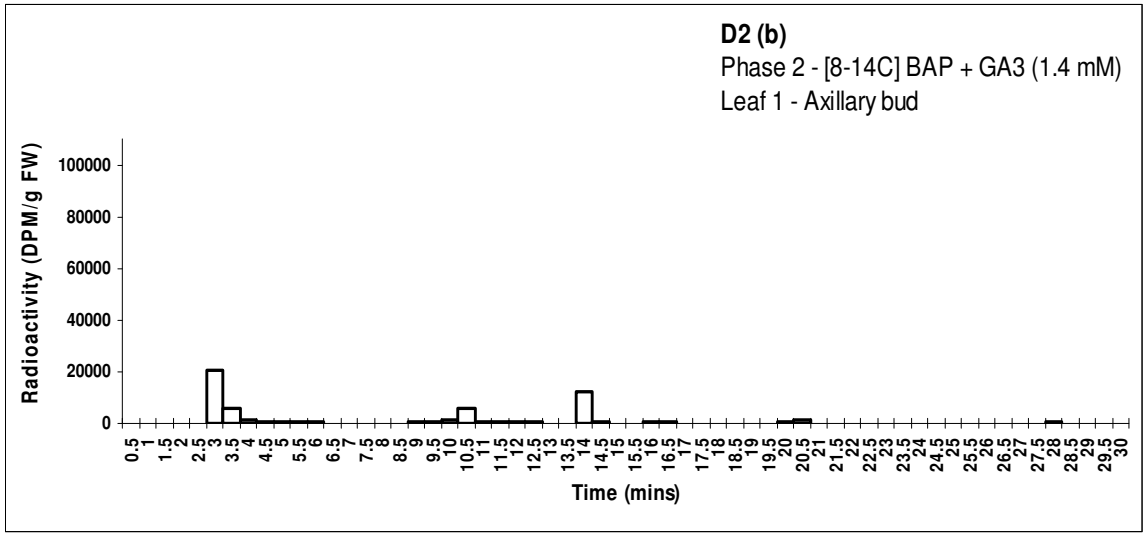

C3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

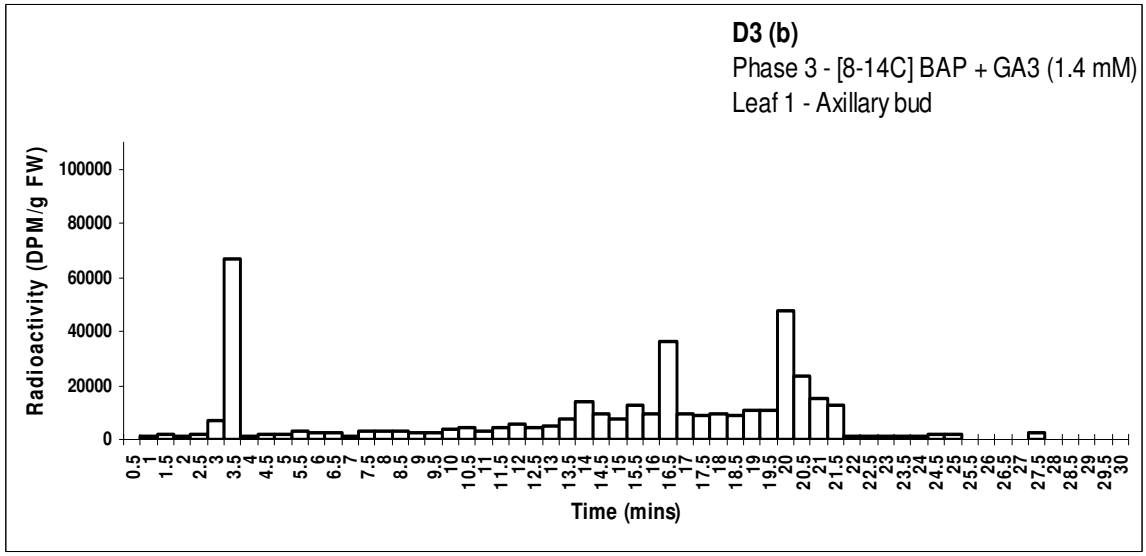

D1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Axillary bud
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

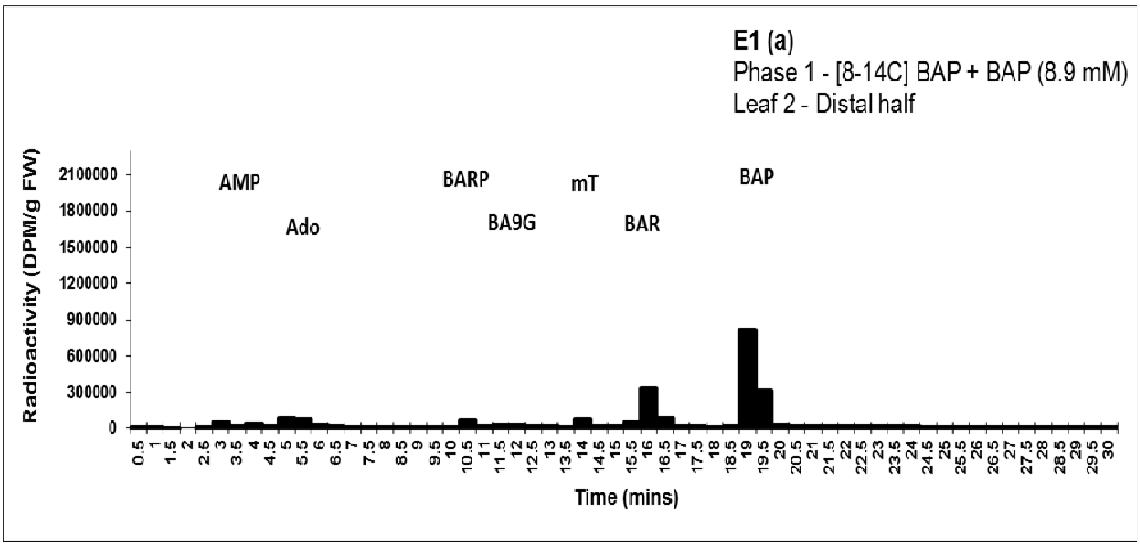

D2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

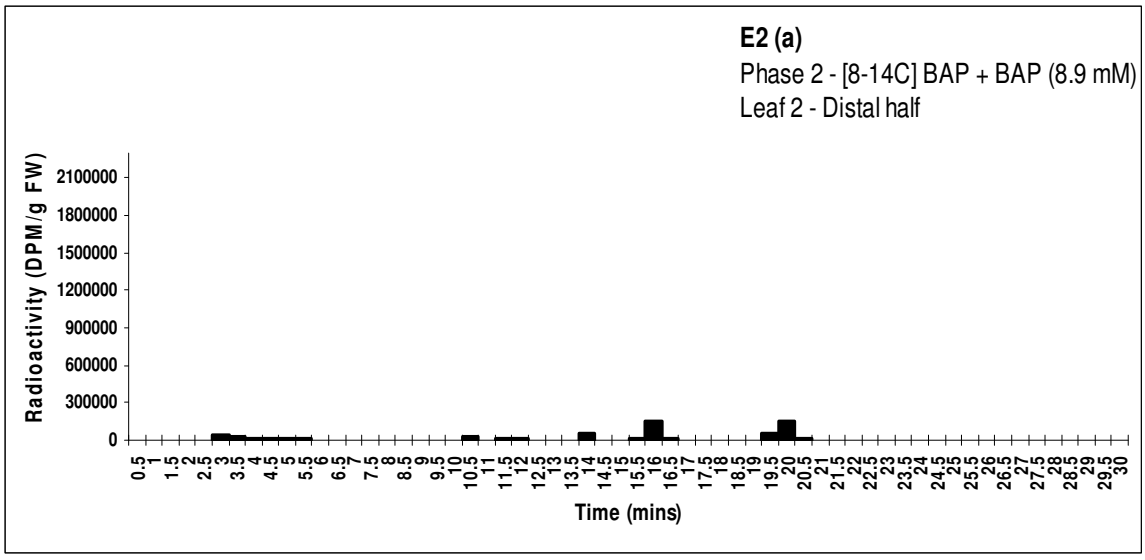

D3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 1 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

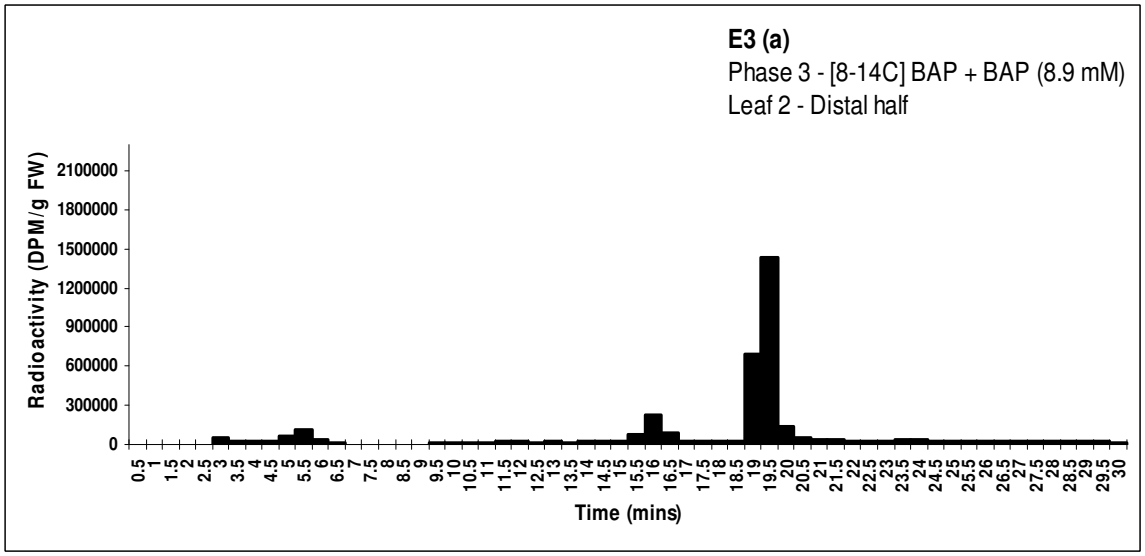

D1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Axillary bud
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

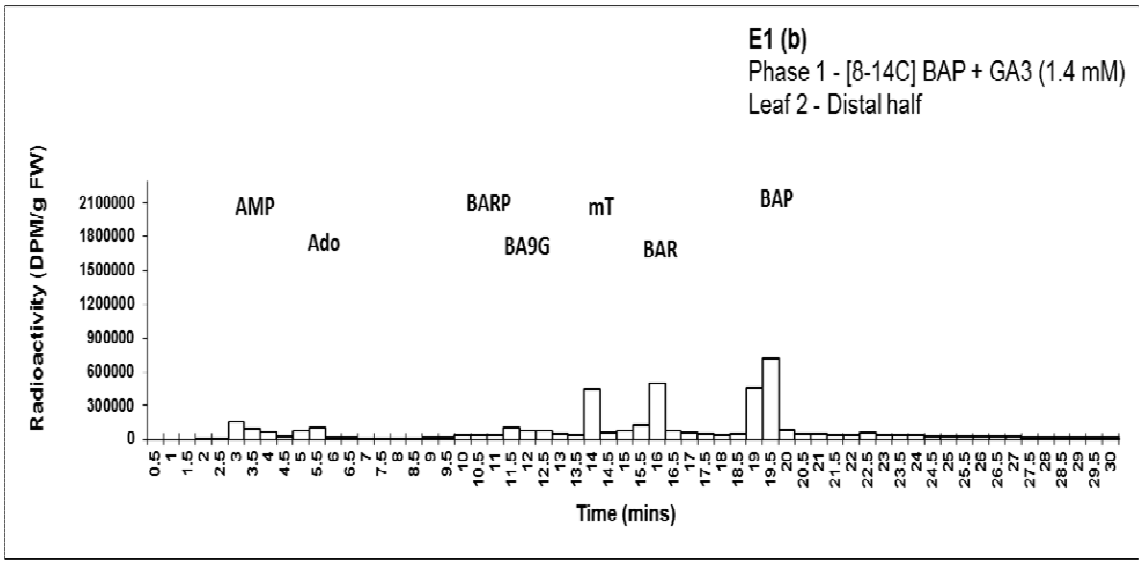

D2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

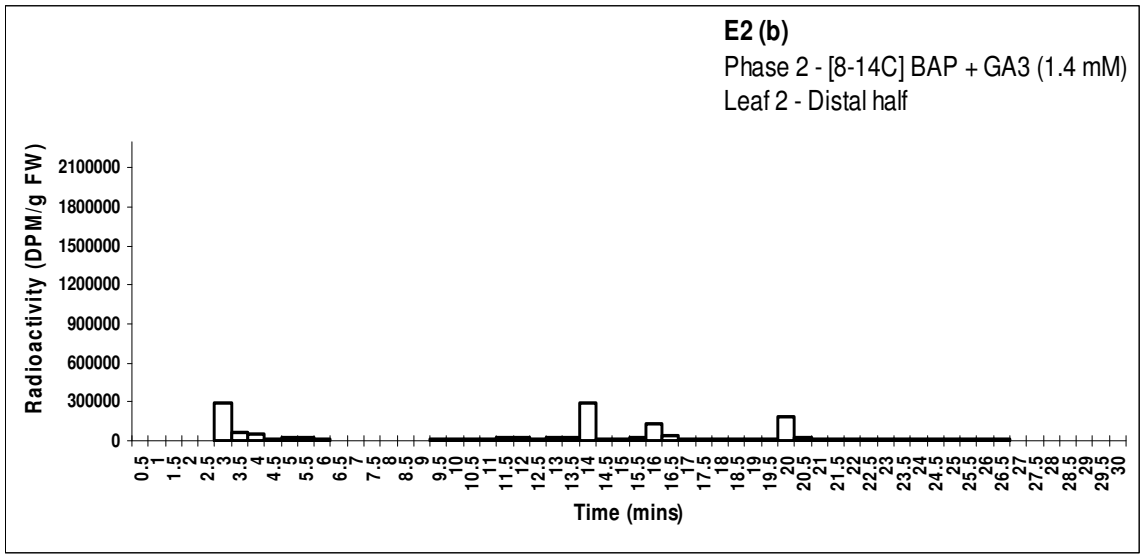

D3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 1 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

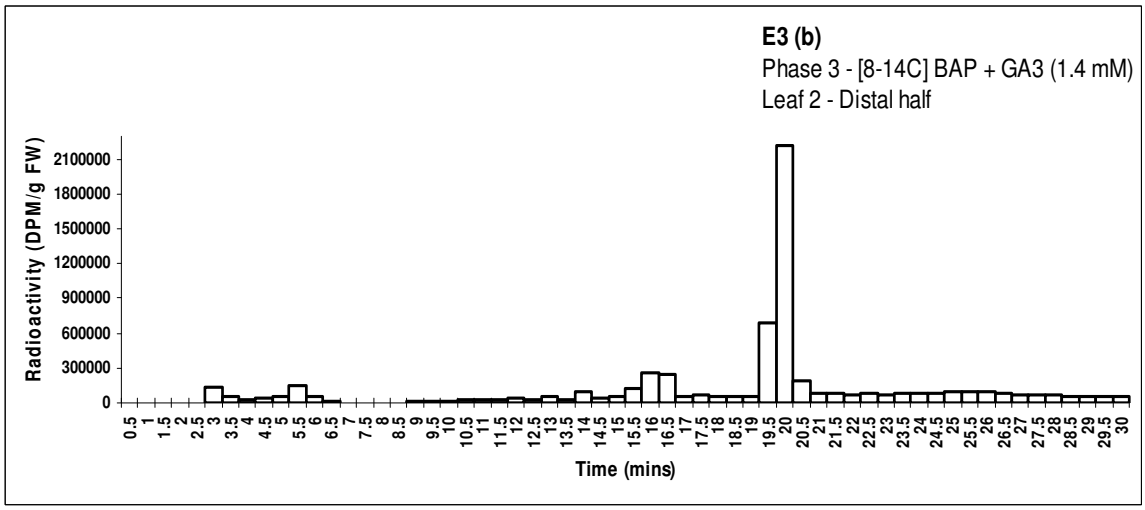

E1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Distal half
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

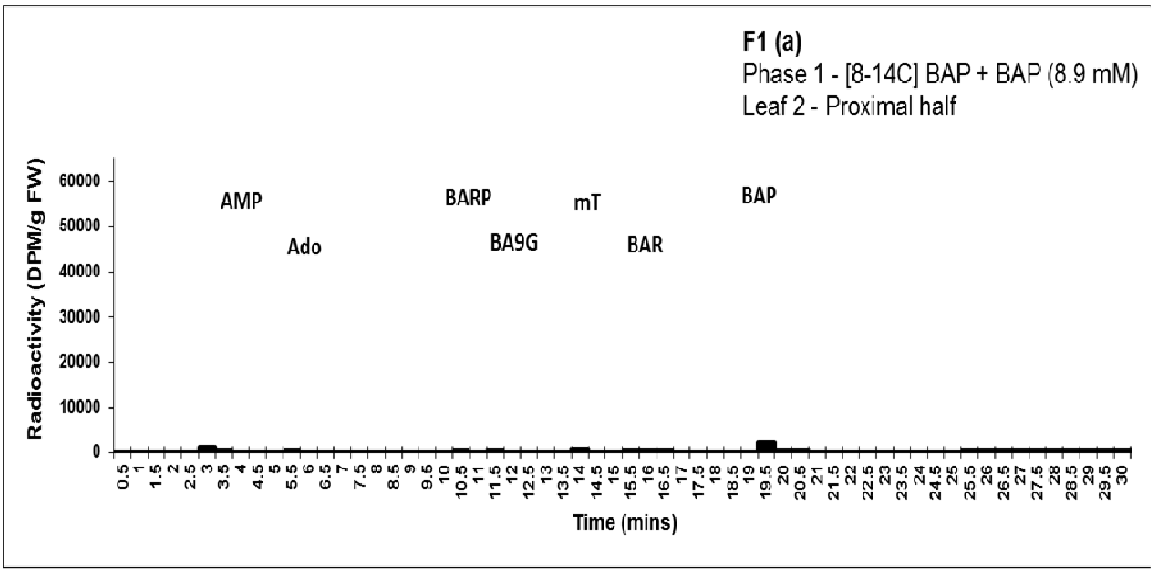

E2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

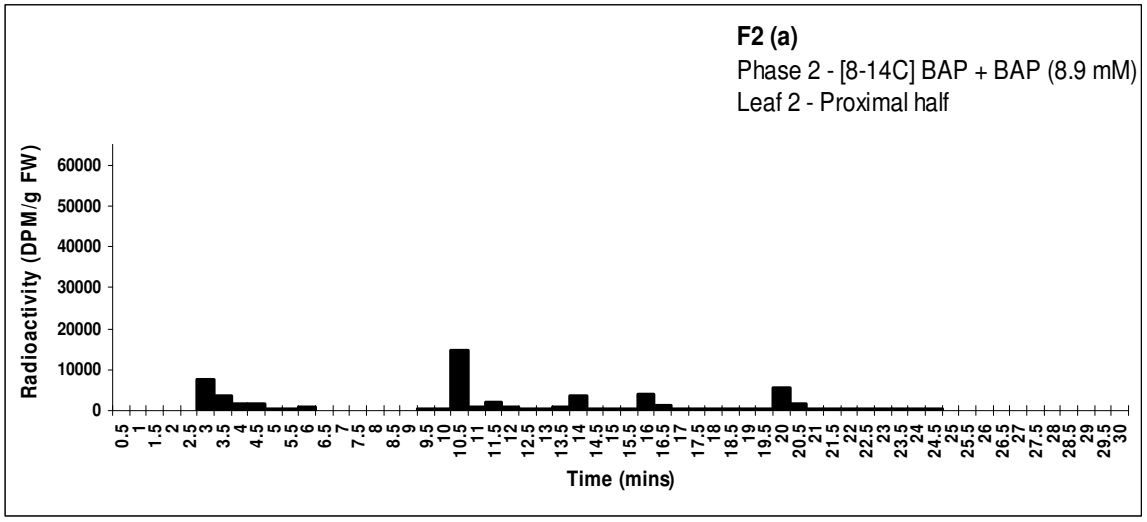

E3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

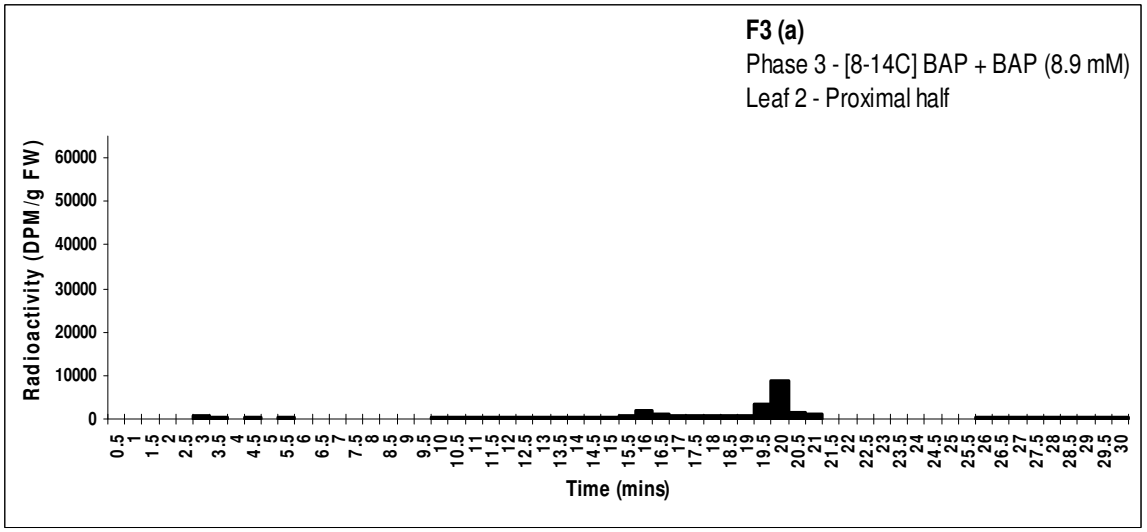

E1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Distal half
Radioactivity (DPM/g FW)
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Time (mins)

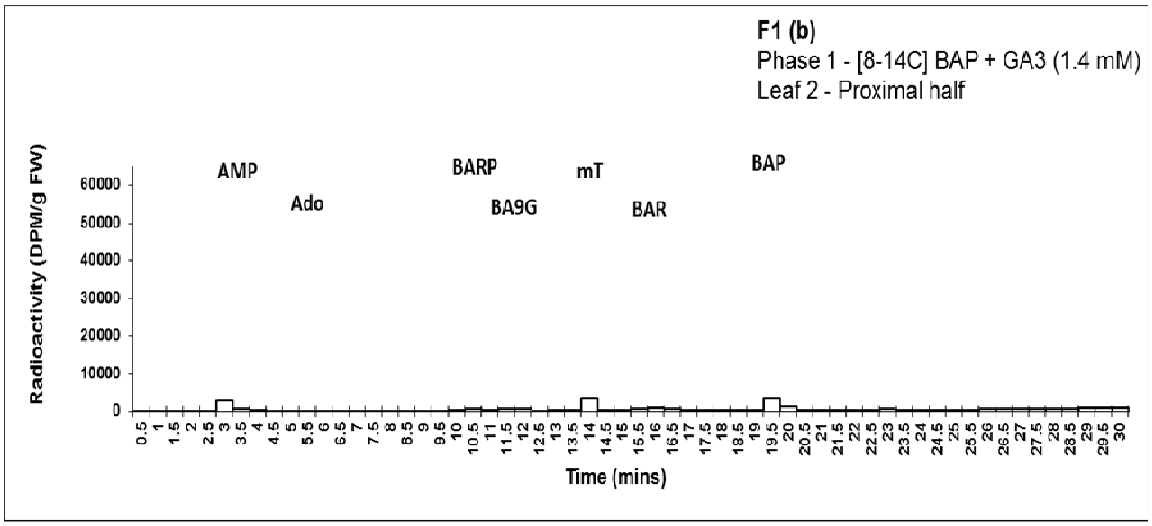

E2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

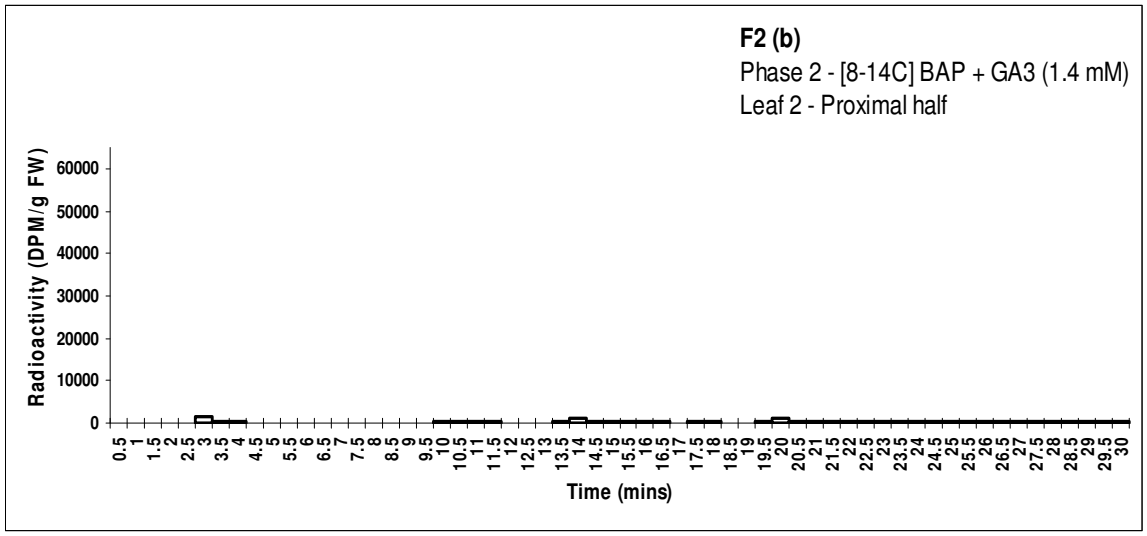

E3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Distal half
Radioactivity (DPM/g FW)
Time (mins)

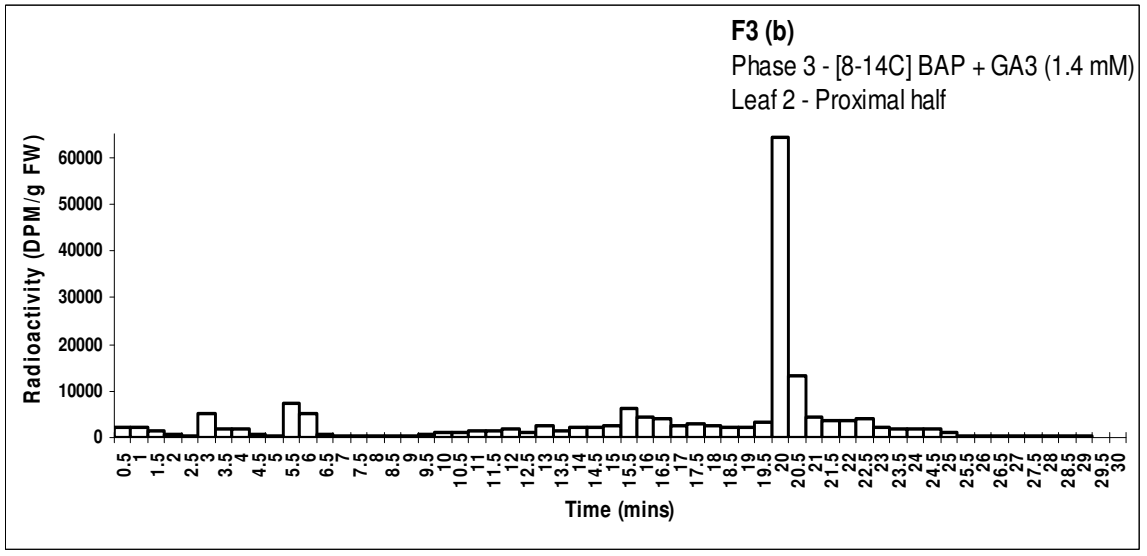

F1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Proximal half
Radioactivity (DPM/g FW)
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Time (mins)

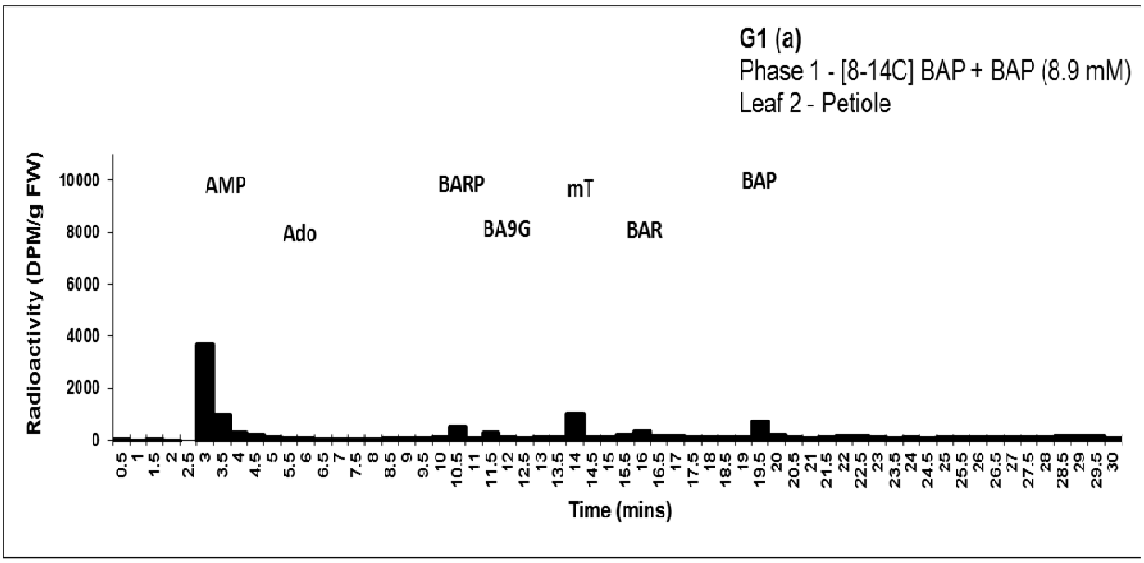

F2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

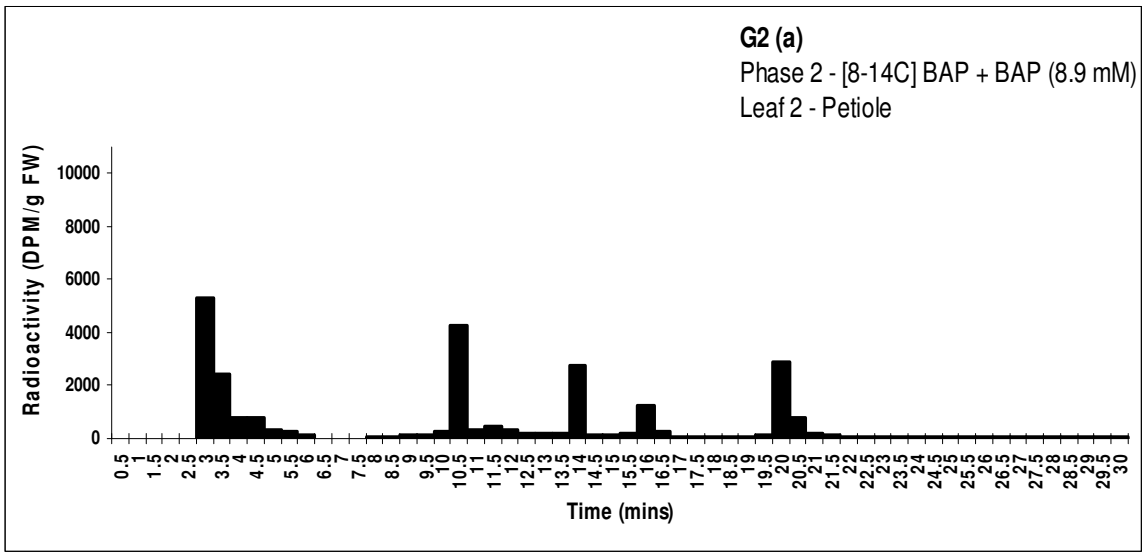

F3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

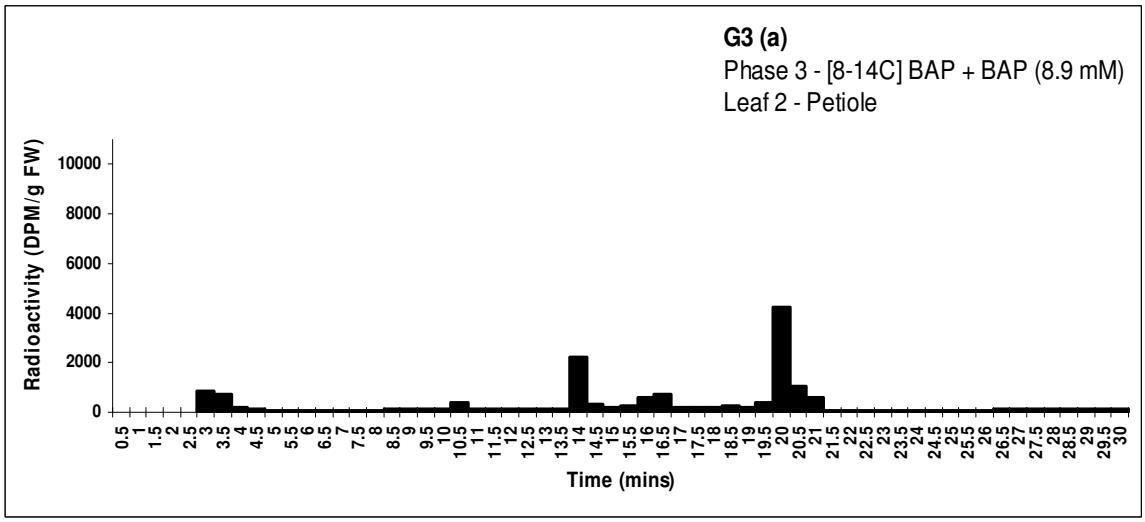

F1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Proximal half
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

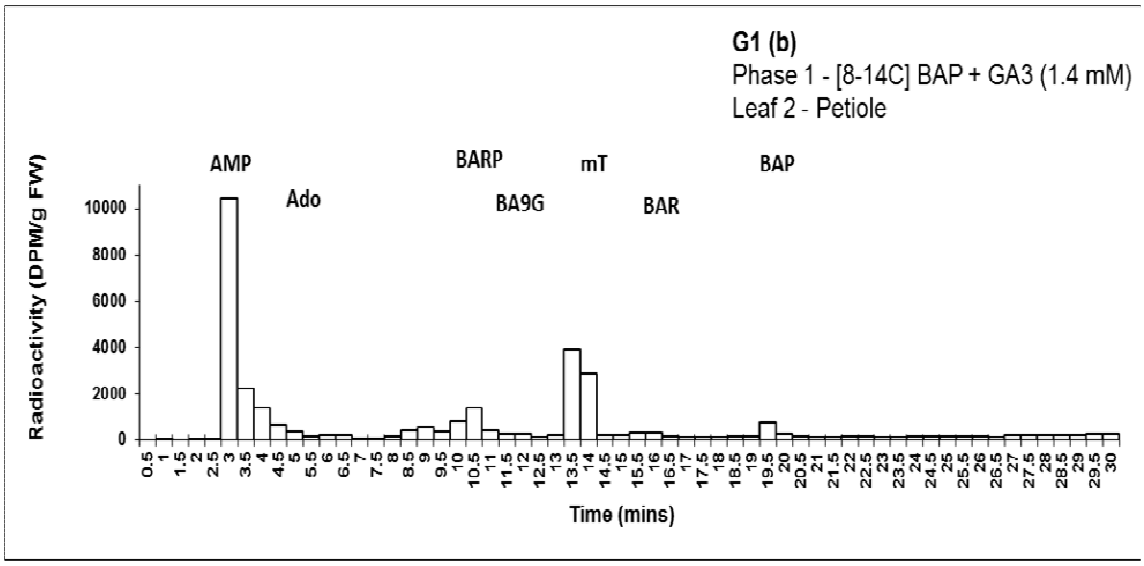

F2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

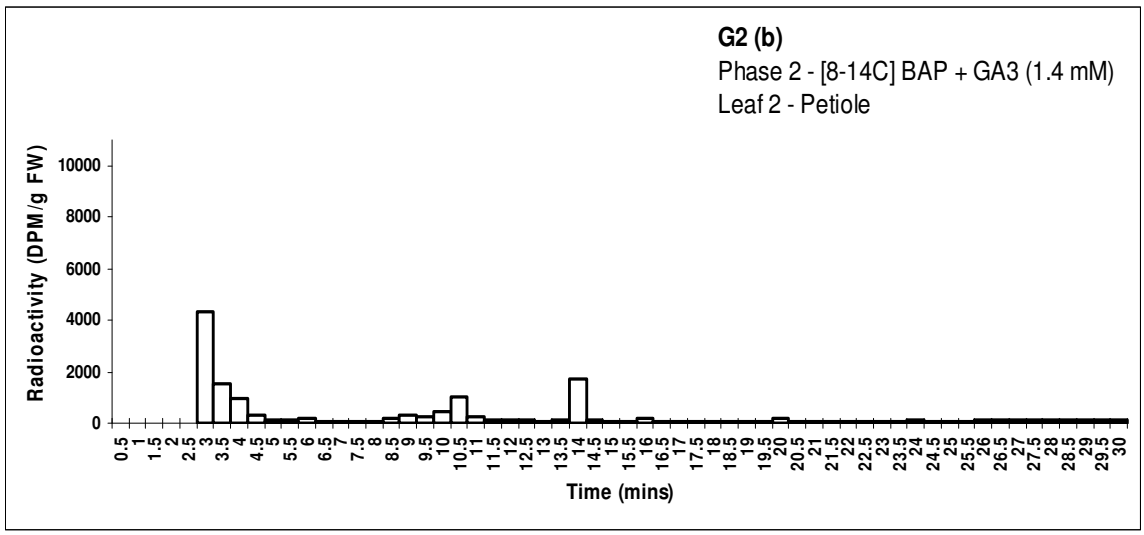

F3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Proximal half
Radioactivity (DPM/g FW)
Time (mins)

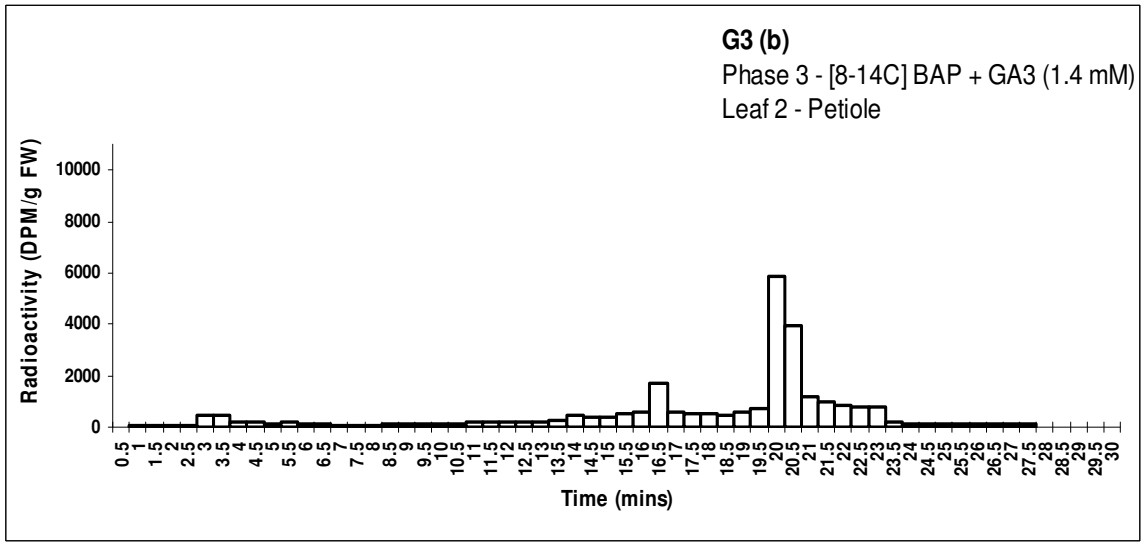

G1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Petiole
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

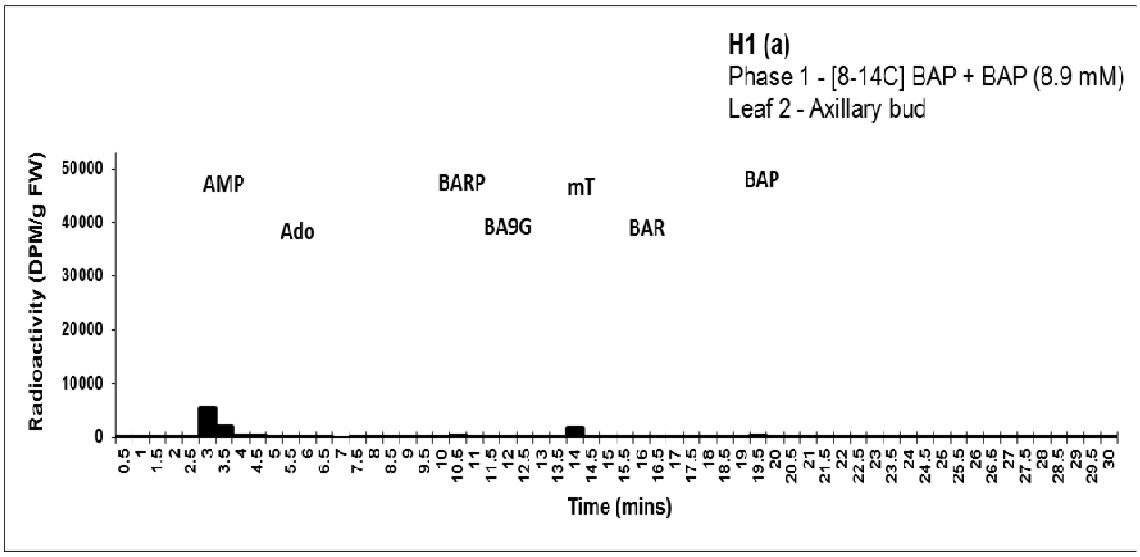

G2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

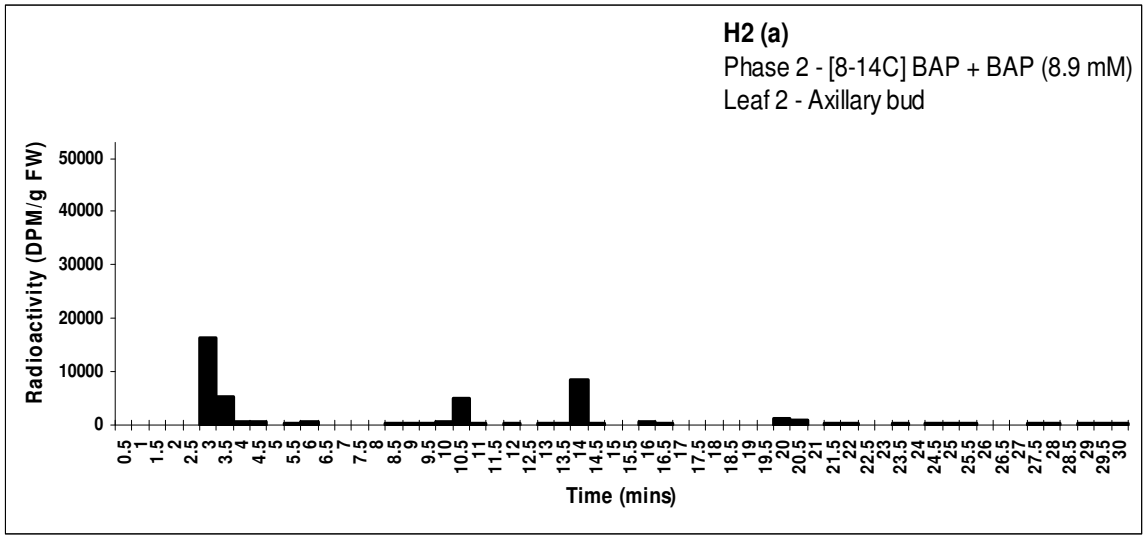

G3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

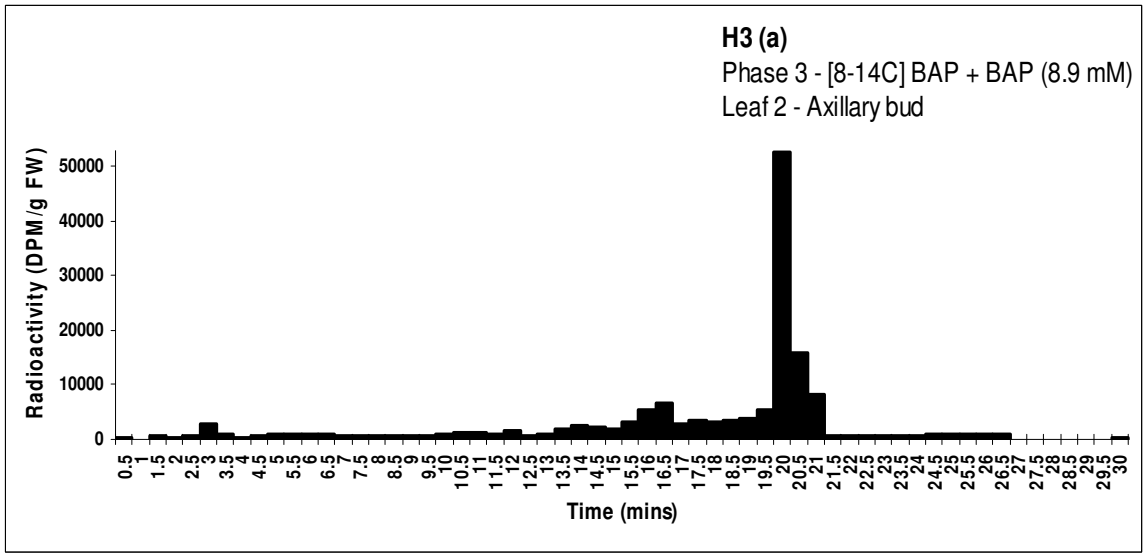

G1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Petiole
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

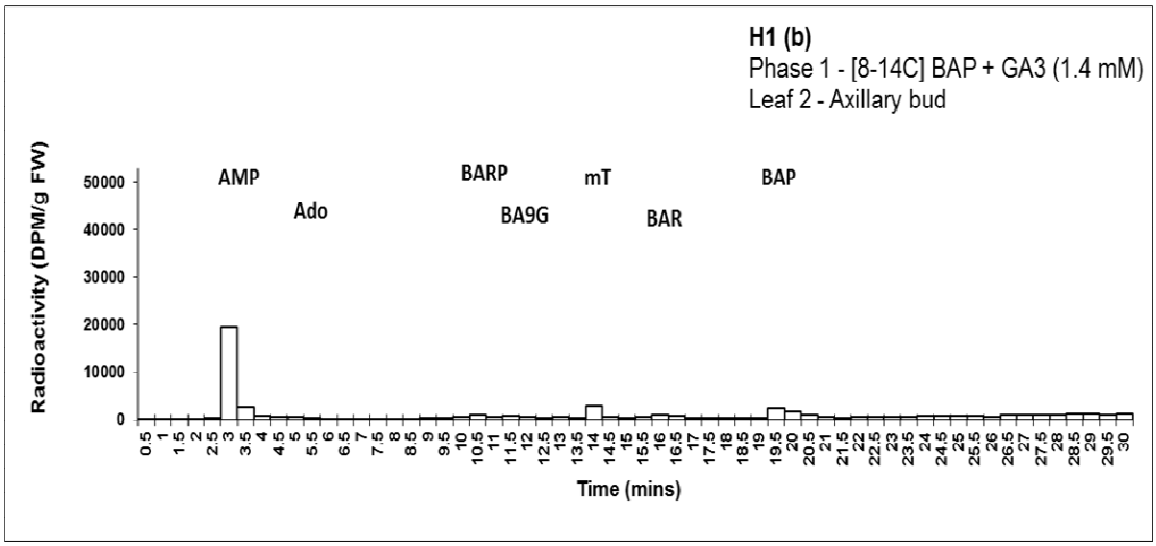

G2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

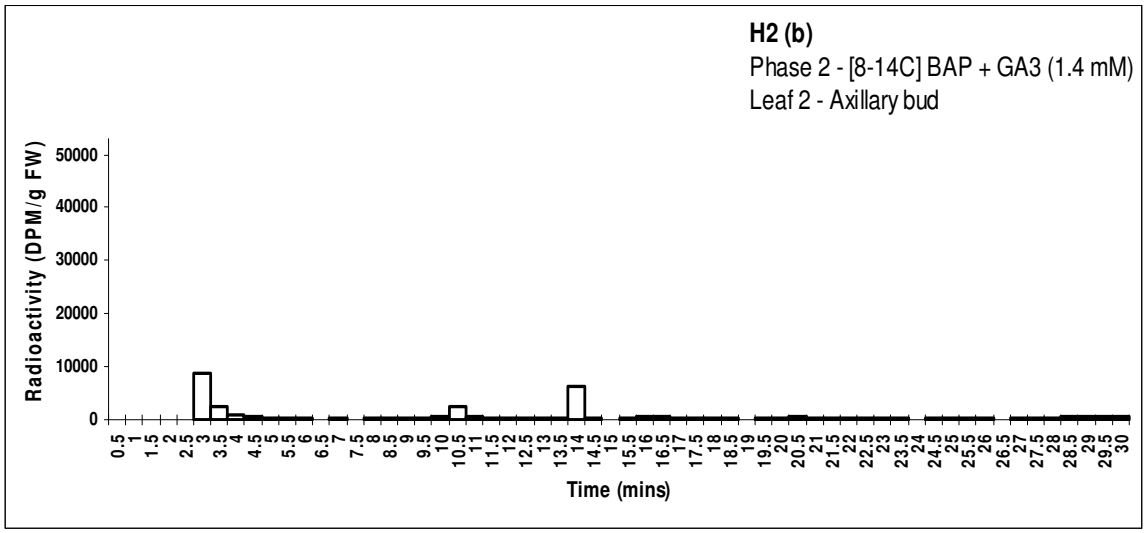

G3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Petiole
Radioactivity (DPM/g FW)
Time (mins)

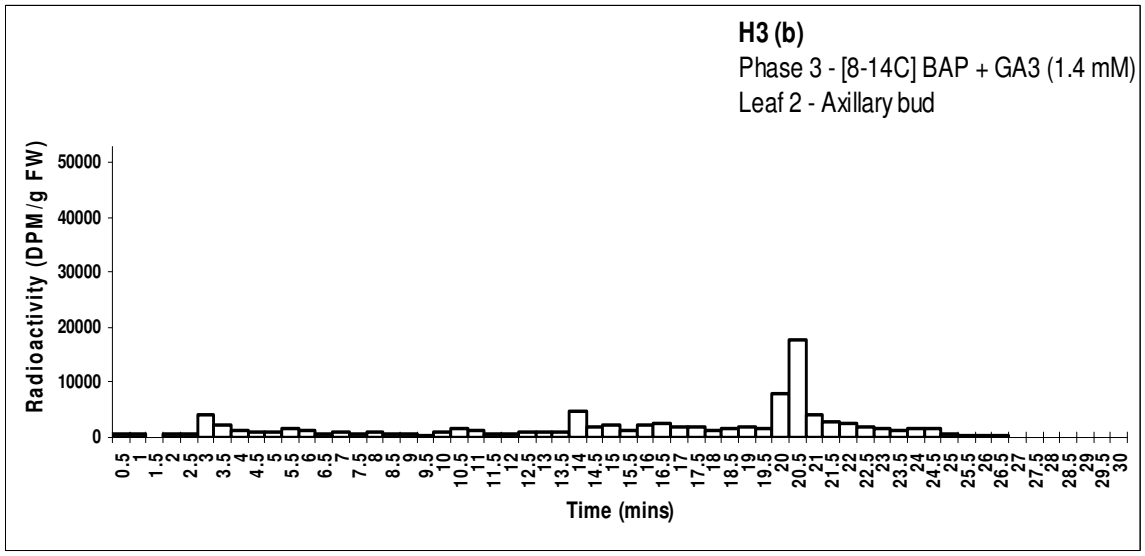

H1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Axillary bud
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

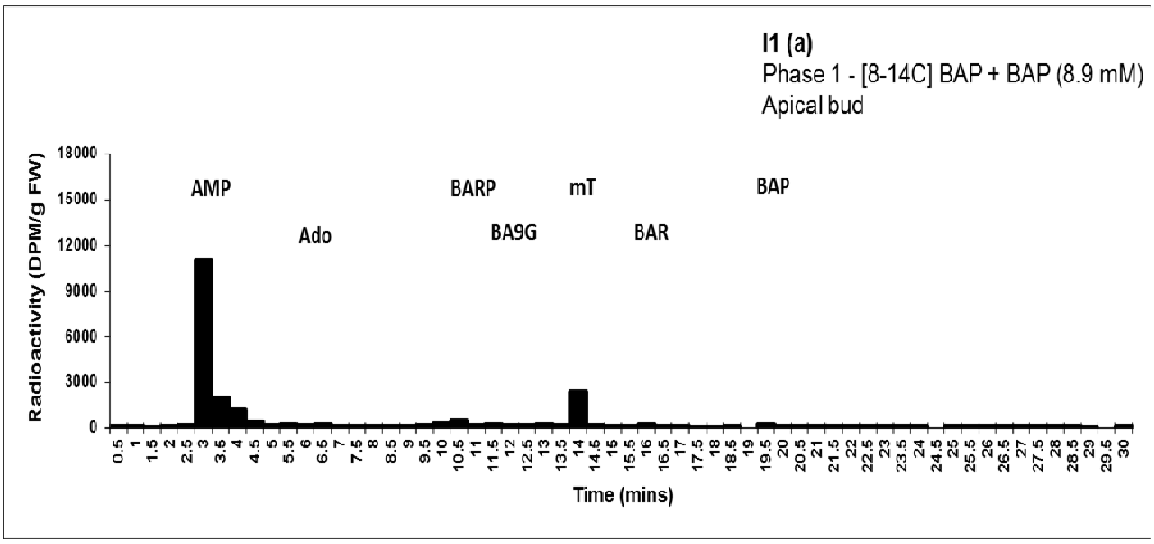

H2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

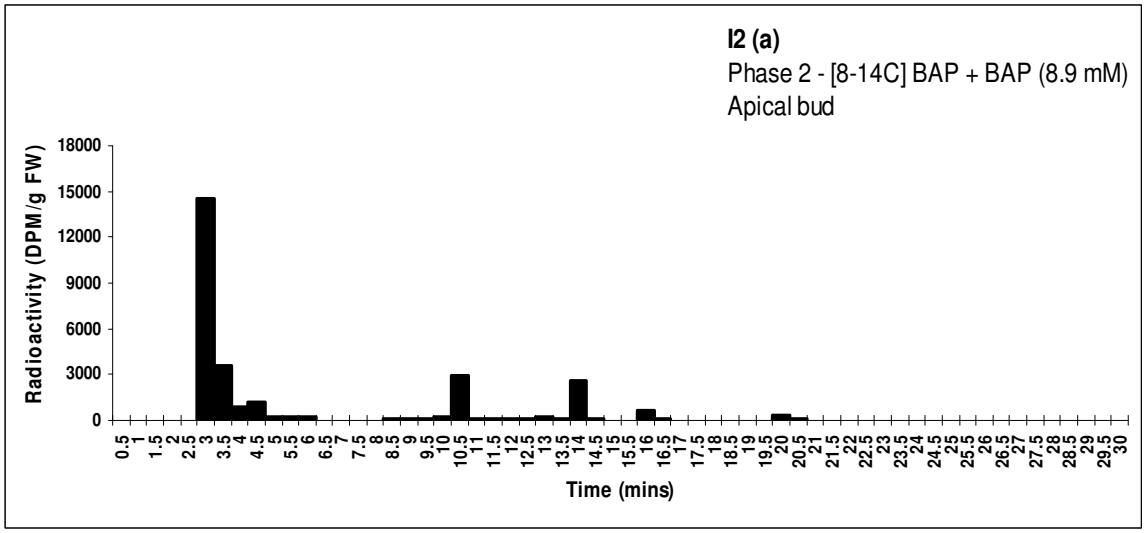

H3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Leaf 2 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

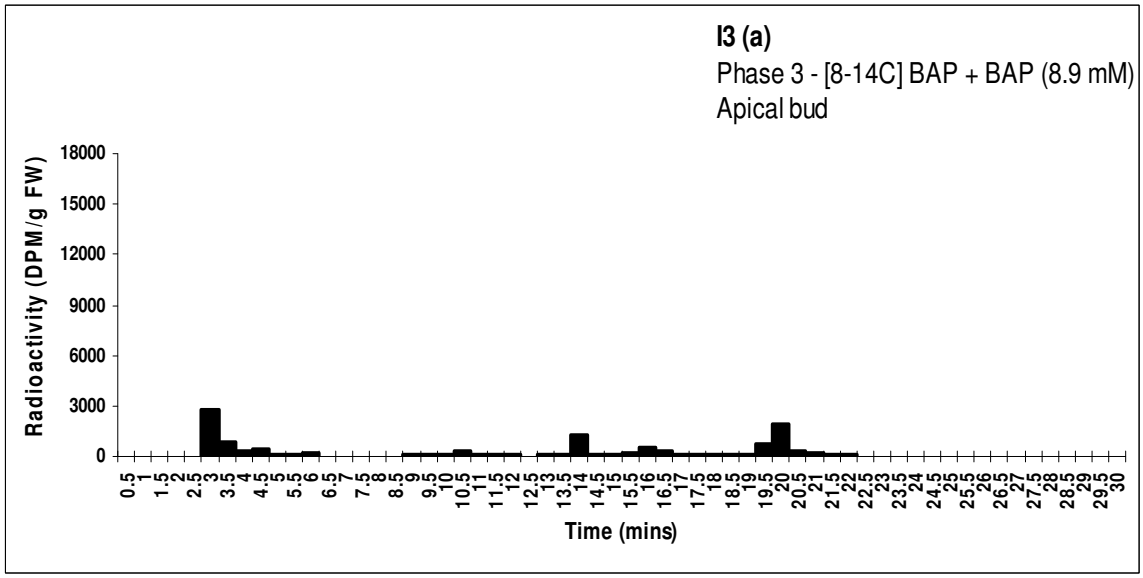

H1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Axillary bud
Radioactivity (DPM/g FW)
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Time (mins)

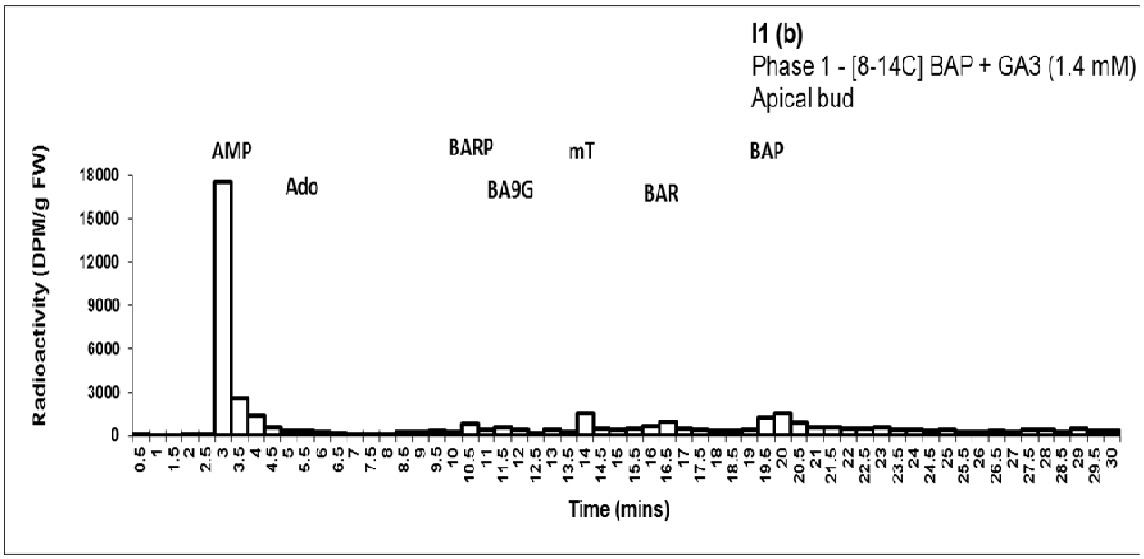

H2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

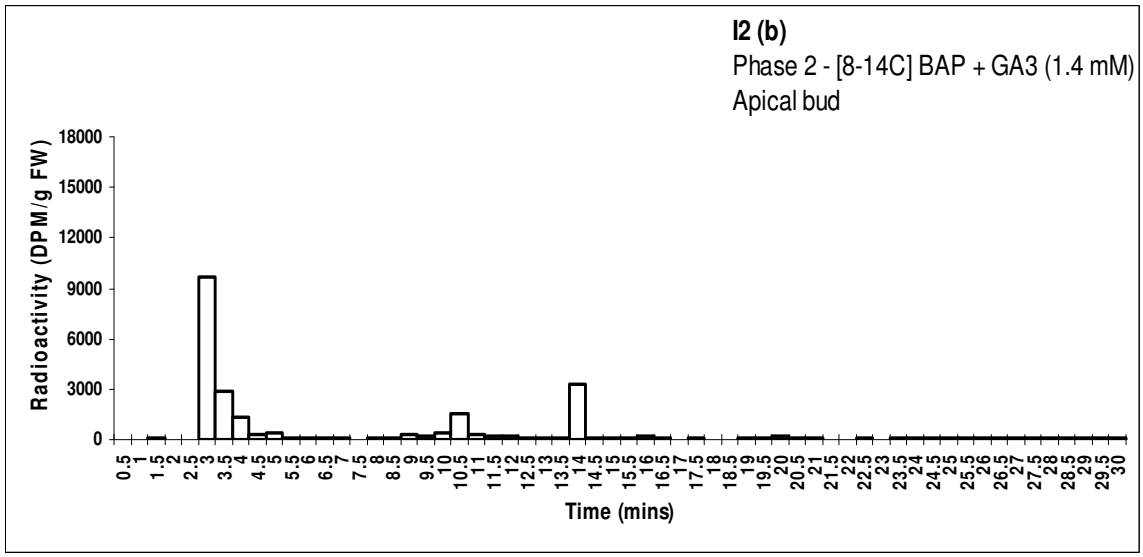

H3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Leaf 2 - Axillary bud
Radioactivity (DPM/g FW)
Time (mins)

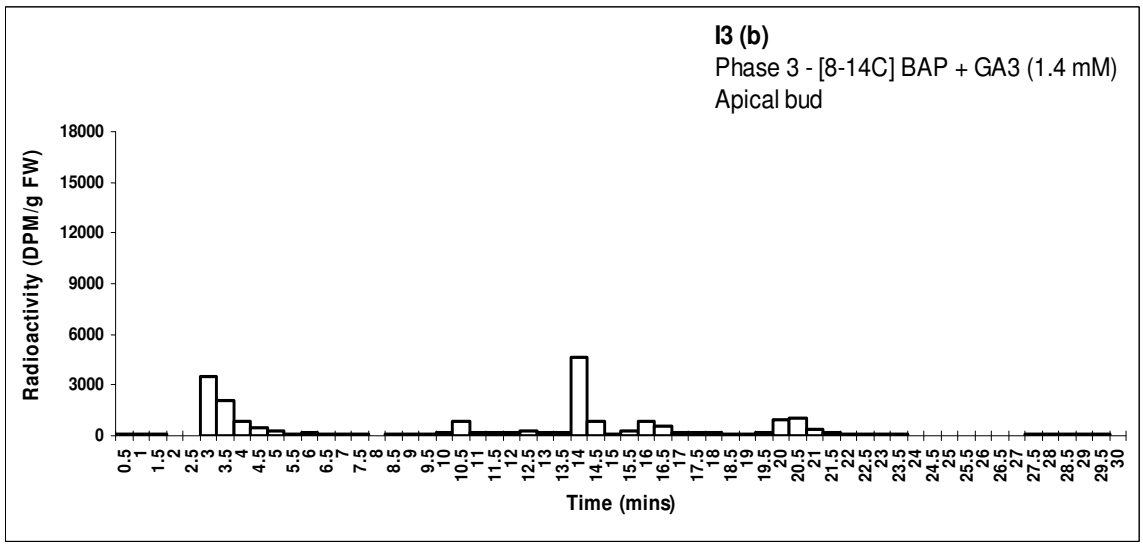

I1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Apical bud
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

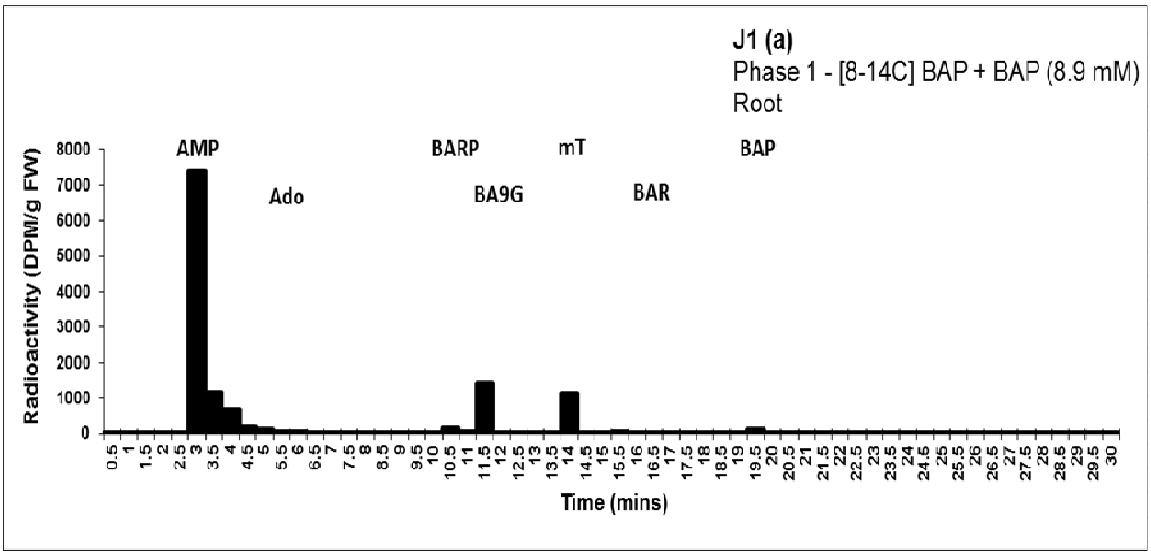

I2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Apical bud
Radioactivity (DPM/g FW)
Time (mins)

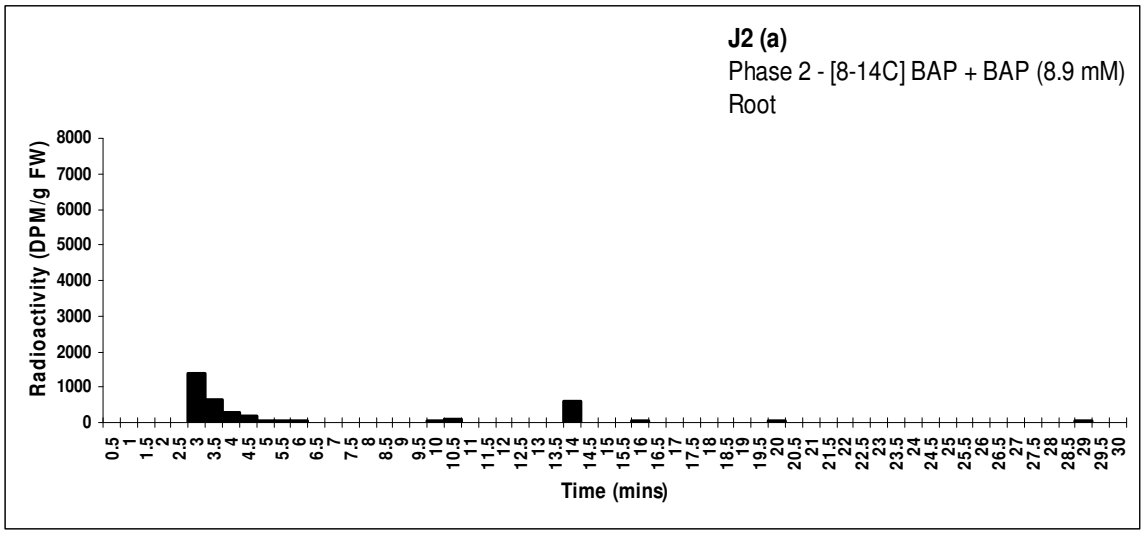

I3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Apical bud
Radioactivity (DPM/g FW)
Time (mins)

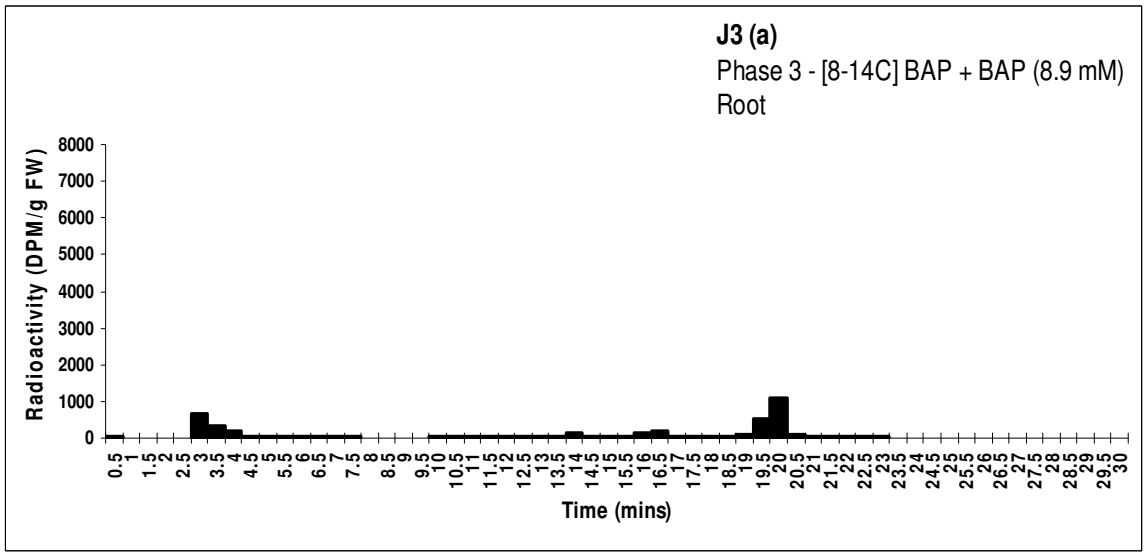

I1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Apical bud
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

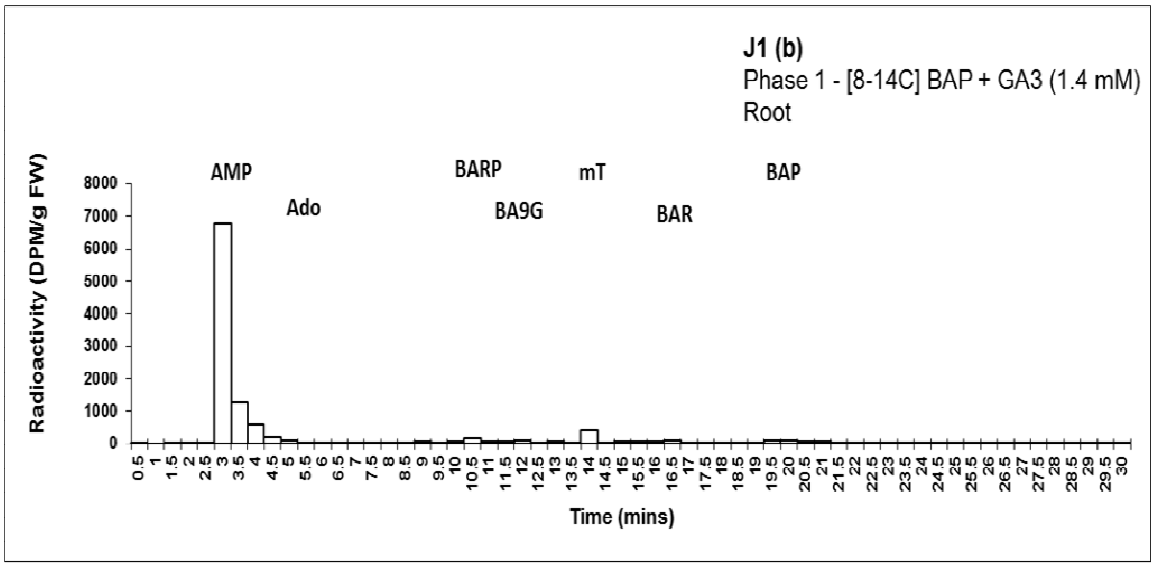

I2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Apical bud
Radioactivity (DPM/g FW)
Time (mins)

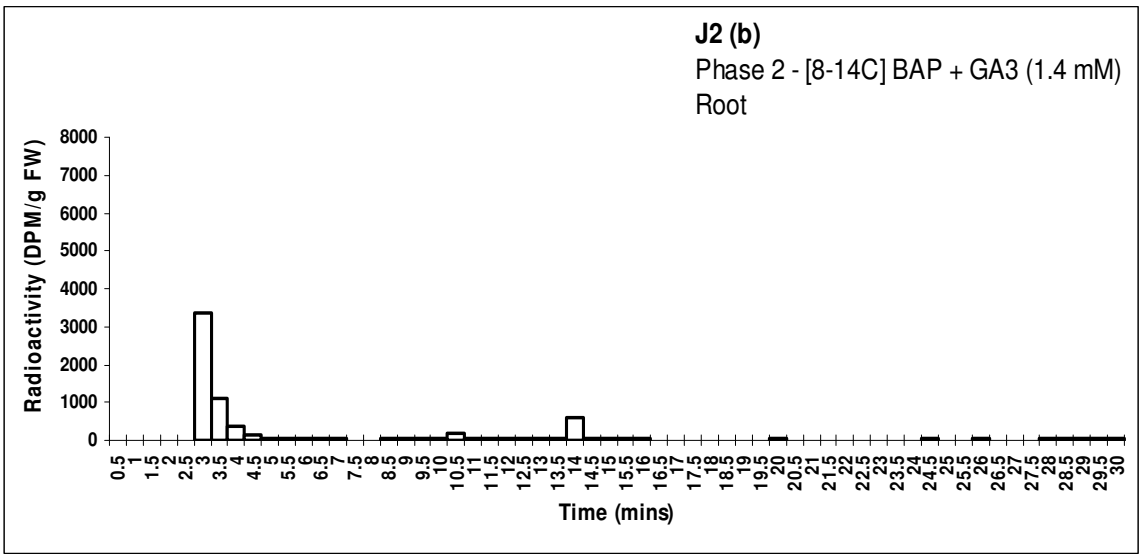

I3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Apical bud
Radioactivity (DPM/g FW)
Time (mins)

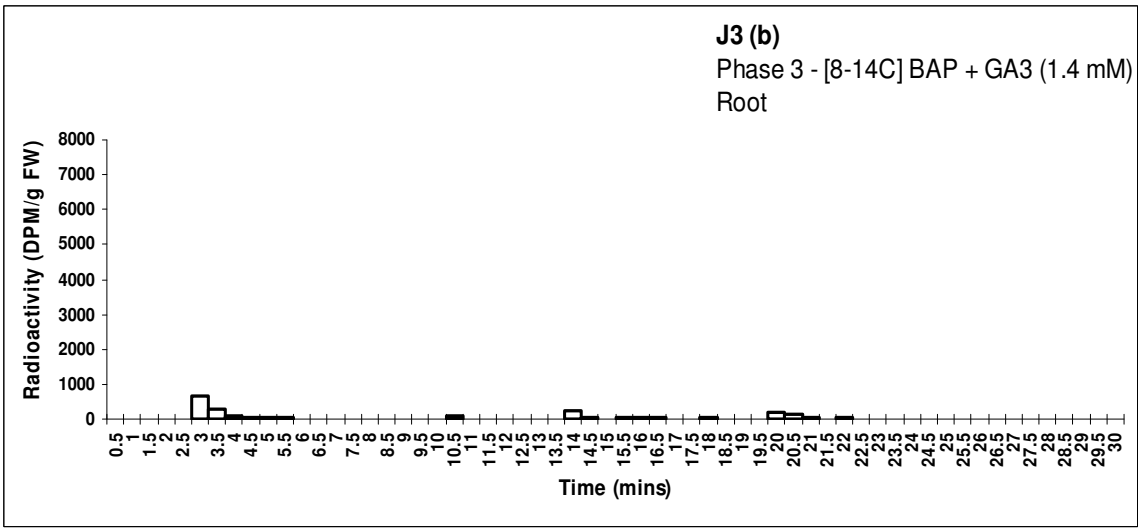

J1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Root
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

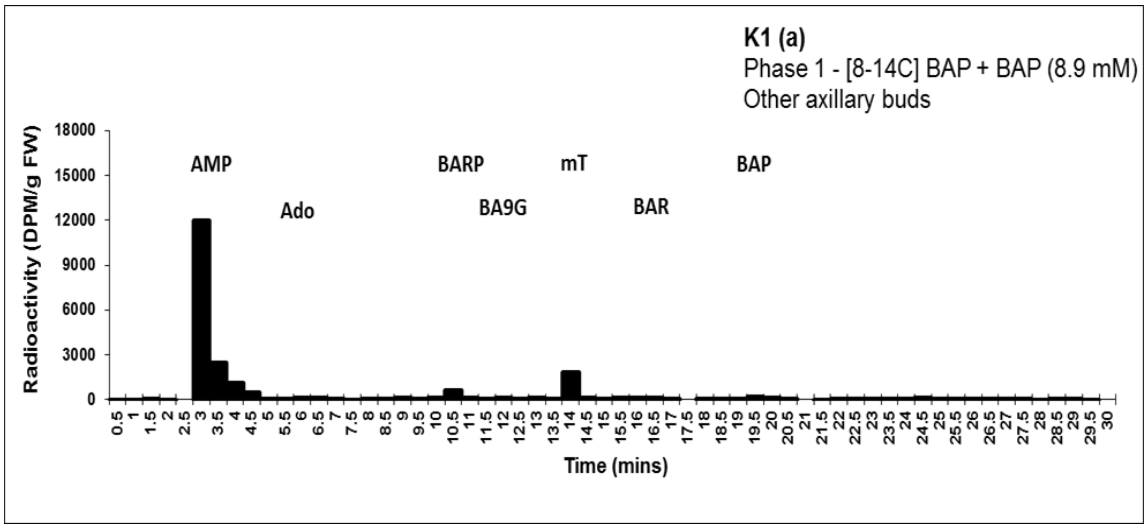

J2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Root
Radioactivity (DPM/g FW)
Time (mins)

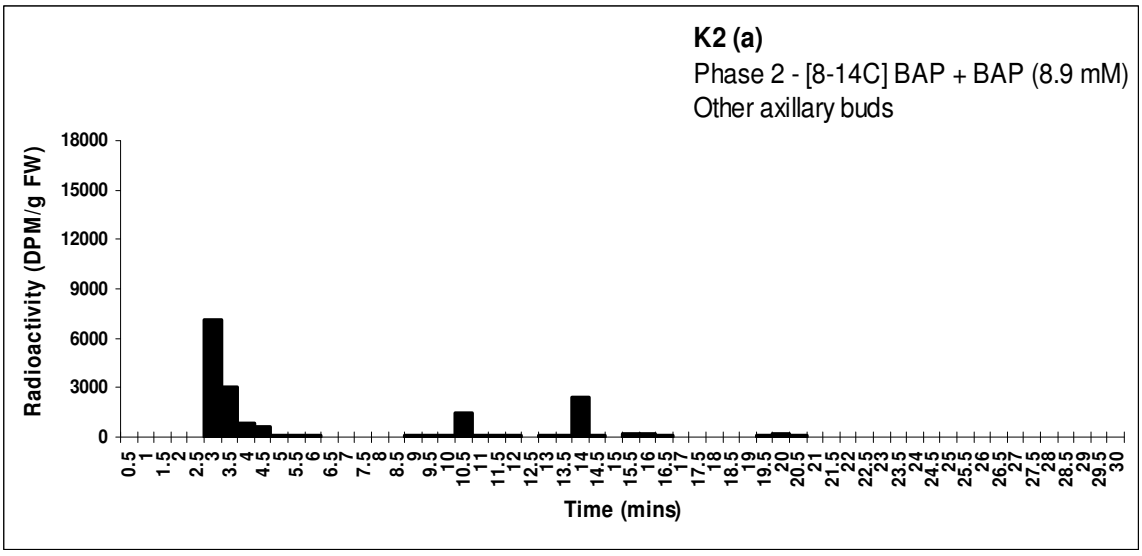

J3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Root
Radioactivity (DPM/g FW)
Time (mins)

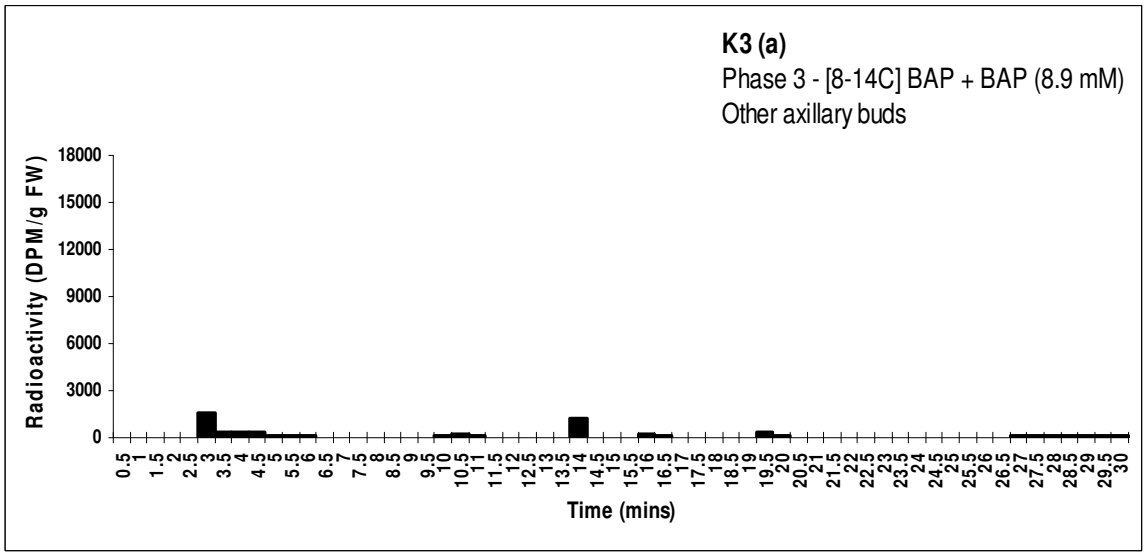

J1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Root
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

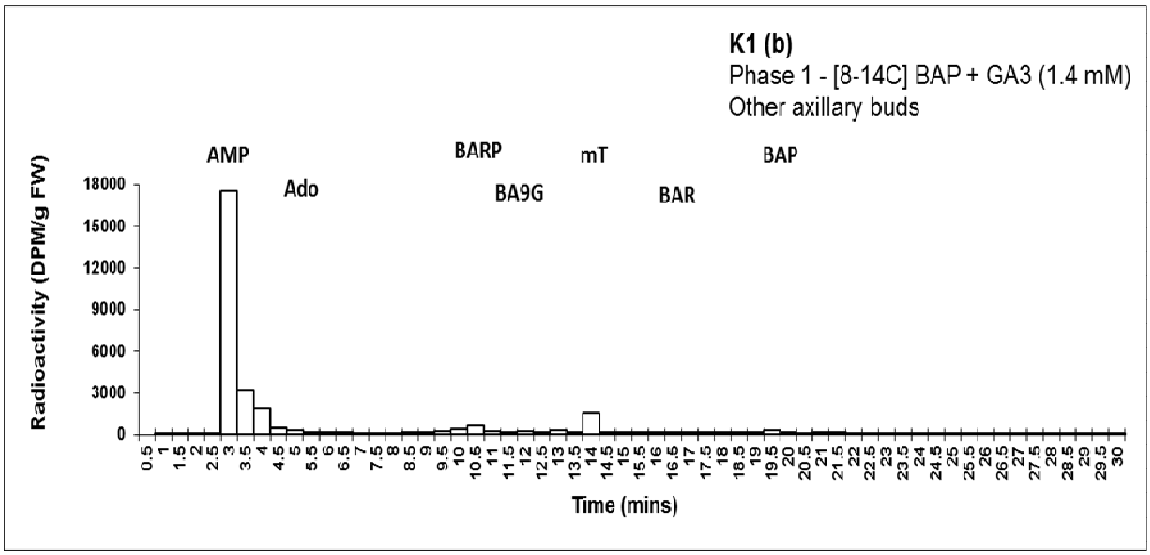

J2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Root
Radioactivity (DPM/g FW)
Time (mins)

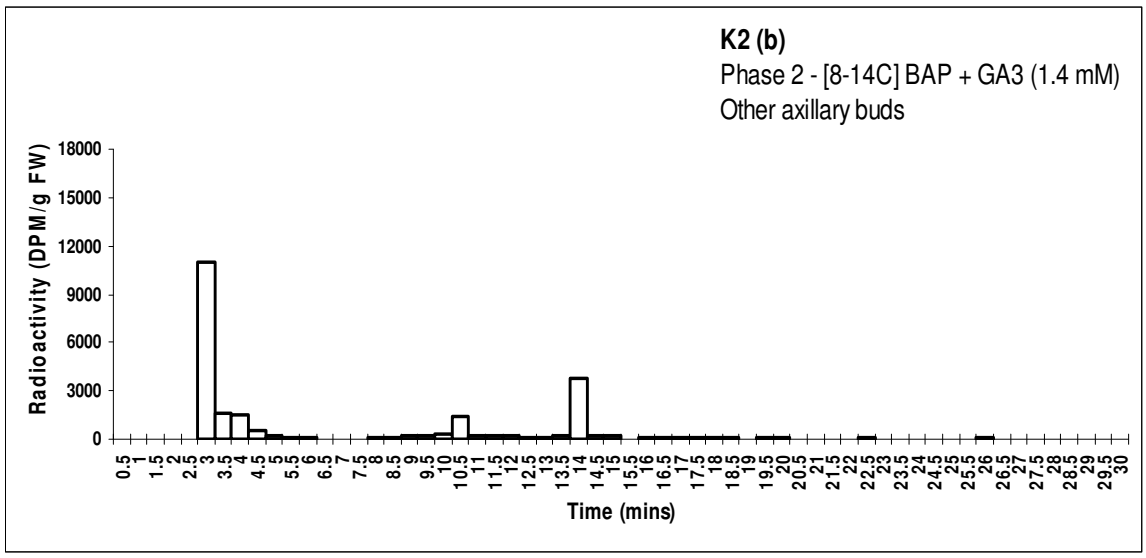

J3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Root
Radioactivity (DPM/g FW)
Time (mins)

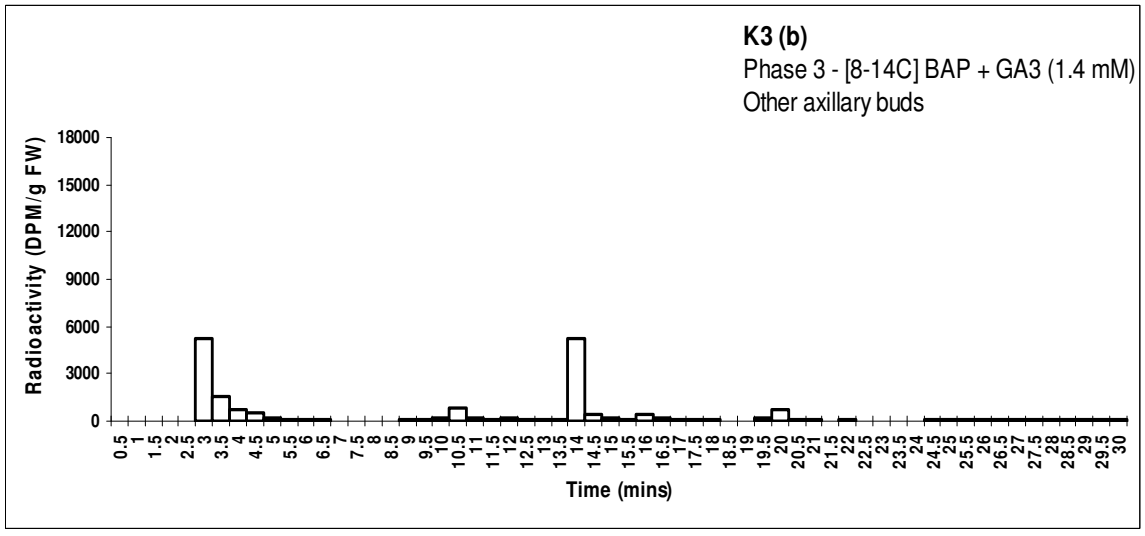

K1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Other axillary buds
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

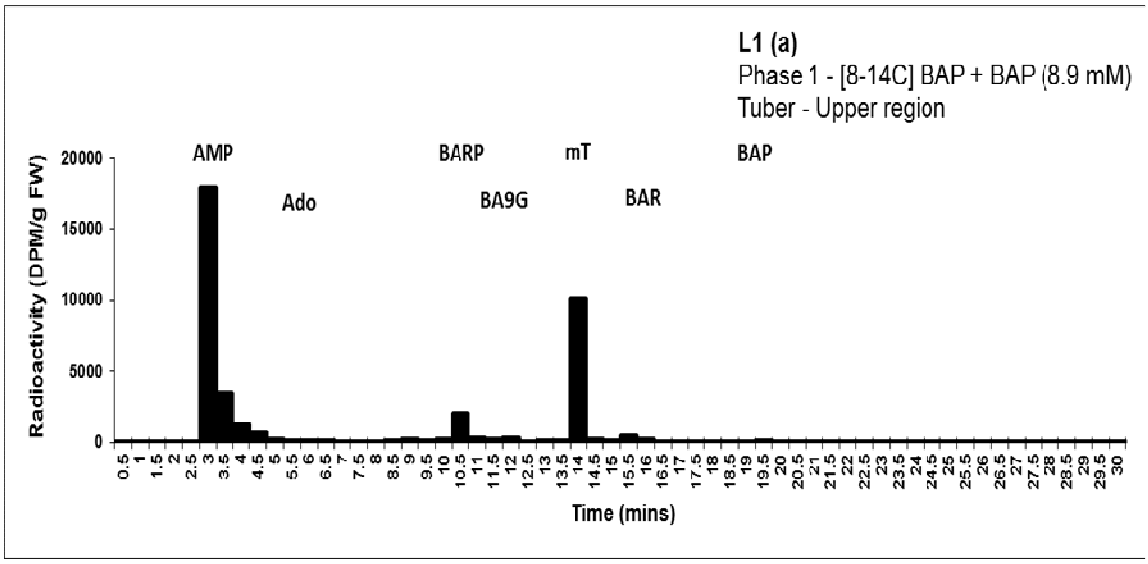

K2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Other axillary buds
Radioactivity (DPM/g FW)
Time (mins)

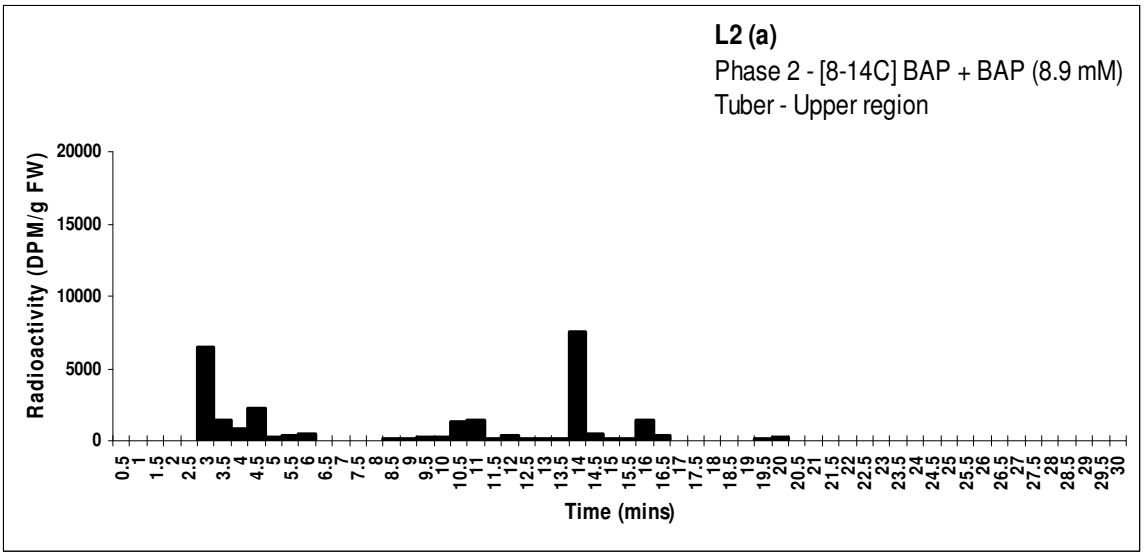

K3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Other axillary buds
Radioactivity (DPM/g FW)
Time (mins)

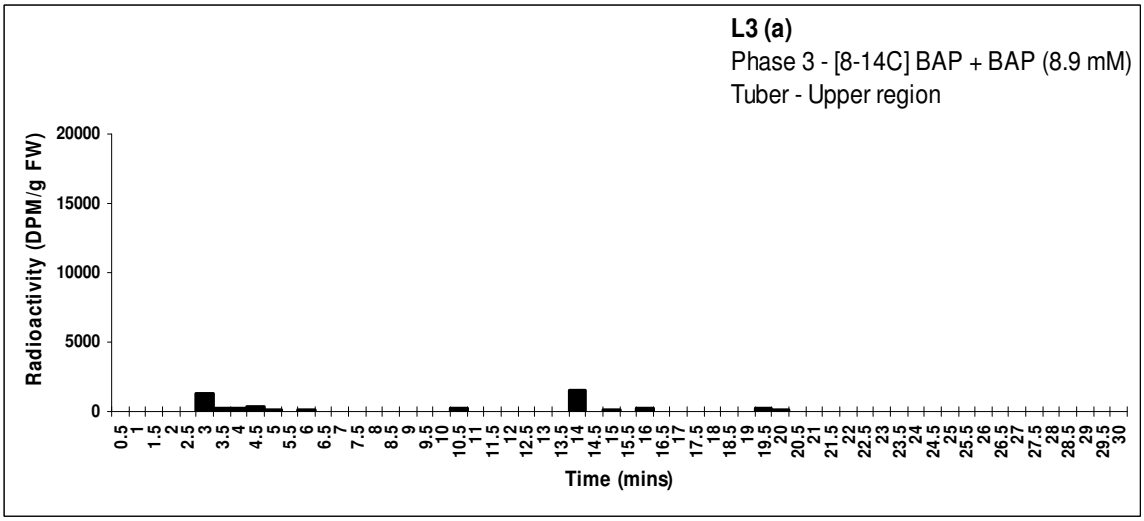

K1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Other axillary buds
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

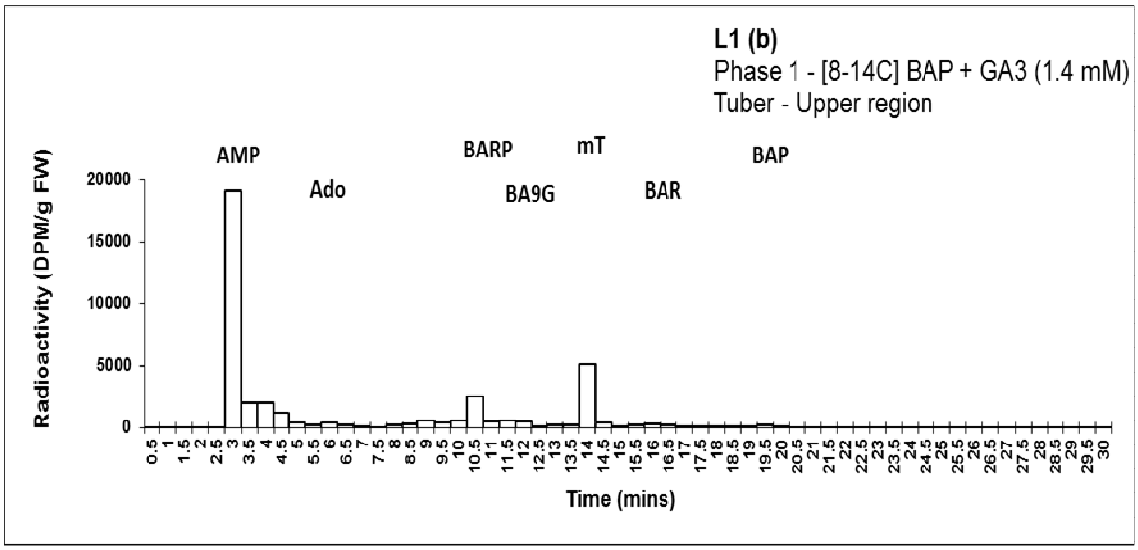

K2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Other axillary buds
Radioactivity (DPM/g FW)
Time (mins)

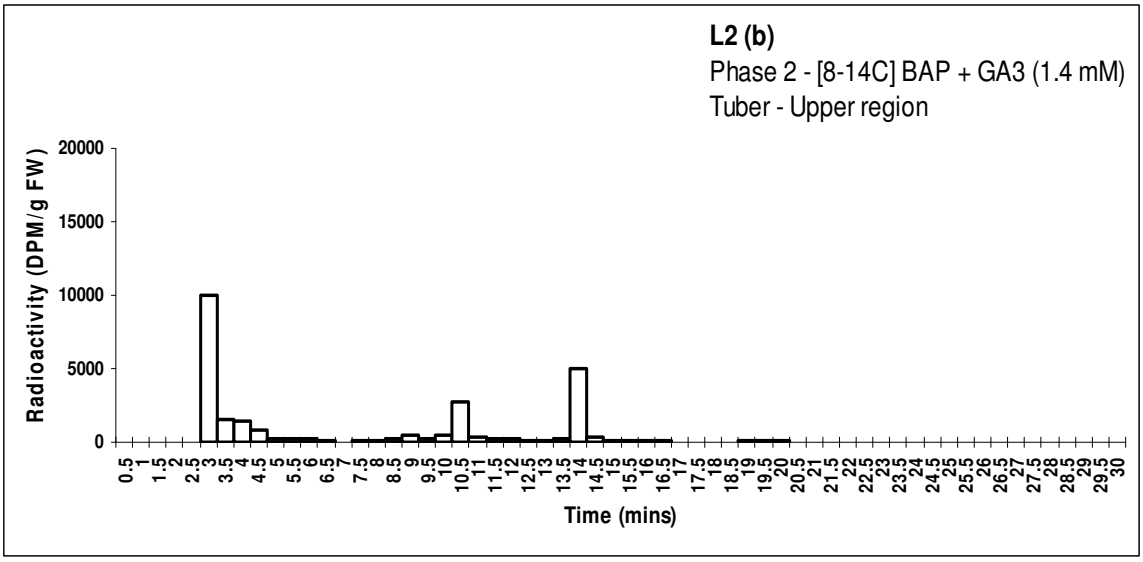

K3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Other axillary buds
Radioactivity (DPM/g FW)
Time (mins)

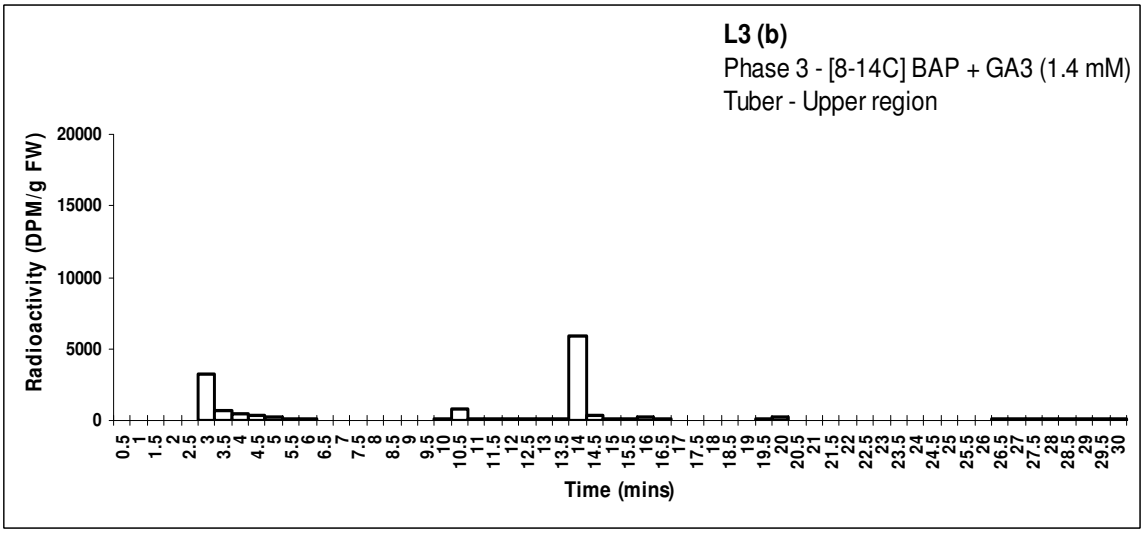

L1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Upper region
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

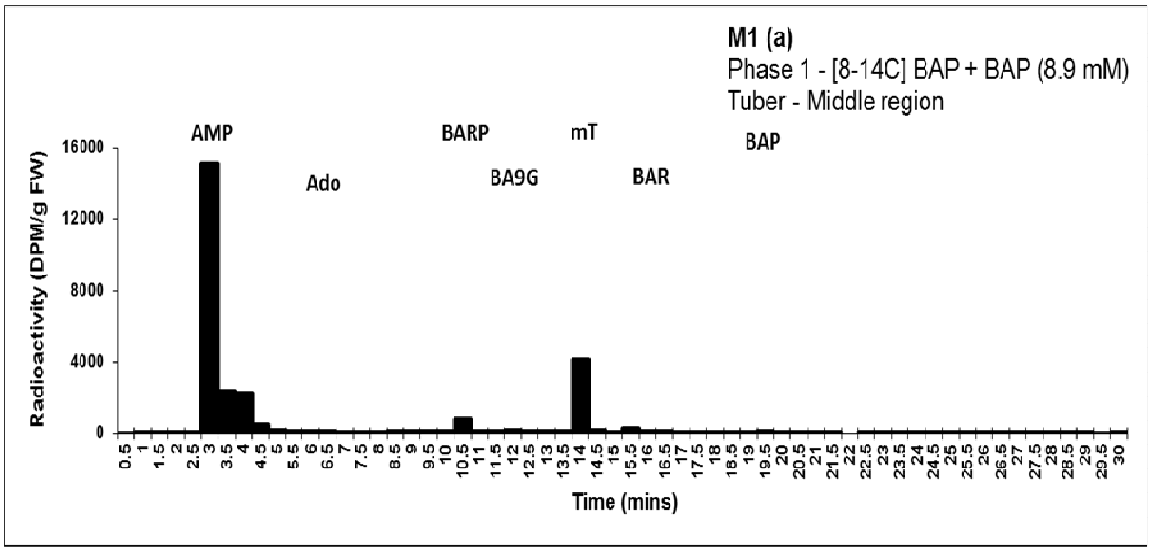

L2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Upper region
Radioactivity (DPM/g FW)
Time (mins)

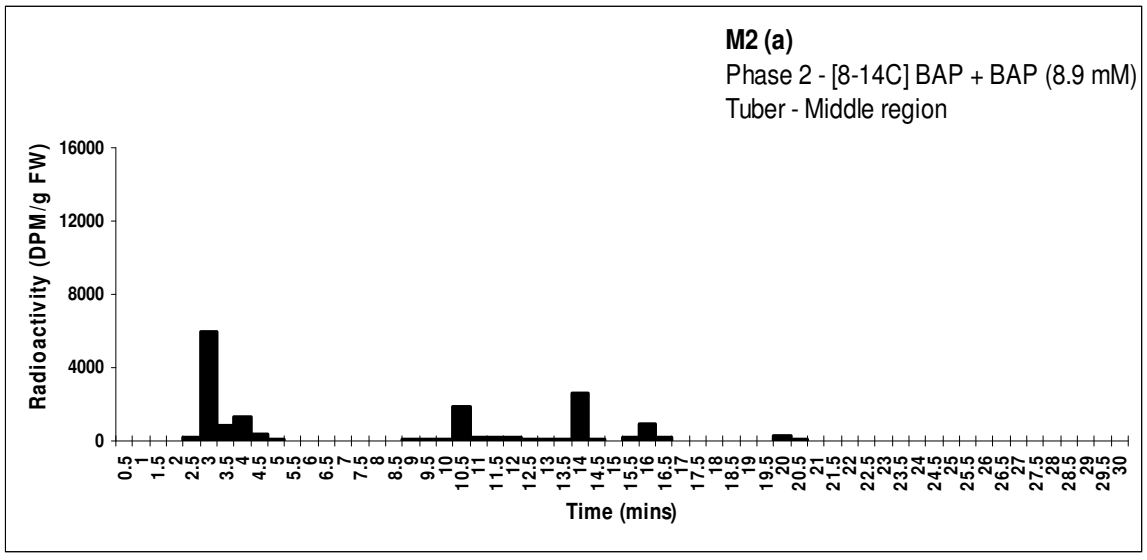

L3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Upper region
Radioactivity (DPM/g FW)
Time (mins)

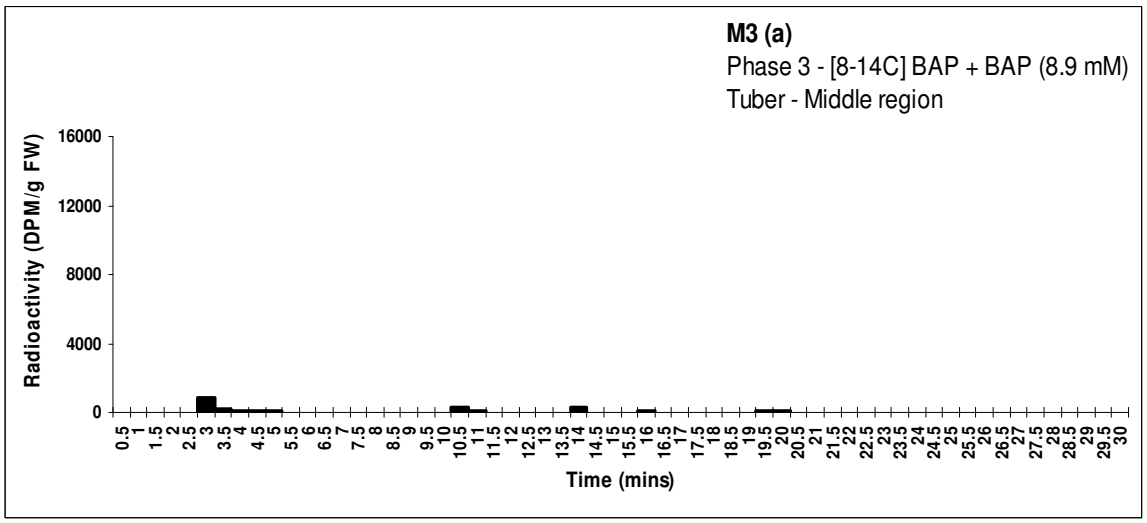

L1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Upper region
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

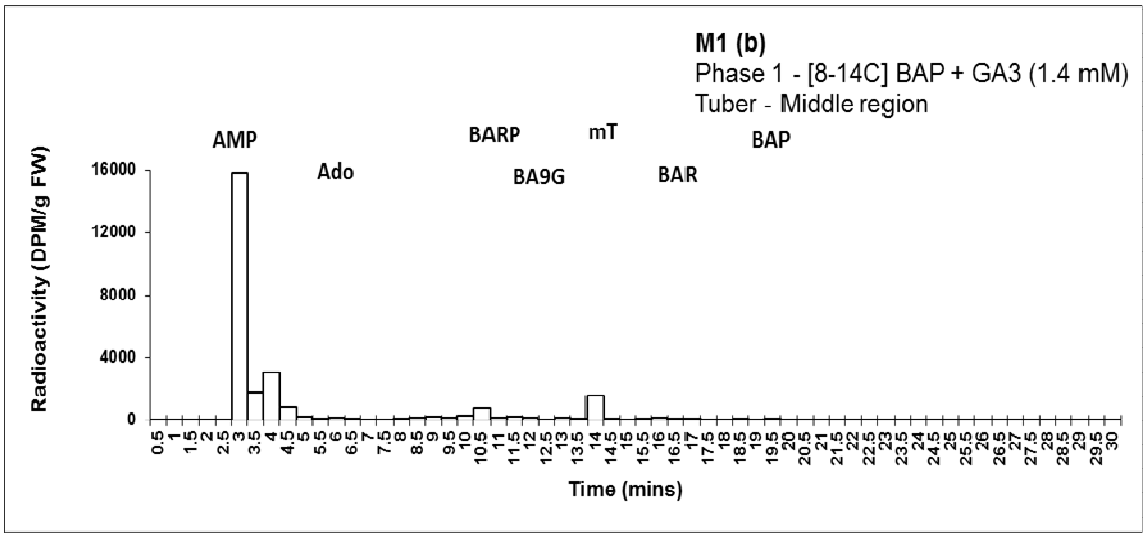

L2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Upper region
Radioactivity (DPM/g FW)
Time (mins)

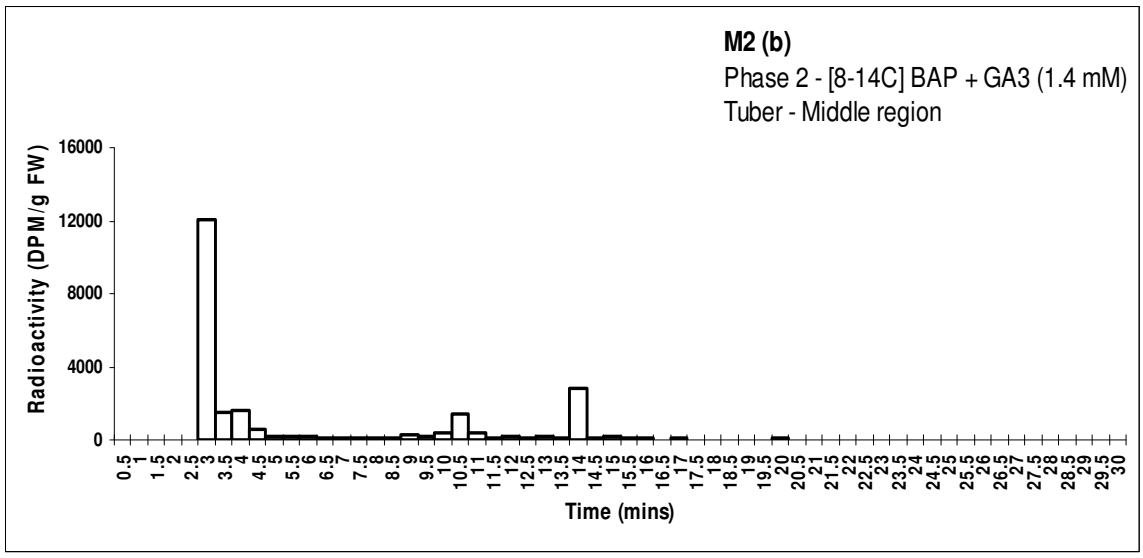

L3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Upper region
Radioactivity (DPM/g FW)
Time (mins)

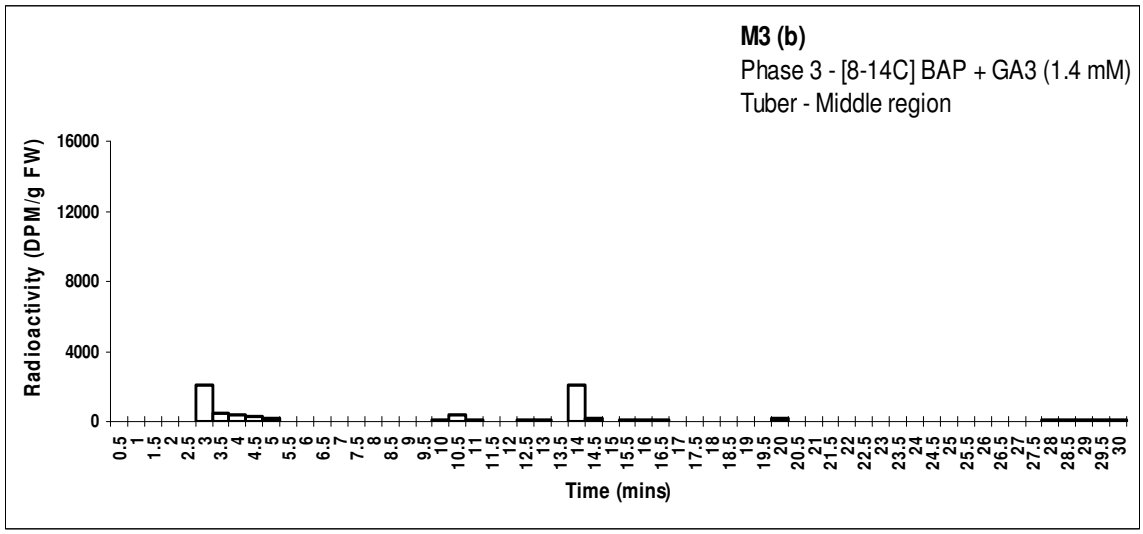

M1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Middle region
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

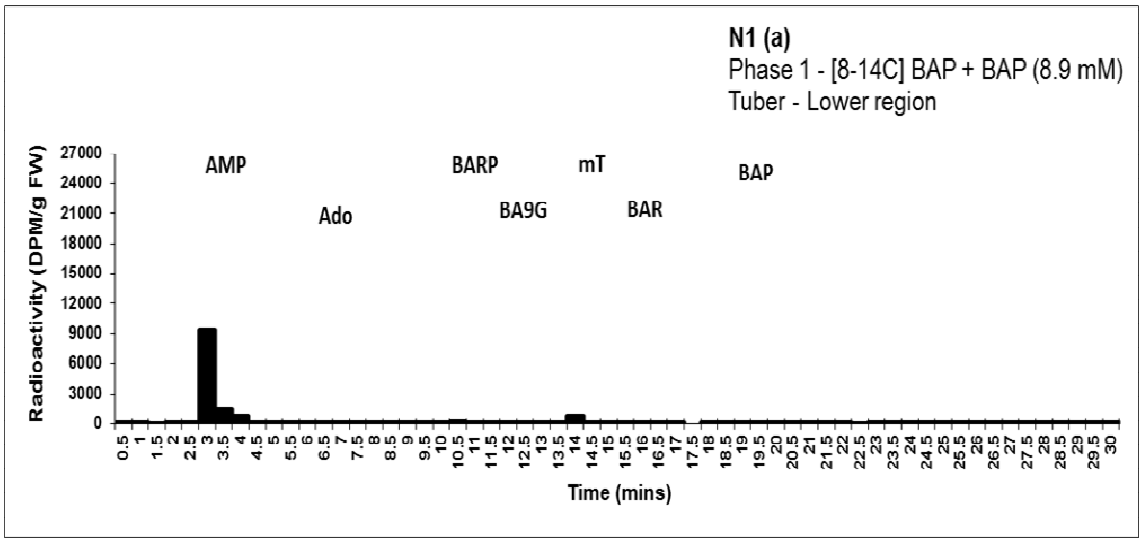

M2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Middle region
Radioactivity (DPM/g FW)
Time (mins)

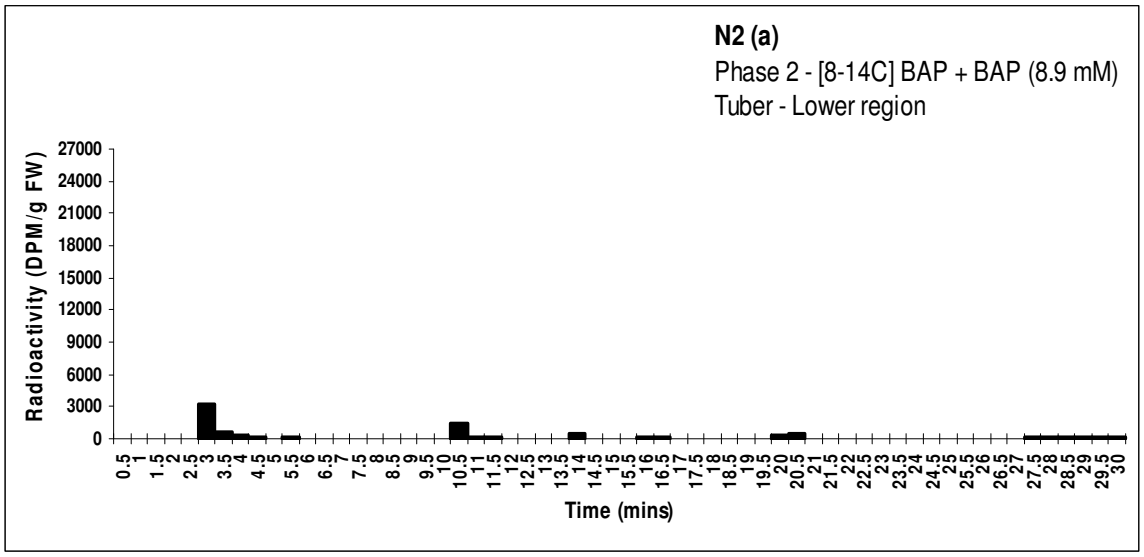

M3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Middle region
Radioactivity (DPM/g FW)
Time (mins)

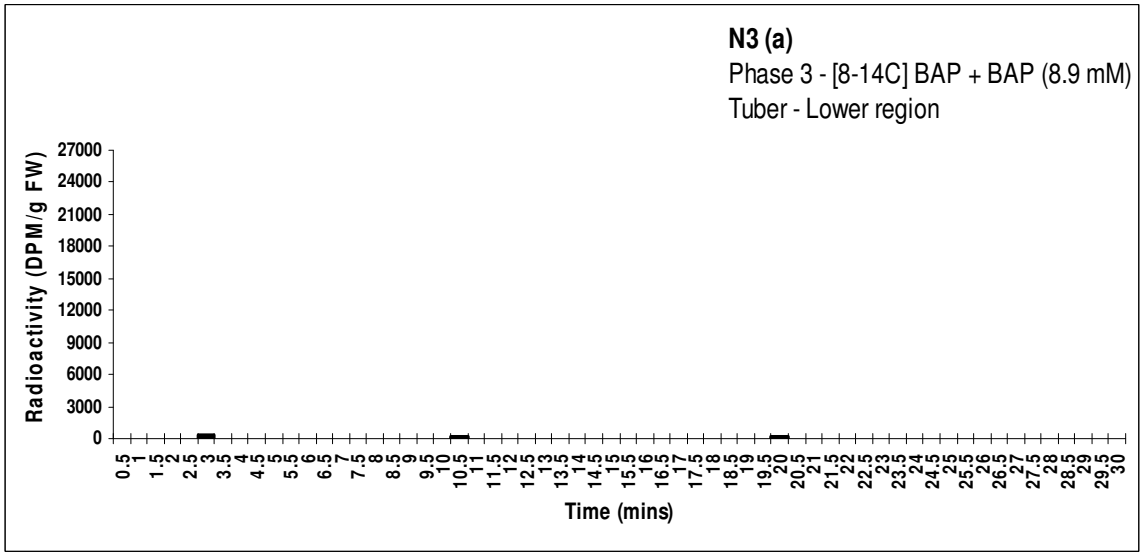

M1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Middle region
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)

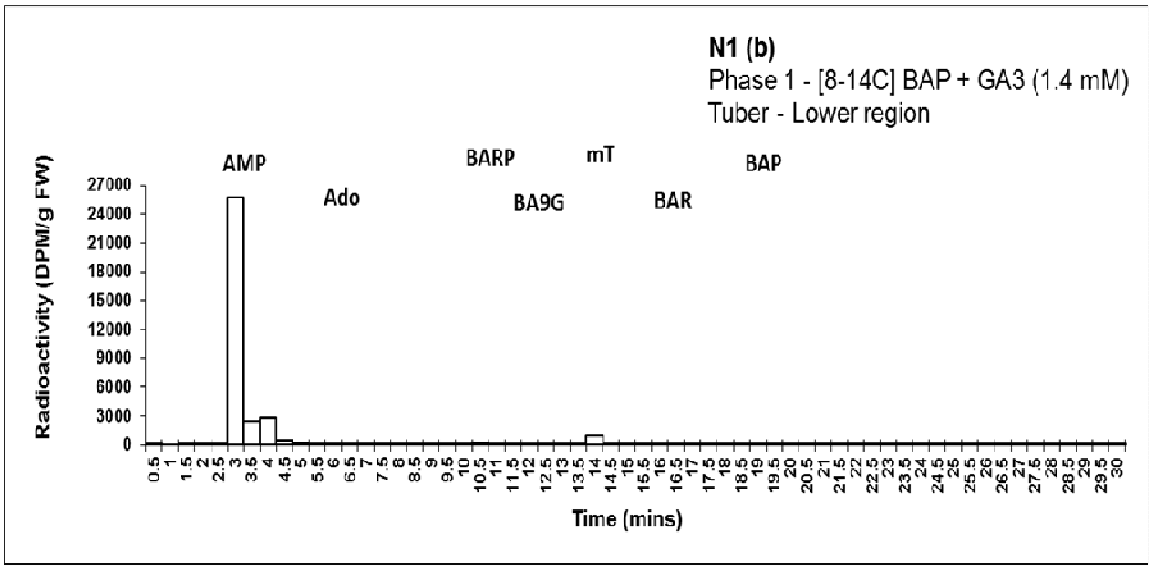

M2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Middle region
Radioactivity (DPM/g FW)
Time (mins)

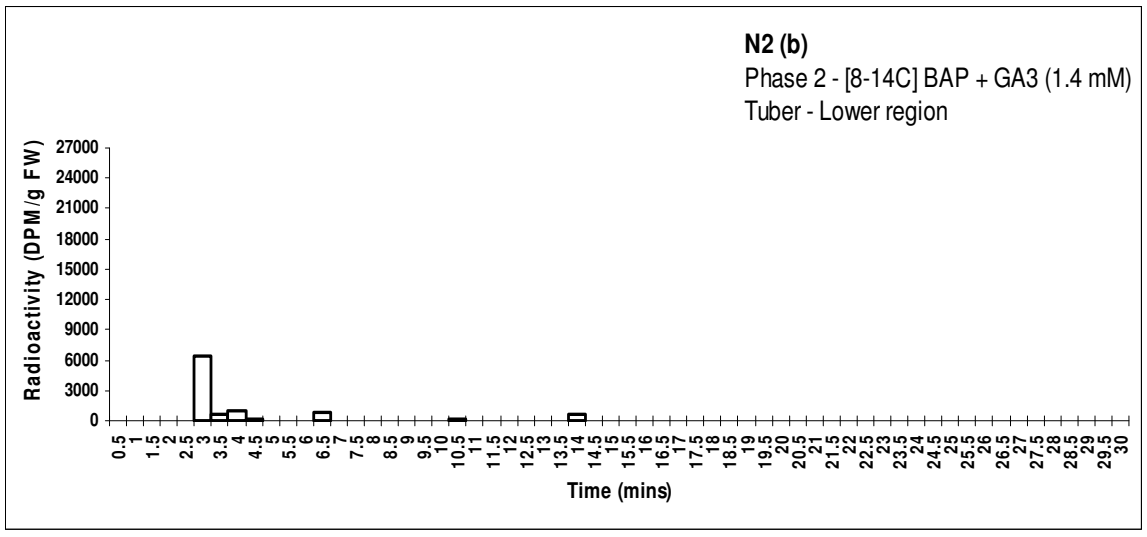

M3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Middle region
Radioactivity (DPM/g FW)
Time (mins)


N1 (a)
Phase 1 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Lower region
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)


N2 (a)
Phase 2 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Lower region
Radioactivity (DPM/g FW)
Time (mins)


N3 (a)
Phase 3 - [8-14C] BAP + BAP (8.9 mM)
Tuber - Lower region
Radioactivity (DPM/g FW)
Time (mins)


N1 (b)
Phase 1 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Lower region
AMP
Ado
BARP
BA9G
mT
BAR
BAP
Radioactivity (DPM/g FW)
Time (mins)


N2 (b)
Phase 2 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Lower region
Radioactivity (DPM/g FW)
Time (mins)

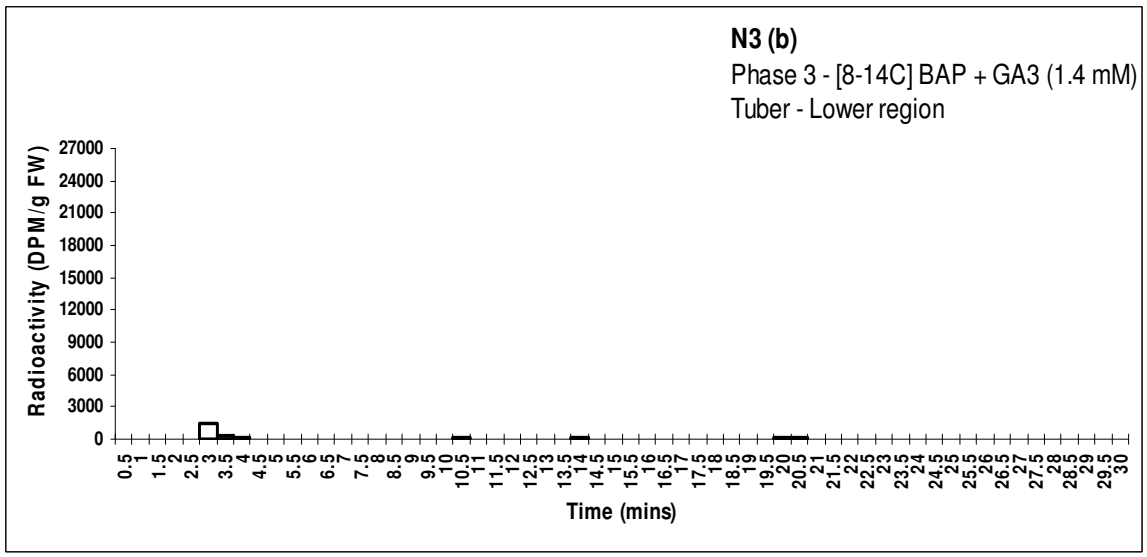

N3 (b)
Phase 3 - [8-14C] BAP + GA3 (1.4 mM)
Tuber - Lower region
Radioactivity (DPM/g FW)
Time (mins)

Fig. 8.6 Metabolic profile of [8-^{14}C] BAP in 1g FW of 14 different plant parts of single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' comprising **A-D** the distal-half, proximal-half, petiole and corresponding axillary bud of leaf 1, **E-H** the distal-half, proximal-half, petiole and corresponding axillary bud of leaf 2, **I** the apical bud, **J** roots, **K** other axillary buds and **L-N** the upper, middle and lower regions of the tuber respectively, constructed along the three phases of growth i.e., phases **1, 2 and 3**, at 10 days after the **(a)** simultaneous application of [8-^{14}C] BAP + BAP (8.9 mM) or **(b)** sequential application of [8-^{14}C] BAP + GA_3 (1.4 mM)

The high radioactivity in the distal halves of leaf 1 (Fig. 8.3A) and leaf 2 (Fig. 8.3E) in phase 3 following the sequential application of GA_3 (1.4 mM), was also due to unmetabolized [8-^{14}C] BAP (Fig. 8.6A3 (b); 8.6E3 (b)). As observed following the application of BAP, mT was the primary metabolite in these halves of leaf 1 in phases 1 (Fig. 8.6A1 (b)) and 2 (Fig. 8.6A2 (b)), whereas BAR was the primary metabolite in phase 3 (Fig. 8.6A3 (b)).

The radioactivity in the proximal halves of leaves 1 and 2 in phase 3, irrespective of the application of BAP (Fig. 8.6B3 (a); F3 (a)) or GA_3 (Fig. 8.6B3 (b); F3 (b)) mainly comprised [8-^{14}C] BAP. The key metabolites identified in this part in phases 1 (Fig. 8.6B1 (a); B1 (b); F1 (a); F1 (b)) and 2 (Fig. 8.6B2 (a); B2 (b); F2 (a); F2 (b)) however mainly comprised AMP, mT, BARP and BA9G (Table 8.2). While the same metabolic profile as observed in the proximal halves was also evident in the petioles of the three phases (Fig. 8.6C1 (a); C2 (a); C3 (a); G1 (a); G2 (a); G3 (a)) following the application of BAP, mT was the key metabolite in this part in phase 3 (Fig. 8.6C3 (a); G3 (a)). This is in contrast to the identification of BAR as the primary metabolite in the distal halves of leaf 1 (Fig. 8.6A3 (a)) and leaf 2 (Fig. 8.6E3 (a)) in phase 3. Following the application of GA_3, the significantly high radioactivity observed in the petiole of leaf 1 in phase 1 than that in phase 2 (Fig. 8.3C) was contributed by AMP (Fig. 8.6C1 (b); C2 (b)). The radioactivity in this part in phase 3 (Fig. 8.3C) was however mainly contributed by mT (Fig. 8.6C3 (b)).

The high radioactivity observed in the corresponding axillary buds of leaf 1 (Fig. 8.3D) and leaf 2 (Fig. 8.3H) in phase 3, following the application of BAP, was mainly contributed by [8-^{14}C] BAP (Fig. 8.6D3 (a); H3 (a)). In these buds in phase 3, the [8-

^{14}C] BAP content was almost 200 times greater than that present in phase 1 (Table 8.3). The key metabolites in these buds in phases 1 (Fig. 8.6D1 (a); H1 (a)) and 2 (Fig. 8.6D2 (a); H2 (a)) however comprised AMP and mT. Following the application of GA_3, a similar metabolic profile as observed following the application of BAP was observed in these buds in their respective phases (Fig. 8.6D1-D3 (b); H1-H3 (b)).

Following the application of BAP, the metabolites in the apical bud across the three phases of growth comprised AMP and mT (Fig. 8.6I1-3 (a)). However the amount of AMP and mT was 2-4 folds higher in phase 1 than in phase 3 (Table 8.3). Following the application of GA_3, AMP and mT were again the key metabolites identified across the three phases (Fig. 8.6I1-3 (b)). In contrast to the application of BAP however, the mT fraction was 3 folds higher in phase 3 than in phase 1 (Table 8.3).

Following the application of BAP, the significantly high amount of radioactivity observed in the roots during phase 1 (Fig. 8.3J), was contributed by an 8-10 fold higher amount of AMP and mT in phase 1 (Fig. 8.6 J1 (a)) than that in phase 3 (Fig. 8.6 J3 (a); Table 8.3). A similar scale of difference in the amount of AMP was also noticed between phases 1(Fig. 8.6J1 (b)) and 3 (Fig. 8.6J3 (b)) following the application of GA_3. However, the difference between the amount of mT between phases 1 and 3 was only 2 fold (Table 8.3).

The significantly high radioactivity observed in the other axillary buds in phase 1, following the application of BAP (Fig. 8.3K) was, as observed in the apical bud (Fig. 8.6I1 (a)), mainly contributed by AMP and mT (Fig. 8.6K1 (a)). While these were the key metabolites identified in phases 2 (Fig. 8.6K2 (a)) and 3 (Fig. 8.6K3 (a)) as well, the amount of AMP and mT in phase 1 was 8 and 1.5 fold higher than that in phase 3, respectively (Table 8.3). In contrast, following the application of GA_3, there was a 2.5 and 1.5 fold increase in the amount of mT between phases 1 (Fig. 8.6K1 (b)) and 2 (Fig. 8.6K2 (b)) and, between phases 2 (Fig. 8.6K2 (b)) and 3 (Fig. 8.6K3 (b)), respectively (Table 8.3). A concomitant decline in the amount of AMP was also observed from phase 1 through to phase 3 (Fig. 8.6K1-3 (b); (Table 8.3).

In the upper region of the tuber, following the application of BAP, the amount of AMP and mT declined concurrently with the progressive transition of the plants from phase 1 through to phase 3 (Fig. 8.6L1-3 (a); Table 8.3). The amount of AMP was 14 times higher in phase 1 than in phase 3, whereas the amount of mT was at least 6 times higher

in phase 1 (Fig. 8.6L1 (a)) than in phase 3 (Fig. 8.6L3 (a); Table 8.3). Following the application of GA_3, the amount of mT in this region in phase 1 (Fig. 8.6L1 (b)) was almost on par with that in phase 3 (Fig. 8.6L3 (b). A similar trend where the amount of AMP and mT declined parallel to the transition of plants from phase 1 through to phase 3 following the application of BAP, and the amount of mT was on par or higher in phase 3 compared to that in phase 1 following the application of GA_3, continued also in the middle (Fig. 8.6M1-M3 (a); M1-M3 (b)) and lower (Fig. 8.6N1-N3 (a); N1-N3 (b)) regions of the tuber (Table 8.3).

Overall, the high radioactivity retained in the distal halves of leaves 1 and 2 in phase 3, irrespective of the application of BAP (8.9 mM) or GA_3 (1.4 mM) was mainly contributed by unmetabolized [8-^{14}C] BAP. While mT was the primary metabolite in this part in phases 1 and 2, following the application of BAP or GA_3, BAR was the primary metabolite observed in phase 3. Irrespective of the application of BAP or GA_3, a high amount of [8-^{14}C] BAP was also found in the corresponding axillary buds of leaves 1 and 2 in phase 3, than in phases 1 and 2. mT was the key metabolite in these buds in phases 1 and 2. In all the remaining subterranean plant parts, following the application of BAP, a higher amount of AMP and mT was identified in phase 1 compared to that in phase 3. On the contrary, following the application of GA_3, a higher amount of mT was identified in all these parts in phase 3, compared to that in phase 1.

8.3.4.2 Metabolism of [8-^{14}C] BAP as influenced by the treatment applications within each phase

Within phase 1, though mT was the major metabolite in the distal halves of leaf 1, irrespective of the application of BAP (8.9 mM) (Fig. 8.6A1 (a)) or GA_3 (1.4 mM) (Fig. 8.6A1 (b)), its amount was at least 4.5 times higher following the application of BAP, compared to that following the application of GA_3 (Table 8.3). In leaf 2 however, in contrast to leaf 1 (Fig. 8.6A1), application of GA_3 caused a 5 fold increase in the amount of mT (Fig. 8.6E1 (b)) compared to the application of BAP (Fig. 8.6E1 (a); Table 8.3). In phase 2 also, application of GA_3 caused a 2.5 and 5 fold increase in mT in leaves 1 (Fig. 8.6A2 (b)) and 2 (Fig. 8.6E2 (b)) compared to the application of BAP (Fig. 8.6A2 (a); E2 (a)), respectively (Table 8.3). In phase 3, BAR was the major metabolite following the application of both BAP (Fig. 8.6A3 (a); E3 (a)) and GA_3 (Fig. 8.6A3 (b); E3 (b)) in both the leaves.

Within each of the three phases of growth, there was no change in the metabolic profile of [8-^{14}C] BAP in the proximal halves of leaf 1 between the application of BAP (Fig. 8.6B1-B3 (a)) and GA_3 (Fig. 8.6B1-B3 (b)). AMP, mT, BARP and BA9G were the key metabolites in phases 1 and 2, whereas BAR was the key metabolite in phase 3. As observed in leaf 1, a considerable change in the metabolic profile was also not observed between the application of BAP (Fig. 8.6F1-F3 (a)) and GA_3 (Fig. 8.6F1-F3 (b)) in the proximal halves of leaf 2. Following the application of BAP or GA_3, AMP and mT comprised the key metabolites in the petioles of leaf 1 across the three phases of growth (Fig. 8.6C1-C3 (a); C1-C3 (b)). However, the amount of mT was at least 2 folds higher after the application of GA_3 in phase 3 (Fig. 8.6C3 (b)), compared to the application of BAP (Fig. 8.6C3 (a); Table 8.3). The significantly high radioactivity present in the petioles of leaf 2, following the application of GA_3 in phases 1 and 3 (Fig. 8.3G) was contributed by AMP and mT in phase 1 (Fig. 8.6G1 (b)) and unmetabolized [8-^{14}C] BAP in phase 3 (Fig. 8.6G3 (b)), respectively.

Between the applications of BAP and GA_3, there was no change in the metabolic profile in the corresponding axillary bud of leaf 1 in phases 1 (Fig. 8.6D1 (a); (b)) and 2 (Fig. 8.6D2 (a); (b)). While AMP and mT were the major metabolites in this bud in phases 1 and 2, BAR was one of the major metabolite in phase 3 (Fig. 8.6D3 (a); (b)). A similar metabolic profile was also observed in the corresponding axillary bud of leaf 2 within phases 1 (Fig. 8.6H1 (a); (b)) and 2 (Fig. 8.6H2 (a); (b)). Within phase 3 however, the amount of unmetabolized [8-^{14}C] BAP in the corresponding axillary buds of both leaves was at least 6 times higher (Fig. 8.6D3 (a); H3 (a)) following the application of BAP compared to that after the application of GA_3 (Fig. 8.6D3 (b); H3 (b); Table 8.3).

As observed in the corresponding axillary buds of leaves 1 and 2, irrespective of the application of BAP or GA_3, AMP and mT were the key metabolites in the apical bud within phases 1 (Fig. 8.6I1 (a); (b)) and 2 (Fig. 8.6I2 (a); (b)). While the amount of mT was 1.5 times higher in phase 1 following the application of BAP (Fig. 8.6I1 (a)) compared to that after the application of GA_3 (Fig. 8.6I1 (b)), GA_3 resulted in a 3.5 fold increase in the amount of mT in phase 3 (Fig. 8.6I3 (b)) compared to the application of BAP (Fig. 8.6I3 (a); Table 8.3).

Apart from a 3 fold increase in the amount of mT observed in phase 1 (Table 8.3), following the application of BAP (Fig. 8.6J1 (a)) compared to that after the application

of GA_3 (Fig. 8.6J1 (b)), a considerable change in the metabolic profile was not observed in the roots (Fig. 8.6J2-J3 (a); (b)).

The high radioactivity observed in the other axillary buds following the application of GA_3 in phase 3, compared to that after the application of BAP (Fig. 8.3K), was contributed by a 4 and 3 fold increase in the amount of mT and AMP, respectively (Fig. 8.6K3 (a); (b); Table 8.3). Within phases 1 and 2 however, a considerable change in the metabolic profile between the application of BAP and GA_3 was not noticed.

A 2 fold increase in the amount of mT was noticed in the upper region of tuber following the application of BAP in phase 1 (Fig. 8.6L1 (a)), compared to that after the application of GA_3 (Fig. 8.6L1 (b); Table 8.3). In contrast, a 4 fold increase in the amount of mT was observed in phase 3 following the application of GA_3 (Fig. 8.6L3 (b)) compared to that after the application of BAP (Fig. 8.6L3 (a); Table 8.3). This trend where a higher amount of mT was present following the application of BAP in phase 1 compared to that after the application of GA_3, and the vice-versa in phase 3, was also observed in the middle (Fig. 8.6M1-M3 (a); (b)) and lower (Fig. 8.6N1-N3 (a); (b)) regions of the tuber.

Overall, AMP and mT were the key metabolites of [8-^{14}C] BAP in most of the plant parts, irrespective of the application of a simultaneous application of BAP (8.9 mM) or a sequential application of GA_3 (1.4 mM). The radioactivity in the distal half of the leaves in phase 3 and their corresponding axillary buds, following the application of BAP or GA_3, was mainly contributed by unmetabolized [8-^{14}C] BAP. In the remaining subterranean plant parts, the application of BAP resulted in a larger amount of mT in phase 1 compared to the application of GA_3. In phase 3, GA_3 resulted in a larger amount of mT in these parts compared to the application of BAP.

Table 8.3 Amount of radioactivity available and the corresponding metabolic profile of [8-^{14}C] BAP in 14 different plant parts of single-shoot seedlings of *Zantedeschia* cv. 'Best Gold' comprising the distal-half, proximal-half, petiole and corresponding axillary bud of leaves 1 and 2, the apical bud, roots, other axillary buds and the upper, middle and lower regions of the tuber respectively, along the three phases of growth i.e., phases **1, 2** and **3**, at 10 days after the **(A)** simultaneous application of [8-^{14}C] BAP + BAP (8.9 mM) or **(B)** sequential application of [8-^{14}C] BAP + GA_3 (1.4 mM)

Phase	Plant part	A [8-14C] BAP +BAP (8.9 mM)									
		Fresh weight (FW in g ± SE)	Availability of radioactivity		Metabolic profile of [8-14C] BAP (DPM × 10^{-3}/g FW of plant part)						
			DPM × 10^{-3}/FW of plant part ± SE	DPM × 10^{-3}/g FW of plant part ± SE	AMP	Ado	BARP	BA9G	mT	BAR	[8-14C] BAP
1	**Leaf 1**										
	Distal half	0.46 ± 0.07	663.3 ± 124.7	1639.3 ± 461.6	36.9	6.5	52.8	20.8	442.3	41.8	94.7
	Proximal half	0.38 ± 0.06	12.9 ± 6.0	30.4 ± 10.4	4.5	0.2	1.2	0.8	2.8	0.6	1.7
	Petiole	0.45 ± 0.07	12.7 ± 2.8	28.5 ± 5.9	10.6	0.3	1.2	0.8	1.4	0.4	0.9
	Axillary bud	0.05 ± 0.00	2.5 ± 0.5	57.1 ± 13.6	18.1	0.5	1.2	0.7	5.0	0.8	0.9
	Leaf 2										
	Distal half	0.33 ± 0.05	1142.5 ± 108.6	3780.5 ± 709.7	49.9	87.2	70.1	24.9	80.6	328.7	810.5
	Proximal half	0.32 ± 0.02	10.8 ± 6.7	32.7 ± 19.3	1.2	0.3	0.5	0.5	0.8	0.5	2.4
	Petiole	0.31 ± 0.03	6.9 ± 1.7	22.4 ± 4.9	3.7	0.1	0.5	0.3	1.0	0.3	0.7
	Axillary bud	0.11 ± 0.03	3.7 ± 0.6	39.3 ± 10.8	5.4	0.1	0.3	0.1	1.8	0.1	0.3
	Apical bud	0.13 ± 0.02	8.5 ± 1.0	74.3 ± 15.5	11.0	0.2	0.5	0.2	2.4	0.2	0.2
	Root	0.98 ± 0.02	22.4 ± 1.8	22.9 ± 2.3	7.4	0.1	0.2	1.4	1.1	0.1	0.1
	Other axillary buds	0.41 ± 0.11	19.6 ± 6.6	46.5 ± 6.5	12.0	0.1	0.7	0.1	1.8	0.1	0.2
	Tuber										
	Upper region	0.35 ± 0.04	17.2 ± 1.5	52.0 ± 8.5	17.9	0.2	2.1	0.3	10.1	0.5	0.2
	Middle region	0.77 ± 0.09	26.9 ± 4.8	34.5 ± 3.2	15.1	0.1	0.8	0.1	4.1	0.3	0.1
	Lower region	0.45 ± 0.04	10.3 ± 0.7	23.3 ± 3.0	9.4	0.1	0.2	0.0	0.8	0.1	0.0
2	**Leaf 1**										
	Distal half	0.50 ± 0.05	479.6 ± 251.3	993.7 ± 526.9	36.0	3.7	49.2	14.9	90.6	31.6	29.9
	Proximal half	0.41 ± 0.06	4.9 ± 0.8	12.8 ± 3.1	2.4	0.2	1.5	0.4	1.2	0.6	0.6
	Petiole	0.52 ± 0.03	10.0 ± 2.3	19.2 ± 4.3	4.9	0.2	1.6	0.2	1.4	0.4	0.3
	Axillary bud	0.03 ± 0.01	2.5 ± 0.5	113.2 ± 30.8	11.2	0.0	1.6	0.1	5.6	0.3	0.6
	Leaf 2										
	Distal half	0.71 ± 0.08	822.2 ± 70.9	1232.6 ± 206.7	47.6	17.6	34.5	8.3	48.6	152.8	154.9
	Proximal half	0.54 ± 0.11	15.1 ± 9.0	36.8 ± 26.6	7.6	0.6	14.6	2.0	3.8	4.1	5.4
	Petiole	0.56 ± 0.08	17.3 ± 3.7	31.4 ± 5.7	5.3	0.3	4.3	0.4	2.7	1.2	2.9
	Axillary bud	0.06 ± 0.02	3.0 ± 0.5	58.3 ± 15.1	16.3	0.5	4.9	0.3	8.4	0.6	1.4
	Apical bud	0.43 ± 0.06	15.7 ± 1.7	37.4 ± 2.5	14.5	0.2	3.0	0.1	2.6	0.7	0.3
	Root	1.00 ± 0.00	6.4 ± 0.6	6.4 ± 0.6	1.4	0.0	0.1	0.0	0.6	0.1	0.1
	Other axillary buds	0.67 ± 0.14	20.8 ± 5.7	30.9 ± 3.4	7.1	0.1	1.5	0.1	2.4	0.2	0.2
	Tuber										
	Upper region	1.00 ± 0.00	37.6 ± 4.8	37.6 ± 4.8	6.5	0.4	1.4	0.3	7.5	1.4	0.2
	Middle region	1.00 ± 0.00	22.8 ± 2.6	22.8 ± 2.6	6.0	0.0	1.8	0.2	2.6	1.0	0.3
	Lower region	1.00 ± 0.00	8.3 ± 2.2	8.3 ± 2.2	3.2	0.1	1.5	0.1	0.5	0.2	0.4
3	**Leaf 1**										
	Distal half	0.37 ± 0.09	1982.0 ± 583.8	6515.3 ± 1769.5	102.8	106.5	25.1	25.1	25.7	394.9	897.4
	Proximal half	0.28 ± 0.09	6.9 ± 2.4	31.4 ± 10.1	0.8	0.3	0.4	0.4	0.5	1.5	4.8
	Petiole	0.39 ± 0.09	8.2 ± 1.4	24.9 ± 6.7	1.8	0.1	0.5	0.1	2.5	1.0	1.9
	Axillary bud	0.01 ± 0.00	1.4 ± 0.4	106.7 ± 9.9	6.3	60.7	3.5	3.3	8.7	25.1	99.7
	Leaf 2										
	Distal half	0.38 ± 0.06	2298.3 ± 501.9	6761.0 ± 2017.2	47.4	112.5	16.9	23.9	31.3	229.8	1429.6
	Proximal half	0.30 ± 0.02	7.2 ± 2.1	24.4 ± 7.5	0.8	0.5	0.3	0.3	0.5	1.8	8.9
	Petiole	0.46 ± 0.02	7.7 ± 1.4	17.3 ± 3.9	0.8	0.1	0.4	0.1	2.2	0.7	4.2
	Axillary bud	0.04 ± 0.02	2.0 ± 0.5	70.1 ± 21.7	2.7	1.0	1.2	1.5	2.4	6.6	52.6
	Apical bud	0.43 ± 0.08	14.1 ± 5.5	30.2 ± 7.9	2.8	0.2	0.3	0.1	1.3	0.5	1.9
	Root	1.00 ± 0.00	5.9 ± 1.5	5.9 ± 1.5	0.7	0.0	0.1	0.0	0.1	0.2	1.1
	Other axillary buds	0.91 ± 0.09	22.4 ± 6.5	24.5 ± 6.0	1.5	0.1	0.2	0.1	1.3	0.2	0.3
	Tuber										
	Upper region	1.00 ± 0.00	19.0 ± 3.8	19.0 ± 3.8	1.2	0.1	0.2	0.1	1.5	0.2	0.2
	Middle region	1.00 ± 0.00	8.0 ± 0.7	8.0 ± 0.7	0.8	0.0	0.2	0.0	0.3	0.1	0.1
	Lower region	1.00 ± 0.00	2.9 ± 0.6	2.9 ± 0.6	0.3	0.0	0.1	0.0	0.0	0.0	0.1

Where SE is provided, *n = 4*; Otherwise *n = 1*.

Phase	Plant part	B [8-14C] BAP +GA3 (1.4 mM)									
		Fresh weight (FW in g ± SE)	Availability of radioactivity		Metabolic profile of [8-14C] BAP (DPM × 10^{-3}/g FW of plant part)						
			DPM × 10^{-3}/FW of plant part ± SE	DPM × 10^{-3}/g FW of plant part ± SE	AMP	Ado	BARP	BA9G	mT	BAR	[8-14C] BAP
1	**Leaf 1**										
	Distal half	0.65 ± 0.02	633.1 ± 134.2	972.9 ± 210.5	85.6	4.8	13.0	56.9	96.7	19.7	17.3
	Proximal half	0.44 ± 0.04	18.0 ± 2.6	41.5 ± 7.0	4.7	0.4	0.9	2.0	1.6	1.0	1.7
	Petiole	0.64 ± 0.04	28.3 ± 2.1	44.8 ± 4.4	14.4	0.3	0.7	0.5	0.9	0.2	0.4
	Axillary bud	0.03 ± 0.01	2.7 ± 0.6	116.8 ± 29.6	25.5	0.7	1.7	0.8	3.6	1.2	1.4
	Leaf 2										
	Distal half	0.31 ± 0.01	2087.4 ± 335.2	6809.9 ± 1010.4	155.3	106.8	37.6	106.1	446.8	499.9	724.2
	Proximal half	0.32 ± 0.02	12.3 ± 3.0	39.5 ± 10.7	2.9	0.2	0.7	0.7	3.5	1.2	3.5
	Petiole	0.36 ± 0.02	16.8 ± 1.5	46.8 ± 4.2	10.5	0.2	1.4	0.3	3.9	0.3	0.8
	Axillary bud	0.04 ± 0.01	2.8 ± 0.5	73.8 ± 13.5	19.5	0.4	1.1	0.8	3.0	1.0	2.4
	Apical bud	0.07 ± 0.01	4.5 ± 0.9	72.2 ± 11.6	17.5	0.3	0.8	0.5	1.6	0.9	1.6
	Root	1.00 ± 0.00	19.5 ± 1.0	19.5 ± 1.0	6.8	0.1	0.2	0.1	0.4	0.1	0.1
	Other axillary buds	0.71 ± 0.18	24.4 ± 4.8	36.7 ± 4.3	17.6	0.2	0.7	0.3	1.6	0.2	0.3
	Tuber										
	Upper region	0.58 ± 0.08	34.6 ± 5.3	59.7 ± 4.1	19.2	0.4	2.6	0.5	5.1	0.3	0.2
	Middle region	0.98 ± 0.03	46.3 ± 4.8	47.2 ± 4.0	15.8	0.2	0.8	0.2	1.6	0.2	0.1
	Lower region	0.69 ± 0.10	31.0 ± 6.8	43.6 ± 4.7	25.7	0.1	0.2	0.1	1.0	0.1	0.1
2	**Leaf 1**										
	Distal half	0.40 ± 0.05	540.6 ± 124.6	1340.2 ± 291.1	161.3	6.0	13.3	25.2	240.0	18.3	13.6
	Proximal half	0.32 ± 0.04	4.9 ± 1.0	15.1 ± 2.7	1.4	0.1	0.4	0.2	0.7	0.2	0.1
	Petiole	0.46 ± 0.07	11.7 ± 2.6	26.2 ± 6.3	4.2	0.2	1.2	0.2	2.0	0.2	0.1
	Axillary bud	0.02 ± 0.01	2.0 ± 0.7	100.0 ± 14.8	20.4	0.4	5.6	0.7	12.2	0.7	1.2
	Leaf 2										
	Distal half	0.55 ± 0.05	1242.7 ± 487.6	2396.5 ± 1035.4	285.6	21.1	18.1	27.0	286.1	137.6	181.3
	Proximal half	0.46 ± 0.01	9.2 ± 2.6	19.6 ± 4.8	1.7	0.1	0.4	0.2	1.0	0.4	1.0
	Petiole	0.61 ± 0.04	14.1 ± 2.9	22.9 ± 4.3	4.3	0.2	1.0	0.1	1.7	0.2	0.2
	Axillary bud	0.05 ± 0.01	2.3 ± 0.8	64.3 ± 29.6	8.9	0.3	2.6	0.5	6.3	0.7	0.5
	Apical bud	0.27 ± 0.01	9.3 ± 1.2	34.6 ± 4.5	9.6	0.1	1.6	0.2	3.3	0.2	0.2
	Root	1.00 ± 0.00	11.2 ± 2.1	11.2 ± 2.1	3.4	0.0	0.2	0.1	0.6	0.1	0.0
	Other axillary buds	0.74 ± 0.14	24.6 ± 6.3	32.4 ± 4.1	11.0	0.1	1.4	0.2	3.8	0.1	0.1
	Tuber										
	Upper region	1.00 ± 0.00	35.9 ± 6.5	35.9 ± 6.5	10.0	0.3	2.7	0.2	4.9	0.2	0.1
	Middle region	1.00 ± 0.00	33.6 ± 7.0	33.6 ± 7.0	12.0	0.2	1.4	0.2	2.9	0.1	0.1
	Lower region	1.00 ± 0.00	18.7 ± 3.2	18.7 ± 3.2	6.4	0.8	0.2	0.0	0.6	0.1	0.0
3	**Leaf 1**										
	Distal half	0.48 ± 0.07	1605.5 ± 727.4	3858.1 ± 2028.8	204.1	153.1	32.8	55.9	141.2	624.8	684.7
	Proximal half	0.36 ± 0.08	12.0 ± 3.4	49.4 ± 28.3	1.2	0.6	0.5	0.7	1.5	3.5	6.2
	Petiole	0.54 ± 0.06	21.3 ± 4.3	39.4 ± 6.4	1.2	0.2	1.6	0.3	5.4	2.0	2.0
	Axillary bud	0.01 ± 0.00	1.8 ± 0.3	147.6 ± 21.9	66.6	3.4	4.6	5.6	14.2	36.0	47.8
	Leaf 2										
	Distal half	0.33 ± 0.08	2969.7 ± 1275.0	14902.6 ± 10064.1	1135.2	154.2	21.7	40.3	90.9	263.8	2214.7
	Proximal half	0.31 ± 0.09	40.9 ± 24.9	41.7 ± 10.7	5.1	7.4	1.2	1.7	2.2	6.1	64.1
	Petiole	0.44 ± 0.12	20.2 ± 7.3	41.5 ± 6.7	0.4	0.2	0.1	0.2	0.4	1.7	5.9
	Axillary bud	0.02 ± 0.00	1.8 ± 0.4	122.6 ± 14.2	4.2	1.7	1.6	0.8	4.9	2.7	17.8
	Apical bud	0.27 ± 0.07	12.8 ± 4.0	47.2 ± 6.8	3.5	0.2	0.9	0.2	4.7	0.8	1.0
	Root	1.00 ± 0.00	7.0 ± 1.5	7.0 ± 1.5	0.7	0.0	0.1	0.0	0.2	0.1	0.2
	Other axillary buds	0.80 ± 0.11	37.2 ± 5.8	46.7 ± 6.3	5.2	0.1	0.9	0.2	5.2	0.4	0.7
	Tuber										
	Upper region	1.00 ± 0.00	46.9 ± 7.6	46.9 ± 7.6	3.2	0.1	0.8	0.1	5.9	0.2	0.3
	Middle region	1.00 ± 0.00	28.1 ± 5.4	28.1 ± 5.4	2.1	0.0	0.4	0.0	2.1	0.1	0.2
	Lower region	1.00 ± 0.00	8.7 ± 1.2	8.7 ± 1.2	1.4	0.0	0.1	0.0	0.1	0.1	0.2

8.4 Discussion

8.4.1 Uptake of [8-^{14}C] BAP

The uptake of a hormone constitutes the first step in signal perception, which affects the subsequent downstream signal transduction processes (Heyl and Schmülling, 2003). The establishment of endodormancy in phase 3 of the growth cycle resulted in a significant decline in the uptake of [8-^{14}C] BAP from both the leaves (1 and 2) following the application of BAP (Fig. 8.2A; B). CK receptors are found in the leaves (Heyl and Schmülling, 2003) and mutants deficient in these receptors lost their sensitivity to CK, resulting in sterility and low viability (Romanov, 2009). It is therefore possible that in phase 3, the decline in the number of CK receptors resulted in poor uptake of [8-^{14}C] BAP. The number of CK receptors could in turn be determined by the age of the leaf governed by senescence related processes, which are known to affect the uptake and transport of plant hormones (Ray and Choudhuri, 1984). Between the youngest (leaf 1; Fig. 8.2A) and the penultimately youngest leaf (leaf 2; Fig. 8.2B) within phase 1 itself, a 7% decline in the uptake of [8-^{14}C] BAP was observed in leaf 2. Therefore, the ontogenetic age of the leaves (1 and 2) in phase 3, compared to that in phase 1, may have rendered the significantly lower uptake of [8-^{14}C] BAP. This decline in the uptake of [8-^{14}C] BAP following the application of BAP after the establishment of endodormancy in phase 3 may have in turn affected the amount of radioactivity available to the other parts of the plant including the buds. Ultimately, the decline in the uptake of BAP in phase 3 probably comprised the first factor that contributed to the decline in the sensitivity of the buds to BAP during the transition of buds from apical dominance to endodormancy, which manifested as a decline in branching (Fig. 7.4). Probably because phase 2 involved the transitional stage of the buds between apical dominance and endodormancy, a significant decline in the uptake of [8-^{14}C] BAP was not observed between phases 1 and 2 (Fig. 8.2A; B).

Following the sequential application of GA_3 however, a significant decline in the uptake of [8-^{14}C] BAP was not observed in the leaves in phase 3, compared to those in phases 1 and 2 (Fig. 8.2A; B). The lack of a decline in the uptake means that in plants treated with [8-^{14}C] BAP + GA_3 in phase 3, the availability of radioactivity to other parts of the plant (especially the buds) was on par with that in phases 1 and 2. This in turn may have contributed to the sensitivity changes of the buds to CK and/or GA, resulting in an

increase in branching observed in phase 3 by the sequential application of BAP followed by GA_3 (BAP → GA_3) (Fig. 7.4c).

A comparison of the uptake between the application of BAP and GA_3 in the leaves showed that the presence of GA_3 did not enhance the uptake of [8-^{14}C] BAP from the leaves in phase 1 (Fig. 8.2A; B). This result partly explains the lack of a significant difference in the branching response between the application of BAP alone and BAP → GA_3 in phase 1 (Fig. 7.4). However, a significant difference in uptake was not noticed also between the application of BAP and GA_3 in phases 2 and 3 (Fig. 8.2A; B), which suggests that factors other than the uptake, such as differences in the transport and/or metabolism, were involved in the stimulation of branching in these phases following the sequential application of GA_3 (BAP → GA_3), compared to the application of BAP alone (Fig. 7.4b; c).

8.4.2 Availability of radioactivity

BAP (Black and Osborne, 1965) and its metabolites (Strnad, 1997) are capable of acropetal and basipetal transport. Therefore, apart from the direct application of CK to the suppressed buds to stimulate branching (Sachs and Thimann, 1967; Ali and Fletcher, 1970), foliar-fed CK was also successful in stimulating axillary bud outgrowth (Van Staden, 1982; Abo-Hamed et al., 1984). In *Zantedeschia* also, evident by the presence of radioactivity (DPM) in different plant parts (Fig. 8.3), foliar applied [8-^{14}C] BAP or its metabolites were transported from the point of application i.e., leaves to the subterranean parts in all three phases of growth. Differences in the availability of radioactivity as influenced by the three phases and the treatment applications comprising BAP (8.9 mM) and GA_3 (1.4 mM) was the theme of the current study, while the mechanisms that orchestrate these differences were beyond the scope of this study, and were therefore not dealt with.

A significant decline in the uptake of [8-^{14}C] BAP from both the leaves (1 and 2) following the application of BAP in phase 3 (Fig. 8.2A; B), meant that a large amount (2-4 fold) of radioactivity was retained in the distal halves of these leaves (Fig. 8.3A; E). This in turn equates to limited radioactivity available to the axillary buds after the establishment of endodormancy in phase 3, compared to that available for the axillary buds in phase 1. This retention of radioactivity in the distal halves of leaves 1 and 2 in phase 3, thereby minimising the amount of radioactivity available to other parts, may

have in part contributed to the decline in sensitivity of the buds to CK, which was manifested as a decline in branching following the application of BAP alone in phase 3 (Fig. 7.4c).

The lack of a significant decline in radioactivity following the application of GA_3 in phase 3 (Fig. 8.2A; B) suggests that the radioactivity available to other plant parts, including the buds in phase 3 was on par with that in phase 1. This observation is noteworthy, given that only the sequential application of BAP followed by GA_3 (BAP $\rightarrow$ GA_3) was successful in stimulating branching in phase 3 (Fig. 7.4c). Since significant differences in the amount of radioactivity, between the application of BAP and GA_3 was not observed in the distal halves of both the leaves within all the three phases (Fig. 8.3A; E), it is possible that GA_3 influenced axillary bud outgrowth in phase 3 also via other factors such as shoot elongation (Ali and Fletcher, 1970), apart from the regulating the availability of radioactivity to the other plant parts.

Though the proximal halves of the leaves and the corresponding petioles do not directly determine the branching response of the underground axillary buds, these parts comprise key components in the conduit of transport of radioactivity from the distal halves of the leaves to the subterranean plant parts (Fig. 8.1). Therefore, though significant differences in the availability of radioactivity was observed in these parts following the application of BAP and GA_3 in the three phases of growth (Fig. 8.3B; C; F; G), it was thought to carry no significant physiological relevance to the current study which was directed towards sensitivity changes of the buds to CK and/or GA in the three phases of growth.

The corresponding axillary buds of leaf 1 (Fig. 8.3D) and leaf 2 (Fig. 8.3H) are in the direct channel of transport of radioactivity from the leaves to other subterranean plant parts (Fig. 8.1). Therefore, they constitute the first subterranean parts to be exposed to radioactivity from the leaves, and thereby act as a transit point that facilitates distribution of radioactivity from the leaves to other plant parts. Considering the ability of BAP alone to stimulate branching in phase 1, where the buds are primarily governed by apical dominance (Fig. 7.4a), a significantly high amount of radioactivity was expected in the corresponding axillary buds of leaves 1 and 2 during phase 1 (Fig. 8.3D; H). In contrast however, following the application of BAP, 50-80% higher radioactivity was found in these buds in phase 3 i.e., endodormant buds, compared to those in phase

1 (Table 8.3). It can be argued that the relatively lesser amount of radioactivity in phase 1 comprised metabolically active forms of BAP, ideal to promote axillary bud outgrowth, compared to that in phase 3. An alternative argument is that the radioactivity in phase 1 could have been more efficiently distributed to other axillary buds, apical bud and the upper region of the tuber compared to that in phase 3. In phase 3, the retention and thereby accumulation of radioactivity in the axillary buds of leaves 1 and 2, may have limited the availability of radioactivity to the other axillary buds, thereby resulting in declined branching in phase 3 (Fig. 7.4c). Though the application of GA_3 resulted in the availability of a higher amount of radioactivity in these buds in phase 3, compared to the application of BAP,the difference was not significant (Fig. 8.3D; H). As mentioned above in the case of radioactivity in phase 1, the radioactivity following the application of GA_3 in phase 3, may have been distributed to the other axillary buds, and/or GA_3 may have facilitated the metabolism of [8-^{14}C] BAP to derivatives ideal to stimulate branching, and/or may have acted exclusively of regulating the transport and metabolism of CK. Radioactivity in the other subterranean plant parts may therefore demonstrate the role of BAP and GA_3 in regulating the availability of radioactivity between the three phases of growth.

In contrast to the distal halves of both the leaves (1 and 2) (Fig. 8.3A; E) and their corresponding axillary buds (Fig. 8.3D; H), where a lower amount of radioactivity was found in phase 1 compared to that in phase 3 following the application of BAP, a significantly high amount of radioactivity was found in other subterranean parts of the plant (Fig. 8.3I-N) in phase 1 compared to that in phase 3. This decline in the radioactivity available to the other axillary buds, apical bud and the upper region of the tuber where these buds reside after the establishment of endodormancy, are cumulatively more likely to contribute to the decline in the sensitivity of the buds to CK, which was consequently manifested as a decline in the amount of branching (Fig. 7.4c). This result, as described above, also rationalizes the lower amount of radioactivity found in the corresponding axillary buds of leaf 1 (Fig. 8.3D) and leaf 2 (Fig. 8.3H) in phase 1. The efficiency of these buds in phase 1 to distribute radioactivity to other plant parts has indeed facilitated the availability of radioactivity to the other axillary buds (Fig. 8.3K), manifested as increased branching following the application of BAP alone in phase 1 (Fig. 7.4a).

In the apical bud (Fig. 8.3I), other axillary buds (Fig. 8.3K) and the upper region of the tuber (Fig. 8.3L) significant differences in the radioactivity was not observed between the application of BAP and GA_3 in phase 1. Therefore, irrespective of the presence of GA_3, BAP alone was sufficient to release axillary buds from apical dominance during phase 1 (Fig. 7.4a). In phase 3 however, a higher amount of radioactivity was available in all these parts after the application of GA_3, compared to the application of BAP (Fig. 8.3I; K; L). Therefore, after the establishment of endodormancy in phase 3, the availability of high radioactivity in these parts caused by the application of GA_3, may in part have contributed to the change in the sensitivity of the buds to CK and/or GA, resulting in the significantly high branching caused by the sequential application of BAP followed by GA_3 (BAP → GA_3) compared to the application of BAP alone (Fig. 7.4c).

Since the establishment of endodormancy in *Zantedeschia* is autonomously controlled (Naor et al., 2008), the series of developmental changes in the SAM (Poethig, 1990) manifested as visual clues formed the basis of demarcation of the growth cycle of *Zantedeschia* into three distinct phases (refer Chapter 7). Developmental changes in the SAM are based on internal and external signals (Van Staden and Davey, 1979) and therefore the SAM was suggested to serve as a major sink for signals imported from other plant parts (Hewett and Wareing, 1973). In the current study, the apical bud constituted the subterranean plant part that accumulated maximum radioactivity, followed by the upper region of the tuber and the other axillary buds (refer Section 8.3.3.1). Acropetal and basipetal transport of CK is always directed towards the actively dividing parts of the plant (Abo-Hamed et al., 1984). Since the axillary buds represent one of the most actively dividing parts of the plant, and they are physically located on the upper region of the tuber, it is possible that the radioactivity applied to the distal halves of the leaves was directed to these parts in phase 1, which manifested as increased branching (refer Chapter 7). In phase 3, on the other hand, while the availability of radioactivity to these actively dividing parts was diminished following the application of BAP, the application of GA_3 significantly enhanced the availability of radioactivity in these parts. Though cross-talk between CK and GA (Weiss and Ori, 2007) is evident in the control of dormancy (refer Chapter 7), the exact mechanism behind the enhancement of radioactivity in these key subterranean plant parts (Fig. 8.3I; K; L) following the application of GA_3 in phases remains unknown.

While the roots are considered to be the primary site of CK biosynthesis (Letham, 1994), in the context of long-distance transport of CK (Kudo et al., 2010), the roots can also act as major sinks for phloem transported CK (Van Staden and Davey, 1979). In the control of branching, CK may regulate axillary bud outgrowth via root (Bangerth, 1994) or local synthesis in the stem (Tanaka et al., 2006). Owing to the significance of roots in the synthesis and transport of CK, the distribution and accumulation of radioactivity in the roots was measured in the current study. However, inspite of their close proximity to the buds in *Zantedeschia* (Fig. 8.1), the roots, amongst all subterranean plant parts, recorded the lowest amount of radioactivity (refer Section 8.3.3.1). Whether the radioactivity was distributed to the buds via the roots remains unclear. However, considering results from previous studies involving [8-^{14}C] BAP transport (Abo-Hamed et al., 1984), it is more likely that the radioactivity was preferentially transported directly to the buds, rather than through the roots.

8.4.3 Metabolism of [8-^{14}C] BAP

Spatial and temporal changes in the metabolism of CK have been observed (Jameson, 1994). Therefore, the mere availability of radioactivity in different plant parts executed through transport does not guarantee the desired physiological response. Accordingly, the high amount of radioactivity observed in the distal halves of leaf 1 (Fig. 8.6A3 (a); (b)) and leaf 2 (Fig. 8.6E3 (a); (b)), irrespective of the application of BAP or GA_3 in phase 3, mainly comprised unmetabolized [8-^{14}C] BAP in contrast to mT in this part in phases 1 (Fig. 8.6A1 (a); (b)) and 2 ((Fig. 8.6A2 (a); (b)). The scarce presence of [8-^{14}C] BAP in this part in phase 1 was either due to the transport of [8-^{14}C] BAP to other plant parts or the metabolism of [8-^{14}C] BAP to mT. This remarkable difference in the metabolic profile of [8-^{14}C] BAP observed between phase 1 and phase 3 may have contributed to the decline in the sensitivity of the buds to CK, which may have in turn manifested as a decline in the amount of branching during the transition of buds from apical dominance to endodormancy (refer Chapter 7).

Several metabolites of exogenously applied BAP such as BAR, BARP, BA3G, BA7G, BA9G, BA9RG, BA9Ala, Ade, Ado, AMP, GMP and ureides have been identified previously (Strnad, 1997). However, the biosynthetic and metabolic pathways of ARCK are still inconclusive in comparison to their ISCK counterparts, and several metabolites of BAP are yet to be identified (Strnad, 1997). Though the hydroxyl derivatives of BAP

i.e., topolins have been identified naturally in several plant species (refer Section 2.3.1.2.1) their identification as metabolites of BAP has been elusive until date. In the current study, identification of one of the six metabolic radioactive peaks as mT, based on coincidence with the retention time of the authentic standard of mT (Table 8.1) and further supported by results from LC-MS (Table 8.2), suggests that mT could be a metabolic product of BAP. However, in the current study, CK extracts from plants treated with unlabelled BAP (8.9 mM) were used for the identification of metabolites using LC-MS (refer Section 8.2.2). Therefore, it is possible that mT or the other metabolites identified through LC-MS/MRM (Table 8.2) occurred naturally in *Zantedeschia* cv. 'Best Gold'. Hence, more rigorous MS studies aimed at verifying that the [8-^{14}C] of mT was in fact derived from [8-^{14}C] BAP need to be conducted. Given that oTR and its derivative have been identified naturally in winter flowering (refer Section 1.1) *Zantedeschia aethiopica* (das Neves and Pais, 1980a; b), it is possible that topolins also exist in the summer-flower group (refer Section 1.1). Amongst the three isomers of topolins i.e., oT, mT and pT (Strnad, 1997), mT exhibits higher biological activity in biological assays (Holub et al., 1998), and compared to BAP, mT was more successful in increasing the branching of Poinsettia (*Euphorbia pulcherrima* Willd.) and Gerbera (*Gerbera jamesonii* Hook.) (Kaminek et al., 1987). In this context, the probable identification of mT as a metabolic product of BAP provided further scope to investigate the natural existence of topolins in summer-flowering *Zantedeschia* cv. 'Best Gold' (refer Chapter 9).

Irrespective of the application of BAP or GA_3, a similar metabolic profile as observed in the distal half of leaves 1 and 2, was also observed in the corresponding proximal halves (Fig. 8.6B1-B3, F1-F3 (a); (b)) and petioles (Fig. 8.6C1-C3, G1-G3 (a); (b)). AMP and mT constituted the major metabolites in these parts in phases 1 and 2 with the occasional occurrence of BARP, whereas the radioactivity in these parts in phase 3 mainly comprised [8-^{14}C] BAP and BAR. Interconversions between the CK free base i.e., BAP, its riboside (BAR) and ribotide (BARP) are well known in CK metabolism, and these interconversions help in maintaining the overall CK pool of the plant (Jameson, 1994). Since the riboside and ribotide are capable of transport (Auer et al., 1992), they could facilitate the availability of CK to other parts of the plant.

In the context that clear evidence supporting the biosynthetic pathway of ARCK is still lacking, it is not unreasonable to use the biosynthetic pathway of the ISCK as a template (refer Section 2.3.1.2.1). In the *de novo* biosynthetic pathway of ISCK, the prenylation of an AMP/ADP/ATP substrate by *isopentenyltransferase* (IPT) constitutes the rate-limiting step of CK biosynthesis (Kamada-Nobusada and Sakakibara, 2009). While ADP/ATP is the preferred substrate in plants, AMP is preferred by bacteria (Kakimoto, 2003). In the current study, AMP was one of the key metabolites of [8-^{14}C] BAP, identified in almost all parts of the plant following the application of BAP or GA_3 (Fig. 8.6). The availability/transport of AMP to the subterranean plant parts, especially the axillary buds, may have acted as the substrate for the biosynthesis of the active form of CK, which may in turn have regulated the sensitivity of the buds to CK during the transition of buds from apical dominance to endodormancy.

The result supporting the hypothesis that the corresponding axillary buds of leaf 1 (Fig. 8.3D) and leaf 2 (Fig. 8.3H) in phase 1 were more efficient in diverting the radioactivity to the other subterranean plant parts such as the apical bud, other axillary buds and the upper region of the tuber, compared to those in phase 3 (refer Section 8.4.2) was also corroborated through the metabolic studies. Though AMP and mT were amongst the key metabolites of these buds in phases 1 (Fig. 8.6D1 (a), (b); H1 (a), (b)) and 2 (Fig. 8.6D2 (a), (b); E2 (a), (b)), following the application of BAP or GA_3, their presence was low in comparison to the amount of [8-^{14}C] BAP present in the corresponding buds in phase 3 (Fig. 8.6D3 (a), (b); E3 (a), (b)). As hypothesized with the distribution of radioactivity (refer Section 8.4.2), it is possible that in phase 1, AMP and mT were transported to the other subterranean plant parts that are pivotal to producing a branching response.

In contrast to the distal half of leaves 1 and 2 and their corresponding axillary buds, unmetabolized [8-^{14}C] BAP was scarcely found in the other subterranean plant parts (Fig. 8.1) during phase 3 of the growth cycle, following the application of BAP or GA_3 (Fig. 8.6I3-N3). In all the three phases, AMP and mT comprised the major metabolic products of [8-^{14}C] BAP. However, as predicted, the amount of AMP and mT was higher in the apical bud, other axillary buds and the upper region of the tuber following the application of BAP in phase 1 i.e., apical dominance (Fig. 8.6I1 (a); K1 (a); L1 (a), respectively), compared to that in the endodormant buds in phase 3 (Fig. 8.6I3 (a); K3 (a); L3 (a), respectively). Given the significance of the biological activity of mT in

comparison to BAP (Kaminek et al., 1987) and the other isomers of topolins (Holub et al., 1998), the lack of mT in the endodormant buds could have been a significant contributing factor for the decline in branching observed during phase 3 of the growth cycle, following the application of BAP alone (Fig. 7.4c). The higher amount of mT identified in these parts during phase 1 following the application of BAP can partly be attributed to the presence of the genes specific for this conversion in these parts. In the biosynthetic pathway of ISCK, the *LOG* gene (*Lonely Guy*) which encodes the enzyme that catalyses the release of active *trans*-zeatin (tZ) from the corresponding nucleotide was expressed exclusively in the apical bud (Kurakawa et al., 2007). Likewise, the enzyme product of this gene or its homolog, that catalyses the synthesis of mT possibly through BAP, could be present in the buds. Accordingly, the highest amount of mT amongst all the subterranean plant parts was found in the upper region of the tuber where the buds reside.

In the apical bud (Fig. 8.6I3 (a)), other axillary buds (Fig. 8.6K3 (a)) and upper region of the tuber (Fig. 8.6L3 (a)), while the application of BAP resulted in a concomitant decline in the amount of mT between phases 1 and 3, the application of GA_3 in phase 3 enhanced the amount of mT (3-4 fold; Table 8.3) in the apical bud, other axillary buds and the upper region of the tuber (Fig. 8.6I3 (b); K3 (b); L3 (b)), respectively. This result plays a significant role in the context that the sequential application of BAP followed by GA_3 significantly increased branching in phase 3, compared to the application of BAP alone (Fig. 7.4c). This increase in the amount of mT may have been accomplished by increasing the availability of radioactivity in these parts by the application of GA_3, compared to the application of BAP (Fig. 8.3I; K; L). By enhancing the amount of mT in these parts in phase 3 therefore, the application of GA_3 may have contributed to the enhanced branching observed in phase 3 (Fig. 7.4c). The exact role of GA in increasing the availability of radioactivity in these key subterranean plant parts, which in turn increased the amount of mT, remains unknown. Further studies using radioactively labelled GA in conjunction with [8-^{14}C] BAP may provide further insights into this regulation.

Though the roots are a key plant component in CK biosynthesis (Letham, 1994), in contrast to other subterranean parts in the current study, the application of GA_3 did not alter the amount of mT in phase 3 (Fig. 8.6J3 (b)) compared to the application of BAP (Fig. 8.6J3 (a)). This, as reported earlier (refer Section 8.4.2), reiterates the significance

of the buds in preference to the roots in regulating the uptake, availability and metabolism of [8-^{14}C] BAP.

8.5 Conclusions

An integral of changes in the uptake, availability and metabolism of [8-^{14}C] BAP contributed to the sensitivity changes of the buds to CK and/or GA, which was manifested as a differential branching response in the three phases of growth, following the application of BAP and BAP → GA_3 (refer Chapter 7).

In phase 1, where the buds are predominantly under apical dominance, following the simultaneous application of BAP (8.9 mM), higher uptake of [8-^{14}C] BAP from the leaves (Fig. 8.2A; B) resulted in higher availability of radioactivity in the apical bud, other axillary buds and upper region of the tuber (Fig. 8.3I; K; L), and subsequently higher amount of metabolic mT in these parts (Fig. 8.6I1 (a); K1 (a); L1 (a)). The lack of significant differences in the uptake, availability and metabolism of [8-^{14}C] BAP, between the simultaneous application of BAP (8.9 mM) and the sequential application of GA_3 (1.4 mM) in phase 1, corroborated the lack of a need for the sequential application of GA_3 (BAP → GA_3) to enhance branching in phase 1 (Fig. 7.4a).

In phase 3, where the buds are predominantly endodormant, following the simultaneous application of BAP (8.9 mM), lesser uptake of [8-^{14}C] BAP from the leaves (Fig. 8.2A; B) resulted in lesser availability of radioactivity in the apical bud, other axillary buds and upper region of the tuber (Fig. 8.3I; K; L), and subsequently lesser amount of metabolic mT in these parts (Fig. 8.6I3 (a); K3 (a); L3 (a)). This decline translated to a concomitant decline in the degree of branching in phase 3, following the application of BAP alone (Fig. 7.4c). The lack of a significant decline in the uptake of [8-^{14}C] BAP following the sequential application of GA_3 (1.4 mM), resulted in an increase in the availability of radioactivity in these key subterranean plant parts (Fig. 8.3I; K; L), and subsequently a higher amount of metabolic mT in these parts, compared to the application of application of BAP (8.9 mM). These factors translated to an increase in branching in phase 3, when GA_3 was applied sequentially to BAP (BAP → GA_3) (Fig. 7.4c).

Though the current experiment corroborated changes in the sensitivity of the buds to CK and/or GA during the transition of buds from apical dominance to endodormancy, the mechanism of a sequential application of GA in enhancing the availability of

radioactivity i.e., [8-^{14}C] BAP or its metabolic products, in the key subterranean plant parts after the establishment of endodormancy in phase 3 is unknown.

Meta-topolin was identified as a metabolic product of BAP. Further studies were therefore aimed at the identification of mT and/or other ARCK naturally in summer-flowering *Zantedeschia* (Chapter 9).

9. Topolins in summer-flowering *Zantedeschia*

9.1 Introduction

The identification of a radioactive metabolic peak coinciding with the retention time of an authentic standard of *meta*-topolin (mT) following the application of radioactive 6-benzylaminopurine ([8-^{14}C] BAP) (Fig. 8.6), and the subsequent identification of mT in the corresponding fraction using liquid chromatography-mass spectrometry (LC-MS) analyses (Table 8.2), led to the assumption that mT and probably other topolins could exist naturally in summer-flowering *Zantedeschia* (refer Section 8.5).

Topolins are hydroxylated analogues of 6-benzylaminopurine (BAP) and belong to a group of aromatic cytokinin (ARCK), characterized by the presence of an aromatic side chain (Strnad, 1997). This group differs from the isoprenoid CK (ISCK) group, largely represented by zeatin (Z) and isopentenyl adenine (iP) type of compounds, which possess an aliphatic side chain (Mok and Mok, 2003). Topolins have been identified naturally in a diverse range of plant species and plant parts (refer Section 2.3.1.2.1), including fruits of winter-flowering *Zantedeschia aethiopica* (das Neves and Pais, 1980a; b). However, owing to differences in their chemical structure, they differ significantly from the ISCK group in their biological activity, metabolism and probably biosynthesis (refer Section 2.3.1.2.1). Even within the ARCK group, hydroxylation in the *meta* position i.e., mT was found to possess higher biological activity in CK bioassays compared to the other positional isomers, *ortho*-topolin (oT) and *para*-topolin (pT) (Holub et al., 1998). In a bacterial system expressing CK receptors, mT showed the highest binding activity compared to BAP and oT (Spichal et al., 2004). Therefore, the identification of *ortho*-topolin riboside (oTR) in winter-flowering *Z. aethiopica* (das Neves and Pais, 1980a) and the probable occurrence of mT in summer-flowering *Zantedeschia* could have significant physiological implications.

Preliminary studies using enzyme linked immunosorbent assays (ELISA) and LC-MS analyses were therefore conducted to detect the natural occurrence of ARCK, primarily represented by *ortho*-topolin (oT), *ortho*-topolin riboside (oTR), *meta*-topolin (mT), *meta*-

topolin riboside (mTR), BAP and 6-benzylaminopurine riboside (BAR), in summer-flowering *Zantedeschia* cv. ‘Best Gold’.

9.2 Materials and Methods

9.2.1 Plant material and culture

Single-shoot seedlings of *Zantedeschia* cv. ‘Best Gold’ during their first growth cycle (Fig. 1.1) were grown as described in Chapter 7. Growth and culture conditions were also similar to that described in Chapter 7.

9.2.2 Cytokinin extraction and purification

For immunodetection (refer Section 9.2.4) and detection by LC-MS using the Multiple Reaction Monitoring (MRM) method (refer Section 9.2.5), 1 g and 5 g fresh weight (FW) of leaves from natural plants (90 days after planting i.e., phase 1) were harvested, respectively. From the leaf samples, cytokinin (CK) extraction and purification was performed as described in Section 8.2.4.

9.2.3 Chromatographic separation

A mixture of authentic standards of different CK, mainly comprising topolins was prepared as described in Section 8.2.7.1, and their retention times was determined using the chromatography system described before (refer Section 8.2.5). Subsequently, the natural plant CK extracts prepared for immunodetection and detection by LC-MS/MRM (refer Section 9.2.2) were passed through the chromatography system and six fractions corresponding to the retention times of the authentic standards of oT, oTR, mT, mTR, BAP and BAR were collected.

9.2.4 ELISA

Polyclonal rabbit anti-CK antibodies for oTR, mTR and BAR, and the corresponding alkaline phosphatase tracers were a gift from Prof. Miroslav Strnad and Dr. Karel Dolezal, Laboratory of growth regulators, Palacky University, Olomouc, Czech Republic. The

immunization protocol and preparation of the corresponding tracers have been described elsewhere (Strnad et al., 1990).

9.2.4.1 Preparation of standards for ELISA

Standard solutions of oTR, mTR and BAR were prepared by dissolving adequate quantities of the respective ribosides in 70% ethanol, and the final volume was made up using Tris buffered saline buffer (TBS; Tris = 50 mM; NaCl = 10 mM; $MgCl_2$ = 1 mM; pH = 7.5). Serial dilutions of the stock solution of oTR were subsequently prepared so that 50 µl aliquots of the standard solutions contained 1000, 100, 10, 1 and 0.1 pg of oTR respectively. Likewise, standard solutions of mTR and BAR were prepared so that 50 µl aliquots contained 10, 1, 0.1, 0.01 and 0.001 ng of mTR, and 100, 10, 1, 0.1, 0.01 ng of BAR, respectively. TBS buffer was used for all serial dilutions.

9.2.4.2 Preparation of natural plant samples

The six natural plant fractions corresponding to oT, oTR, mT, mTR, BAP and BAR (refer Section 9.2.3) were dried *in vacuo* (refer Section 8.2.5), dissolved in 50 µl of 70% ethanol and the final volume made up to 500 µl using TBS buffer. This volume of the natural plant fraction was then spiked with 500 µl of the corresponding standard solution containing the highest concentration of oTR/mTR/BAR (refer Section 9.2.4.1). For ELISAs, a 1/200 serial dilution was subsequently prepared from the respective stock solutions.

9.2.4.3 Preparation of solvents and reagents

Working dilutions of the antibodies for oTR, mTR and BAR were prepared by diluting 5 µl of the stock solution in 15 ml of 50 mM $NaHCO_3$ buffer (pH = 9.6). Working dilutions of the corresponding alkaline phosphatase tracers were prepared by diluting 3 µl of the stock solution in 5 ml of TBS-bovine serum albumin (BSA) buffer (0.04 g l^{-1}). 5N KOH was used as the stopping solution, and *p*-nitrophenylphosphate solution (1mg ml^{-1} 50 mM $NaHCO_3$ buffer; pH = 9.6) was used as the substrate.

9.2.4.4 ELISA protocol

The assays were performed as described by Strnad (1996). Microtiter plates (Nunc A/S, Denmark) were coated with 150 µl of the working dilution of the respective antibodies i.e., oTR/mTR/BAR. The wells were washed with distilled water and filled with 200 µl of BSA solution. After incubation (25ºC) for 1 h, the BSA solution was decanted and the wells were washed twice with distilled water. The wells were then filled with the following sequence of solutions:

1. 50 µl of TBS
2. 50 µl of standard i.e., oTR/mTR/BAR (refer Section 9.2.4.1) or natural plant sample (refer Section 9.2.4.2)
3. 50 µl of the working dilution of the corresponding alkaline phosphatase tracer solution i.e., oTR/mTR/BAR

Non-specific binding (NSB) was determined by adding an excess of the respective standards, and for maximum tracer binding (B_0), TBS was added instead of the standard or sample.

Subsequently, the plates were shaken for 1 min and incubated for 1 h at 25ºC. The decanted plates were then washed 4 times with TBS and filled immediately with 150 µl of the *p*-nitrophenylphosphate solution. After incubation for 1 h at 25ºC, the reaction was stopped by adding 50 µl of 5N KOH. Absorbance was measured at 405 nm in a spectrophotometer (Perkin Elmer, USA).

9.2.5 LC-MS/MRM

The natural plant fractions corresponding to oT, oTR, mT, mTR, BAP and BAR were dried *in vacuo* (refer Section 8.2.5), and sent to Dr. Colin Turnbull, Imperial College, London (UK) for LC-MS/MRM analyses. LC-MS analysis of a mixture of CK standards and the samples was conducted as described in Section 8.2.7.2.

9.2.6 Data analysis

ELISA for standards of oTR/mTR/BAR and the natural plant fractions corresponding to oT, oTR, mT, mTR, BAP and BAR were conducted in triplicate. Means ± standard errors for absorbance (405 nm) were calculated using MS Excel (Microsoft Corp., USA). The sigmoidal curves for the standards and plant samples were linearized by log-logit transformation as follows (Strnad, 1996):

$$\text{Logit } B/B_0 = \ln [(B/B_0)/(1- B/B_0)]$$

Where B = tracer bound in the presence of standard or sample and B_0 = tracer bound in the absence of standard or sample.

Following two runs in the LC-MS/MRM system (equivalent to 1g FW of leaves each), the corresponding compounds in the natural plant fractions were detected by measuring the peak area of these compounds.

9.3 Results

9.3.1 Overview

Retention times of a mixture of authentic standards of CK obtained through the chromatography system at Massey University, Palmerston North and Imperial College, London were as shown in Fig. 8.4 and Fig. 8.5, respectively.

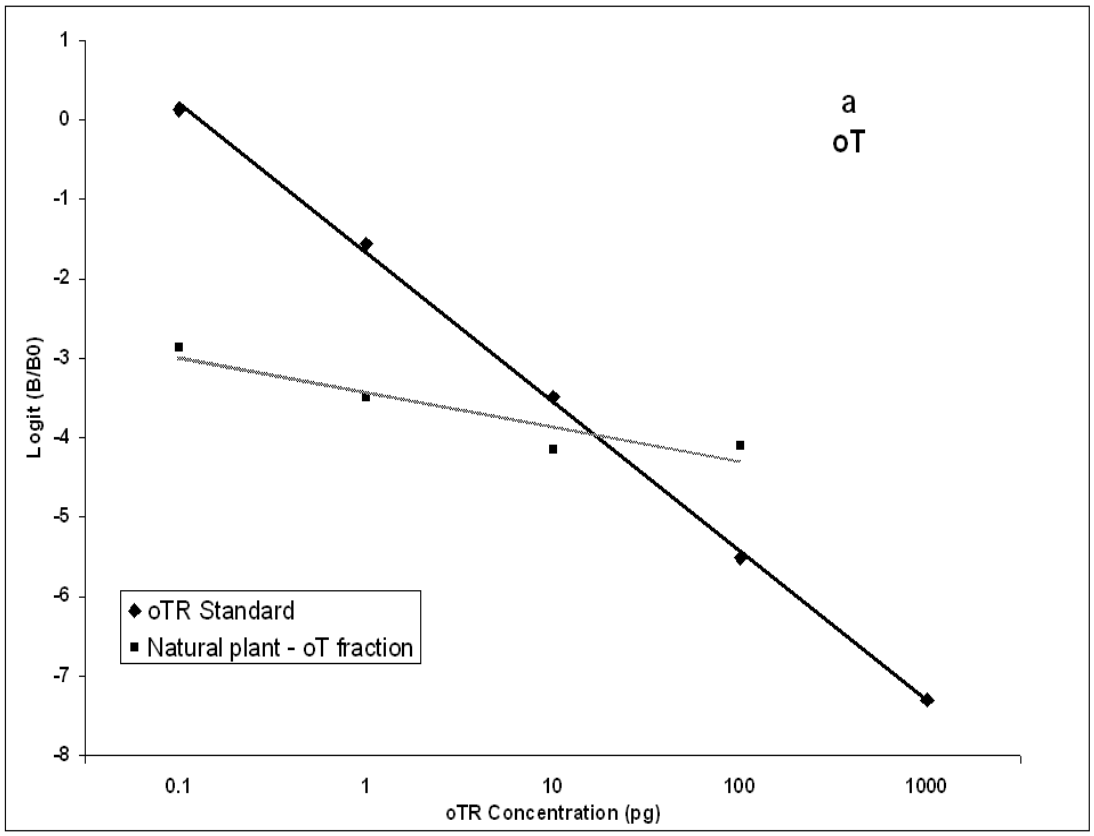
Absorbance (405 nm) for maximum oTR tracer binding (B_0) was 0.7 (n = 3), whereas for BAR and mTR, this value was 1 (n = 3). The regression co-efficient (R^2), which provides an estimate of the fit of a straight line was 0.98-0.99 for the linearized standard curves of oTR, mTR and BAR (Fig. 9.1).

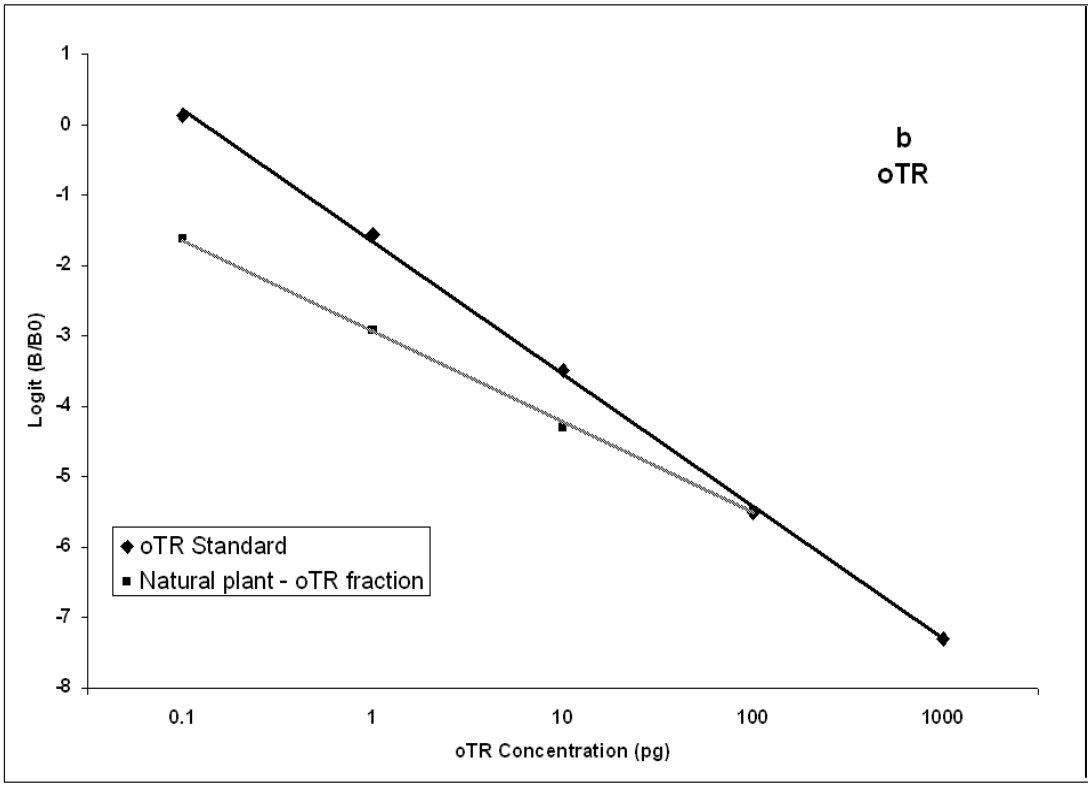
For ELISAs, parallel lines between the standard curves of oTR, mTR or BAR and the response curve obtained from the respective natural plant fraction suggest the absence of substances interfering with the assays, and provide a validation of assay performance. Between the parallel lines, a shift in the y-intercept of the sample curve below that of the standard curve suggests the occurrence of the respective compound in the natural plant fraction.

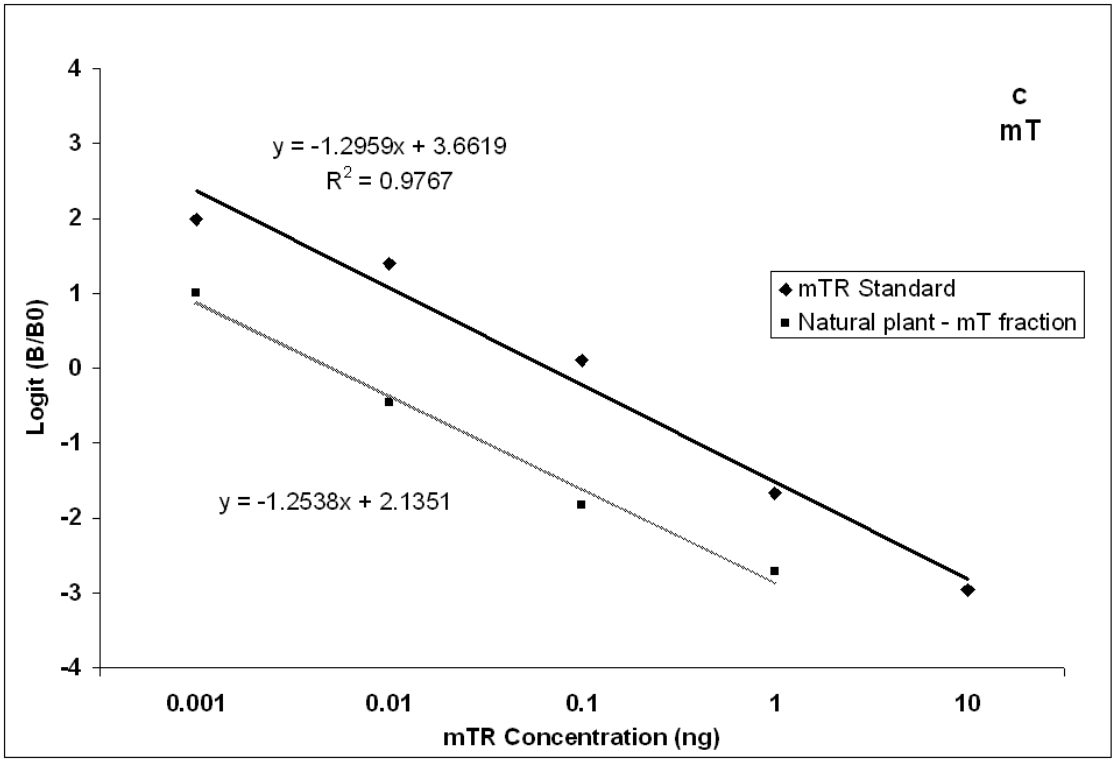
9.3.2 Natural occurrence of topolins

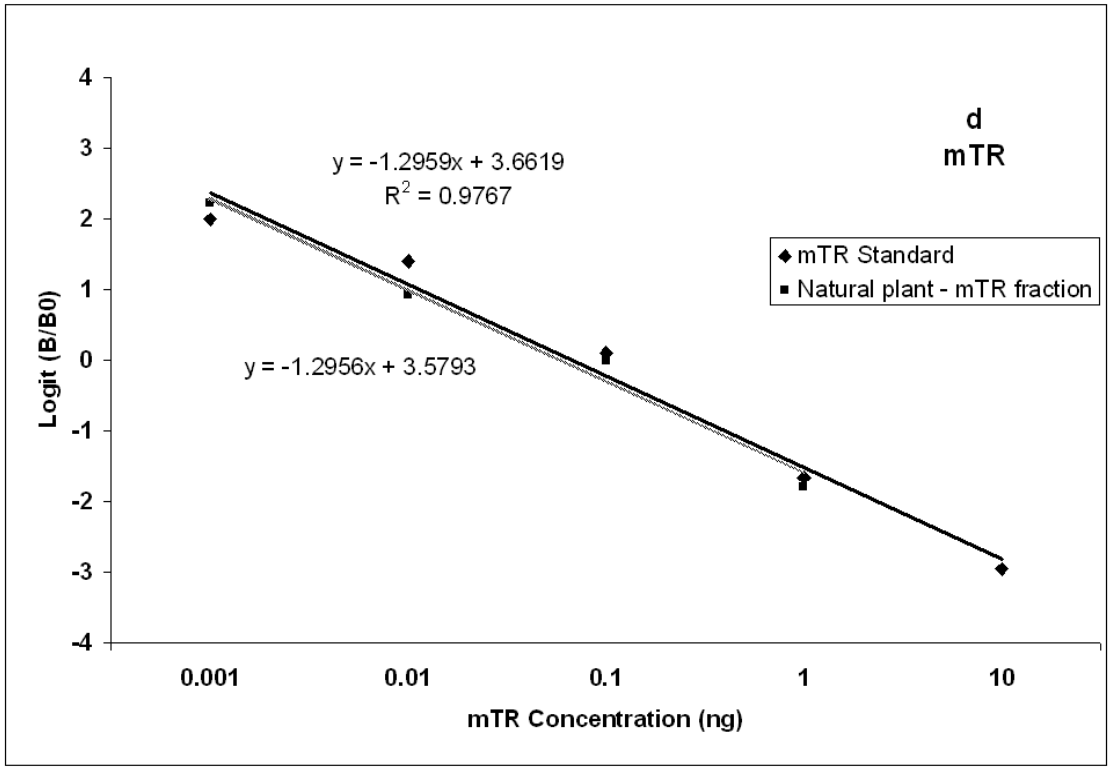
9.3.2.1 ELISA

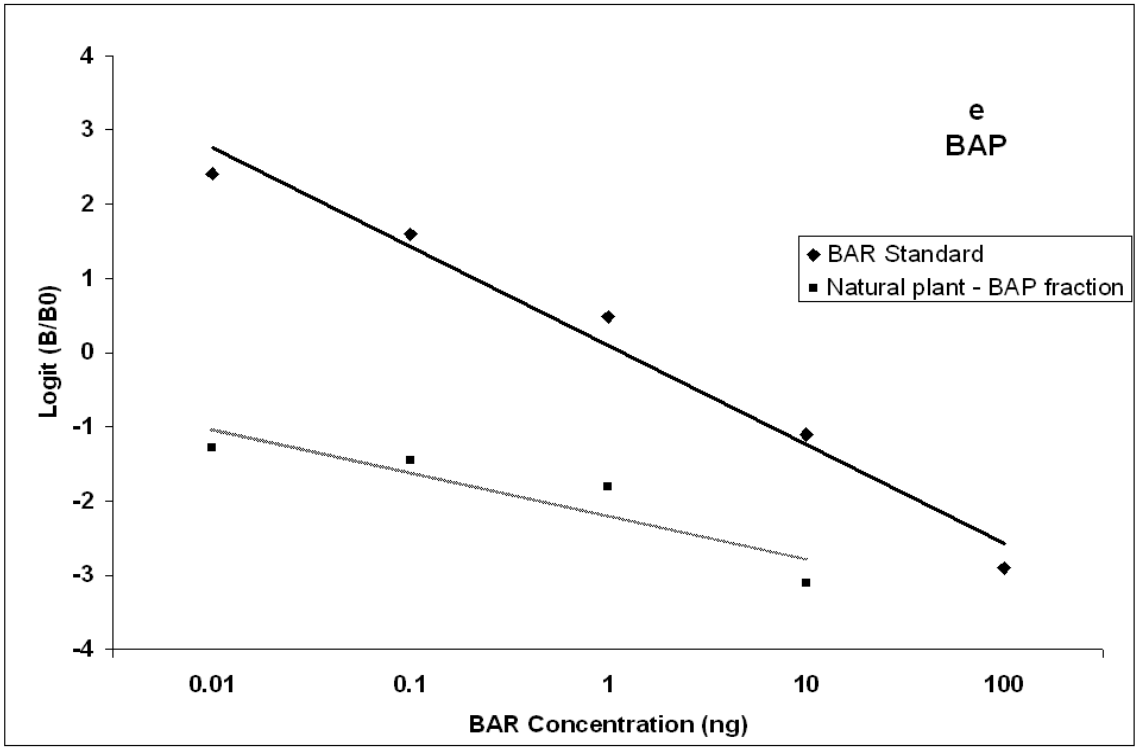
Parallel lines between the standard curves and the corresponding natural plant fractions was obtained with mT (Fig. 9.1c), mTR (Fig. 9.1d) and BAR (Fig. 9.1f). The y- intercept of the natural plant fraction for mTR was similar to that of the mTR standard curve (Fig. 9.1d). A shift in the y-intercept of the natural plant fractions corresponding to mT (Fig. 9.1c) and BAR (Fig. 9.1f) was observed below that of the respective standard curves. Parallel lines were not obtained between the natural plant fractions corresponding to oT (Fig. 9.1a), oTR (Fig. 9.1b) and BAP (Fig. 9.1e) and their respective standard curves.


a
oT
Logit (B/B0)
1
0
-1
-2
-3
-4
-5
-6
-7
-8
0.1
1
10
100
1000
oTR Concentration (pg)
oTR Standard
Natural plant - oT fraction


b
oTR
Logit (B/B0)
1
0
-1
-2
-3
-4
-5
-6
-7
-8
0.1
1
10
100
1000
oTR Concentration (pg)
oTR Standard
Natural plant - oTR fraction


c
mT
y = -1.2959x + 3.6619
R2 = 0.9767
mTR Standard
Natural plant - mT fraction
y = -1.2538x + 2.1351
Logit (B/B0)
mTR Concentration (ng)
4
3
2
1
0
-1
-2
-3
-4
0.001
0.01
0.1
1
10


d
mTR
y = -1.2959x + 3.6619
R2 = 0.9767
mTR Standard
Natural plant - mTR fraction
y = -1.2956x + 3.5793
Logit (B/B0)
mTR Concentration (ng)
4
3
2
1
0
-1
-2
-3
-4
0.001
0.01
0.1
1
10


e
BAP
BAR Standard
Natural plant - BAP fraction
Logit (B/B0)
4
3
2
1
0
-1
-2
-3
-4
0.01
0.1
1
10
100
BAR Concentration (ng)

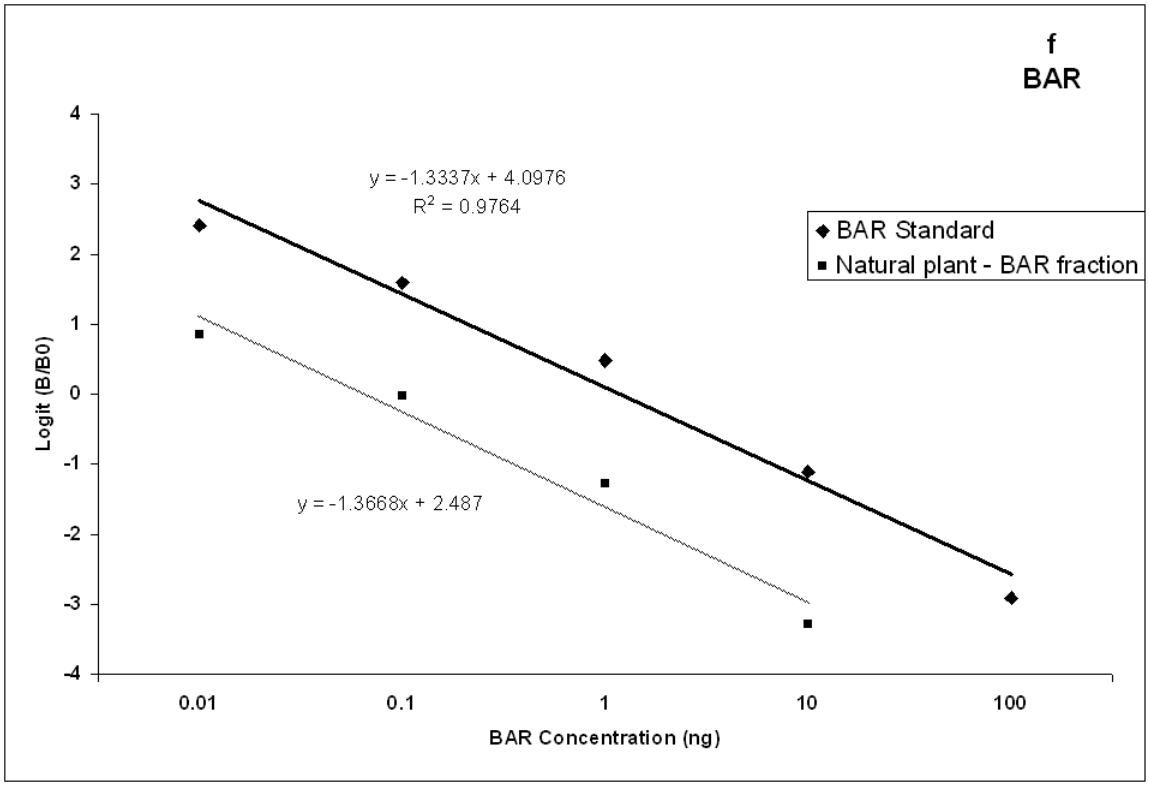

f
BAR
y = -1.3337x + 4.0976
R2 = 0.9764
BAR Standard
Natural plant - BAR fraction
y = -1.3668x + 2.487
Logit (B/B0)
4
3
2
1
0
-1
-2
-3
-4
0.01
0.1
1
10
100
BAR Concentration (ng)

Fig. 9.1 Detection of topolins in natural plant fractions of *Zantedeschia* cv. 'Best Gold' by enzyme linked immunosorbent assay (ELISA). Validation of the ELISA for **a**) oT, **b**) oTR, **c**) mT, **d**) mTR, **e**) BAP, and **f**) BAR based on logit transformation of absorbance data (405 nm) for the respective standard and 1/200 serial dilution of the corresponding natural plant fraction. Each point represents the mean of triplicate analysis of standard or sample. Logit $B/B_0 = \ln [(B/B_0)/ (1- B/B_0)]$

9.3.2.2 LC-MS/MRM

Among the 6 natural plant fractions, oT and oTR were not detected in their respective fractions. *Meta*-topolin (mT), mTR and BAP were scarcely detected, whereas BAR was present in the highest concentration (Table 9.1). The concentration of BAR (measured as the peak area) (Mean = 3158.5; n = 2) was at least 5 times higher than that of mT (Mean = 536.5; n = 2) (Table 9.1).

Table 9.1 Detection of topolins in the natural plant fractions of *Zantedeschia* cv. 'Best Gold', based on the peak area of the respective compounds obtained from the LC-MS/MRM system at Imperial College, London (Courtesy: Dr. Colin Turnbull)

Natural plant fraction corresponding to	**Peak area***	
	1st run	**2nd run**
oT	Not detected	Not detected
oTR	Not detected	Not detected
mT	382	691
mTR	405	303
BAP	Not detected	568
BAR	3683	2634

* An area of 200 is considered the absolute minimum ($\alpha = 0.05$)

9.4 Discussion

9.4.1 Topolins in summer-flowering *Zantedeschia*

In contrast to the identification of derivatives of oT in winter-flowering *Z. aethiopica* (das Neves and Pais, 1980a; b), mT was predominantly identified naturally in summer-flowering *Zantedeschia* cv. 'Best Gold' (Fig. 9.1c; Table 9.1). The lack of parallel curves between the natural plant fractions of oT and oTR and the respective standard curves (Fig. 9.1a; b) suggested the presence of interfering substances and/or the absence of these isomers of topolins in the natural plant fractions. This was further corroborated by the results from LC-MS studies where oT and oTR were not detected in their respective fractions (Table 9.1). Spatial differences in the distribution of CK have been reported (Staden and Davey, 1979), where the zeatin (Z) type of CK are predominantly found in the xylem and isopentenyl adenine (iP) type are prevalent in the phloem (Romanov, 2009). oTR was previously identified in mature leaves of *Populus* × *robusta* (Poplar) in which cell division had ceased (Horgan et al., 1975), and in fruits of *Z. aethiopica* (das Neves and Pais, 1980a). Given that mT has higher biological activity than the other isomers of topolins (Holub et al., 1998), it is possible that mT predominantly occurs during phase 1 of the growth cycle, and oT is prevalent during the later stages of growth i.e., phase 3 and fruitification. Further studies involving additional purification steps and quantification of different isomers of topolins may elucidate the spatial and temporal changes in the distribution of topolins in natural plants of *Zantedeschia*.

BAR has not been identified previously in *Zantedeschia*, and in this study, the concentration of BAR in natural plant fractions was 5-6 times higher than the endogenous mT content (Table 9.1). Since interconversion between the base, nucleoside and nucleotide is a common feature of CK metabolism (Jameson, 1994), and ribosides are capable of transport (Auer et al., 1992), it is possible that BAR contributes to the distribution of BAP/BARP to other plant parts. BAP was however only tentatively detected in the natural plant fractions (Table 9.1) and parallel lines were not obtained during the validation of the respective ELISA (Fig. 9.1e). As mentioned earlier, more rigorous purification of samples may enable the unequivocal identification of BAP in *Zantedeschia*.

A correlation between the bushy/multiple branching syndrome and the endogenous isoprenoid CK (ISCK) concentration could not be established between bushy and control (non-bushy) plants of *Zantedeschia* cv. 'Florex Gold' (D'Arth et al., 2007). Given that the current study has detected the presence of mT and BAR naturally in *Zantedeschia* (Fig. 9.1; Table 9.1), and exogenous application of BAP was successful in stimulating branching in *Zantedeschia* (refer Chapter 7), the endogenous ARCK content may correlate with this syndrome.

9.4.2 Comparison of the natural topolin profile with the metabolic profile of BAP

The identification of mT and BAR as metabolites of exogenously applied BAP (Table 8.2) and their role in the context of sensitivity changes associated with the transition of buds from apical dominance to endodormancy i.e., phases 1, 2 and 3, was established earlier (refer Chapter 8). Given that mT and BAR were also shown to occur naturally in *Zantedeschia* (Fig. 9.1c; f; Table 9.1), a comparison of peak areas of mT and BAR in the natural plant fractions (Table 9.1) and after the application of BAP (8.9 mM) (Table 8.2) was thought to illustrate the effect of BAP on these compounds.

The peak areas of mT and BAR were at least 10 (Fig. 9.2) and 50 times (Fig. 9.3) higher in BAP treated plants than in natural plants, respectively (Table 8.2). Though BAR has been prevalently identified as a metabolic product of BAP (Jameson, 1994; Strnad, 1997), mT has not been identified so far (Strnad, 1997). Though the exogenous application of BAP caused an increase in the concentration of mT (measured as peak area; Fig. 9.2), whether this increase was caused by an influence of BAP on the endogenous mT pool, or mT was derived through the metabolism of BAP still remains unclear. However, as mentioned in Section 8.4.3, the identification of the [8-^{14}C] group of BAP in metabolic mT following the application of [8-^{14}C] BAP may provide some useful insight into the metabolic pathway of ARCK.

Given that mT was found to be more effective in stimulating branching than BAP (Kaminek et al., 1987), and mT was suggested to, among other factors, regulate the degree of branching during the transition of buds from apical dominance to endodormancy i.e.,

phases 1, 2 and 3, following the application of BAP and BAP → GA_3 (refer Chapter 8), this increase in mT concentration identified following the application of BAP (Fig. 9.2) could have significant physiological and commercial implications. The results from these preliminary studies provide further motivation to conduct experiments aimed at quantification of endogenous mT.

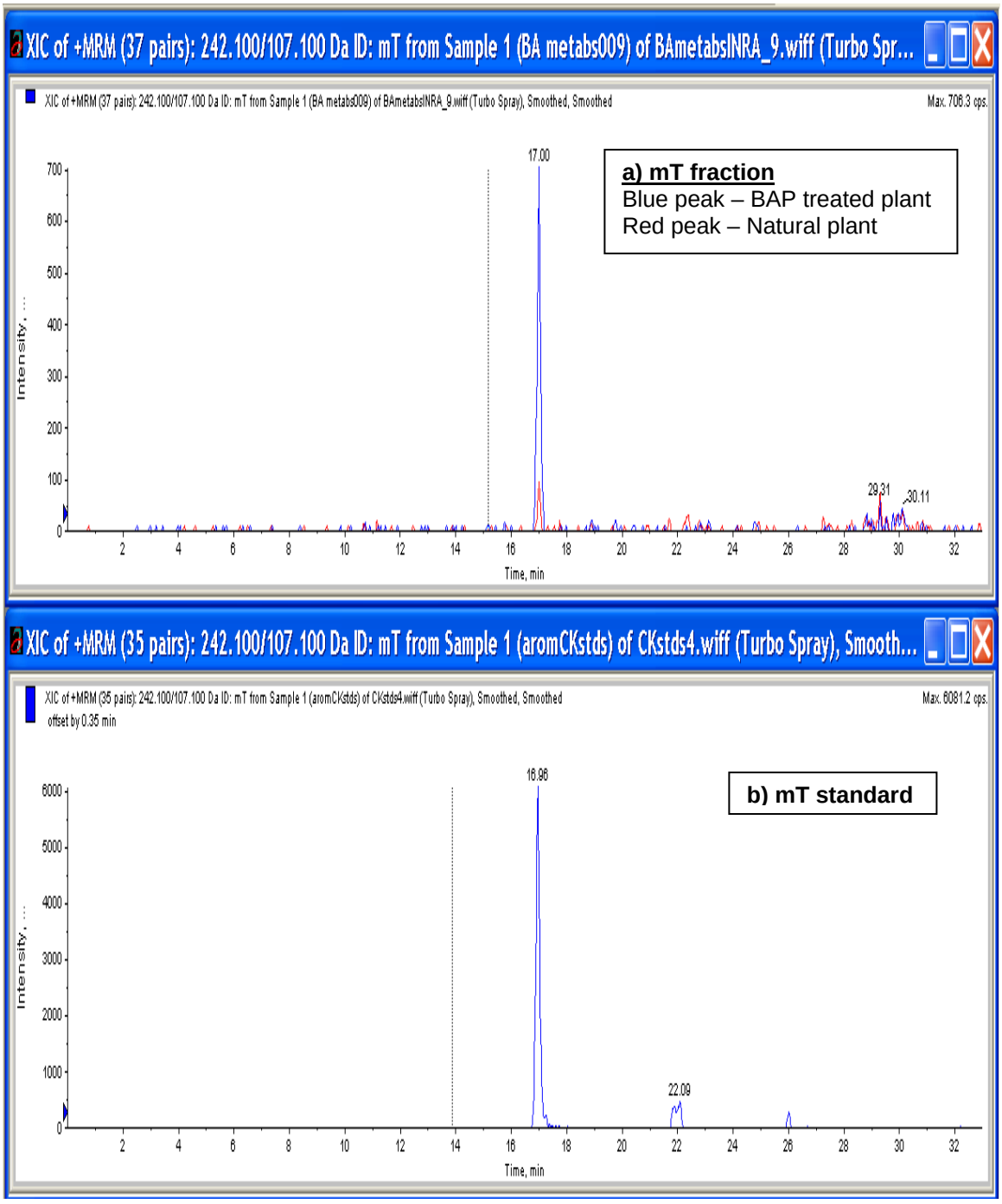


Fig. 9.2 a) Comparison of peak area between the mT fractions of natural plants of *Zantedeschia* cv. 'Best Gold' and plants treated with BAP. **b**) Standard mT peak (Courtesy: Dr. Colin Turnbull)

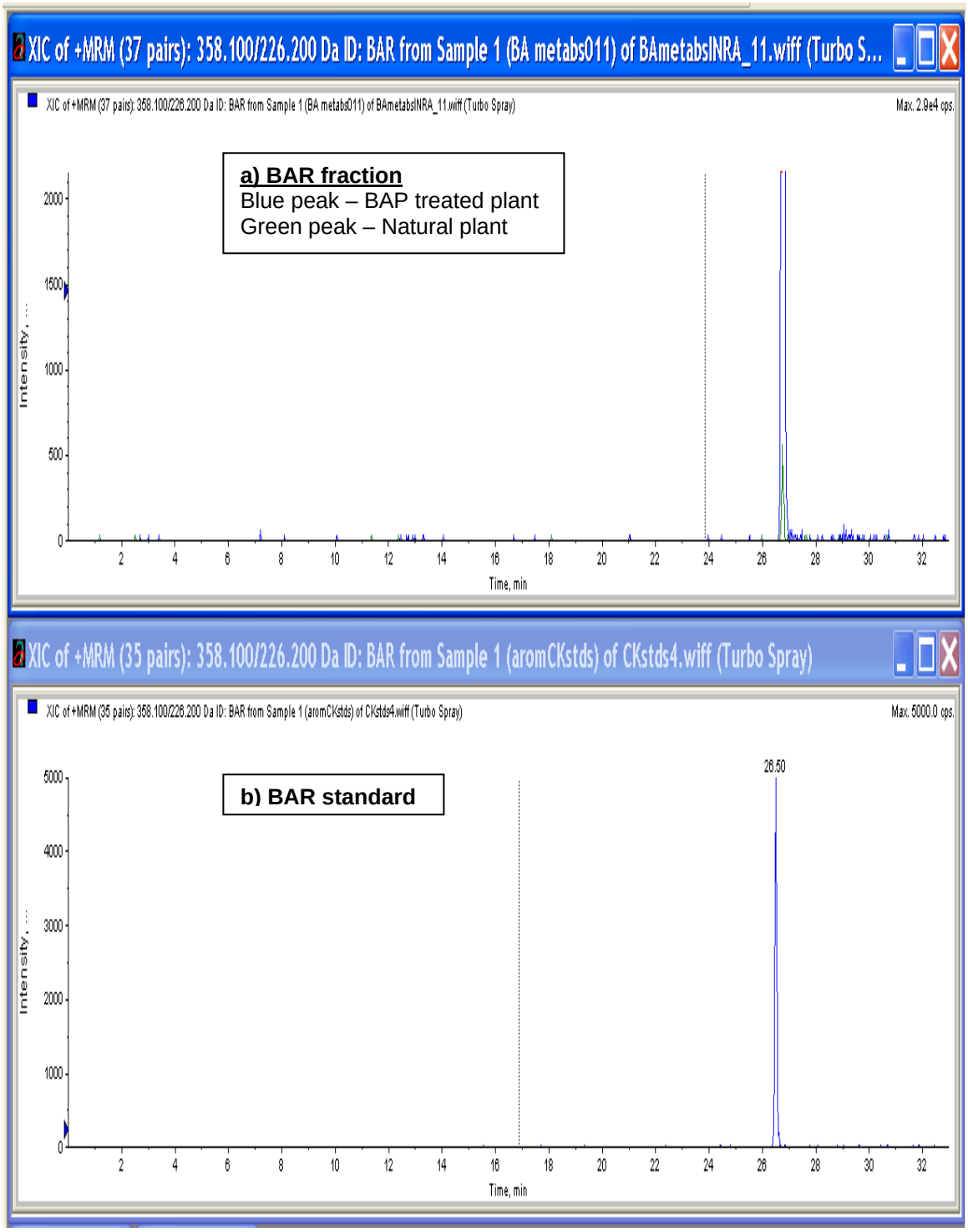


Fig. 9.3 a) Comparison of peak area between the BAR fractions of natural plants of *Zantedeschia* cv. 'Best Gold' and plants treated with BAP. **b**) Standard BAR peak (Courtesy: Dr. Colin Turnbull)

9.5 Conclusions

Meta-topolin (mT) was detected naturally in summer-flowering *Zantedeschia*, in contrast to the identification of oTR in the winter-flowering group. While oT and oTR were not detected naturally, and mTR and BAP were scarcely present, BAR was detected in high concentrations naturally. The scale of mT and BAR increased 10 and 50 fold respectively following the exogenous application of BAP. Given the physiological and commercial significance of mT, further studies aimed at quantification of endogenous mT are recommended.

10. General discussion and conclusions

10.1 The three phases of the growth cycle of Zantedeschia

Though the influence of the three types of dormancy (Lang, 1987) i.e., para-, endo- and ecodormancy, on the growth cycle of *Zantedeschia* has been widely acknowledged (refer Section 1.3), a reliable indicator that signals the transition of buds from apical dominance to endodormancy was elusive. An inherent drawback associated with this judgment is that the different types of dormancy do not occur as discrete units (Tanino, 2004), and the transition of buds through different types of dormancy exists in a continuum (Faust et al., 1997). Accordingly in *Zantedeschia*, while the role of the atrophied leaf (Fig. 1.2) in the context of dormancy was not ascertained (refer Section 1.3), the onset of endodormancy was suggested to occur even prior to the cessation of leaf production (Halligan et al., 2004). Within the context of this thesis, as evident by the pilot studies conducted within Chapters 4 and 5, the lack of knowledge on the establishment of endodormancy rendered the respective branch-inducing treatments ineffective. Given that one of the approaches to attain the commercial objective (refer Section 1.6) comprised the stimulation of branching, identification of the right time to apply branch-inducing treatments during the growth cycle was a major challenge.

A remarkable breakthrough was achieved when the period of active growth, cessation of new leaf production and the formation of an atrophied leaf were placed in the context of underlying changes in the shoot apical meristem (SAM). The notion that morphological and/or physiological changes that plants undergo during their ontogeny are different episodes in the life of the SAM (Poethig, 1990) led to the demarcation of the growth cycle of *Zantedeschia* into three phases i.e., phases 1, 2 and 3 (refer Chapter 7), based on visual signposts that manifest underlying changes in the SAM. Since these visual signposts coincide with the transition of buds from apical dominance to endodormancy (refer Section 1.3), and dormancy is also a facet of altered meristem fate (Horvath et al., 2003), the three phases were suggested to hypothetically track the transition of buds from apical dominance to endodormancy (refer Chapter 7).

Using the decline in branching observed along the three phases of growth following the application of the cytokinin (CK) 6-benzylaminopurine (BAP) as a diagnostic tool, the parallel transition of buds from apical dominance to endodormancy with the three phases of growth was vindicated (refer Chapter 7). Concomitant with the decline in the degree of branching following the application of BAP alone along the three phases of growth, the influence of a sequential application of gibberellin (GA) i.e., GA_3 (BAP → GA_3) on enhancing the degree of branching along the three phases of growth was established (Chapter 7). The decline in flowering along the three phases of growth following the application of GA_3 alone, and the concomitant increase in the degree of flowering with the sequential application of BAP (GA_3 → BAP), further highlighted the significance of the three phases of growth on the regulation of dormancy and flowering in *Zantedeschia*. As developed later in the thesis (Chapter 8), the three phases were also used as a roadmap to elucidate the sensitivity changes of the buds to CK, during their transition from apical dominance to endodormancy.

Though a modeling approach based on heat-unit accumulation was proposed to predict the onset and duration of endodormancy (Carrillo Cornejo et al., 2003), the range of degree-days that specifies the duration of endodormancy varied with the season and cultivar. This modeling approach is further confounded by the logistics of executing this approach at the field level. In contrast, the three phases of the growth cycle based on visual clues can be used as reliable indicators at the grower's level to successfully track the transition of buds from apical dominance to endodormancy, which will in turn facilitate devising further strategies to stimulate branching in *Zantedeschia*. Future studies investigating dormancy and flowering phenomena in *Zantedeschia* can therefore use these three phases as a navigation device.

10.2 Hormonal control of branching in Zantedeschia

10.2.1 Apical dominance

Owing to its sympodial growth habit (Fig. 1.3b), the floral productivity of *Zantedeschia* was hypothesized to be directly proportional to the degree of branching (refer Section 1.3). Therefore, one of the approaches devised to attain the commercial objective of increased floral productivity was aimed at increasing the degree of branching (refer Section 1.6). Historically, two strategies based on hormonal control have been employed to stimulate branching in plants:

1. Inhibition of apically derived auxin (Thimann and Skoog, 1933; Leyser, 2005) and/or,
2. Application of CK to the suppressed buds (Sachs and Thimann, 1967; Shimizu-Sato et al., 2009)

10.2.1.1 Auxin inhibition

Within Chapters 4 and 5 respectively, a manual method of elimination of an auxin source i.e., defoliation and a chemical method of inhibiting polar auxin transport (PAT) (refer Section 2.3.1.1) were explored towards achieving stimulation of branching. In spite of a treatment regime comprising the removal of young and/or old leaves (refer Section 4.2.2), a significant increase in the degree of branching was not stimulated, except a marginal increase in branching (< 1 axillary shoot) achieved following the removal of the two oldest leaves (Table 4.1). A striking feature corroborating the relationship between the leaf area and leaf area duration (LAD) on the resultant tuber size/weight (Funnell et al., 2002) was however evident in this experiment, where the continuous removal of leaves failed to generate a visible tuber at the end of the growth cycle (Table 4.1). This detrimental effect of defoliation, combined with the lack of stimulation of branching stressed the need to seek alternative modes of inducing branching in *Zantedeschia*. In other plant species such as *Fraxinus excelsior* L. (common ash), complete defoliation was more effective in stimulating axillary bud outgrowth compared to partial defoliation (Collin et al., 2000). Nevertheless, the increase in tuber size/weight caused by the marginal increase in

branching by the removal of the oldest leaves (Table 4.1) provided motivation to stimulate branching further. Defoliation therefore cannot be recommended as a commercial practice.

Inhibition of PAT using auxin transport inhibitors (ATI) performs the physiologically intended objective of decapitation minus the physiological and/or morphological drawbacks inherent to decapitation (refer Section 2.4.1). ATI have therefore been successfully used to stimulate branching in several plant species (refer Section 2.4.3). In *Zantedeschia*, a media drench of a highest concentration of 1 – N – Naphthalphthalamic acid (NPA) tested and foliar spray of NPA at a lower concentration were successful in stimulating only a marginal increase in branching (≤ 1 axillary shoot; Table 5.1). However, the scope to stimulate further branching by foliar application was limited due to a concomitant decline in branching with higher concentrations of NPA (Table 5.2). Whatsoever, as observed following defoliation, the marginal increase in branching stimulated by the application of ATI, also equated to an increase in the resultant weight of the tuber (Table 5.2). In other plant species such as legumes (Mitchell et al., 1965) and apples (*Malus domestica* Borkh.) (Hooijdonk, 2009) the application of ATI on the stem below the apical bud was successful in inhibiting PAT and thereby stimulating outgrowth of axillary buds below the point of application of ATI. However in *Zantedeschia*, where the stem is a modified underground structure i.e., the tuber, the dynamics of auxin transport and distribution is largely unknown. Moreover, the application of ATI below the apical bud is impossible after planting (refer Section 5.4.1). While the application of other ATI such as 2, 3, 5–triiodobenzoic acid (TIBA) and 9–hydroxyfluorene–9–carboxylic acid (HFCA) can be attempted in contrast to NPA, the logistics of applying/transporting the ATI to the tuber region below the apical bud will still remain a major challenge. Alternatively, a media drench of these ATIs can be tested.

10.2.1.2 CK application

Following the moderate success of the auxin inhibition strategy to induce branching in *Zantedeschia* (Chapters 4; 5), the historical approach of CK application to stimulate branching (Shimizu-Sato et al., 2009) was devised as an alternative strategy (Chapter 6). Though the exogenous application of BAP was successful in stimulating branching in flowering-sized tubers of *Zantedeschia* (Naor et al., 2005b), these tubers were naturally

prone to branching. The strength of apical dominance of these tubers was therefore considered to be weaker than that of the single-shoot seedlings used throughout this thesis during their first growth cycle (refer Section 1.3). The stimulation of branching in an experimental material and cultivar i.e., 'Best Gold', less prone to branching (D'Arth et al., 2007) was therefore considered a challenge, and the successful stimulation of branching in this experimental material following the foliar-application of BAP during phase 1 of the growth cycle, was a major breakthrough (Chapter 6). Given that a linear branching response was obtained with increasing concentrations of BAP, and 6.6 ± 0.5 additional shoots were stimulated with the highest concentration of BAP tested (8.9 mM) (Chapter 7) as opposed to the ≤ 1 axillary shoot stimulated by the auxin inhibition strategy (Table 4.1; 5.1), there is still further scope to increase branching in *Zantedeschia* by the application of CK. While BAP belongs to the group of aromatic CK (ARCK) (refer Section 2.3.1.2), the influence of other CK such as zeatin (Z) which belongs to the isoprenoid CK (ISCK) group and synthetic CK such as thidiazuron (Mok and Mok, 2003) on the degree of branching were not explored in this thesis. In view of the fact that an increase in branching following the application of BAP resulted in an increase in the tuber size/weight at the end of the growth cycle, which in turn equated to an increase in flowering in the next growth cycle (Chapter 6), the stimulation of additional branching by alternative sources of CK may increase the chance of enhancing the floral productivity of *Zantedeschia* further.

10.2.1.3 GA application

Irrespective of its concentration (refer Chapter 7), GA_3 stimulated 1 additional axillary shoot during phase 1 of the growth cycle (Chapter 7). When applied in sequential combination with BAP (BAP → GA_3), GA_3 failed to enhance branching significantly during phase 1 compared to the application of BAP alone, and the branching stimulated by the reverse order i.e., (GA_3 → BAP) was solely attributed to the BAP component (Chapter 7). The significance of the sequential application of GA_3 on the degree of branching however became prominent only after the establishment of endodormancy in phases 2 and 3 (Chapter 7). The inability of GA_3 to alleviate apical dominance in *Zantedeschia*, at least to the extent that BAP does, has also been acknowledged by Naor et al. (2005a; b). However, gibberellins differ in their biological activity (Crozier et al., 1970) and in other

studies, GA_{4+7} have been found to be more potent than GA_3 (Shoub and De Hertogh, 1974). Though it is unlikely that GA alone would be as effective as BAP in alleviating apical dominance in *Zantedeschia*, exploring the effects of other forms of gibberellins would still be worthwhile, and could potentially replace the role of GA_3 as a hormone that interacts with BAP in phases 2 and 3.

10.2.2 Endodormancy

Unlike other plant species where the inception of endodormancy is governed by environmental factors (Wareing, 1956), endodormancy in *Zantedeschia* is autonomously controlled (Naor and Kigel, 2002). While the *CONSTANS/FLOWERING LOCUS T* (CO/FT) module relates to the photoperiodic control of endodormancy (Bohlenius et al., 2006), the mechanism underlying the autonomous control of day-neutral *Zantedeschia* (Naor et al., 2005b) is unknown. Among other factors, it is possible that the inception of endodormancy in *Zantedeschia* is governed by an internal node counting mechanism. While this mechanism has been shown to indirectly regulate autonomous flowering (Sachs, 1999), its role in the regulation of endodormancy is only speculative at this stage.

10.2.2.1 Cross-talk between CK and GA

The efficacy of BAP alone to stimulate branching declined significantly with the transition of buds from apical dominance to endodormancy i.e., phase 1 through to 3, and GA_3 alone had no effect on branching in phases 2 and 3 (Chapter 7). After the establishment of endodormancy, the sequential order of application of BAP and GA_3 (BAP → GA_3) was the key to the successful stimulation of branching, where BAP → GA_3 was successful in stimulating significant branching compared to the application of GA_3 → BAP (Table 7.1). The significance of the order of application of CK and GA has been stressed before in *Glycine max* L. (soy bean), where the sequential application of CK followed by GA was successful in stimulating branching compared to their simultaneous application (Ali and Fletcher, 1971). The simultaneous application of CK and GA to cuttings of *Solanum andigena* resulted in complete arrest of the axillary bud outgrowth (Woolley and Wareing, 1972), and in *Zantedeschia*, simultaneous application of GA_{4+7} and BAP as Promalin ™ (Valent BioSciences, USA) was not capable of stimulating branching significantly

compared to the application of BAP alone (Funnell and MacKay, 1988a). For these reasons, the sequential application of BAP followed by GA_3 (BAP → GA_3) is recommended for future studies aimed at stimulating branching after the onset of endodormancy. Given the direct relationship between the LAD and the size/weight of the tuber (Funnell et al., 2002), extending the active growth period of *Zantedeschia* by stimulating branching in phases 2 and 3 may result in enhanced tuber size/weight.

Recently, the cross-talk between CK and GA in the control of endodormancy was corroborated in *Solanum tuberosum* L. (potato) via molecular/genetic approaches (Hartmann et al., 2011). In this study, as observed in *Zantedeschia*, the application of BAP to endodormant buds did not support sprout growth until GA_3 was applied sequentially. Transgenic potato plants deficient in endogenous CK exhibited prolonged dormancy and plants deficient in endogenous GA exhibited a delay in sprouting. While GA induced sprouting of buds in wild-type plants, it failed to induce sprouting of buds in transgenic plants with low endogenous CK levels, thereby highlighting the roles of both CK and GA in the regulation of endodormancy. Similar studies in *Zantedeschia* are required to progress our understanding of the hormonal cross-talk in the control of endodormancy.

Given the pleiotropic nature of plant hormones (Davies, 1995), the hormonal regulation of endodormancy described in *Zantedeschia* (Chapter 7) cannot be confined to the cross-talk between CK and GA alone. The antagonistic relationship between auxin and CK is well known in the control of axillary bud outgrowth (Nordstrom et al., 2004), and auxin has also been suggested to regulate the rate limiting step of GA biosynthesis (Ross et al., 2001). Though investigating auxin dynamics during the transition of buds from apical dominance to endodormancy was beyond the scope of this study, cross-talk between auxin-CK-GA cannot be ruled out. Likewise, the antagonistic cross-talk between GA and abscisic acid (ABA) in the control of endodormancy is well known (Tanino, 2004). Though a decline in endogenous ABA content and a concomitant increase in GA were observed during bud sprouting following endodormancy release, the reverse correlation i.e., increase in ABA and a concomitant decline in GA, were not observed during the inception of endodormancy (Naor et al., 2008). Quantification of endogenous ABA, GA (Naor et al., 2008) and ISCK (D'Arth et al., 2007) has been attempted in separate studies within the context of

endodormancy and subsequent sprouting. Given that ARCK also exist naturally in *Zantedeschia* (das Neves and Pais, 1980; Chapter 9), and cross-talk between BAP and GA_3 was involved in the regulation of endodormancy (Chapter 7; 8), revisiting the quantification of ARCK-GA-ABA in the context of endodormancy will provide significant insight into the hormonal control of endodormancy in *Zantedeschia*.

10.2.3 Sensitivity as a controlling factor

The decline in the degree of branching observed with the transition of buds from apical dominance to endodormancy i.e., phases 1 through to 3, following the application of BAP alone, and a subsequent increase in branching following a sequential application of GA_3 (BAP → GA_3) (Chapter 7), indicated an ontogenetic change in the sensitivity of the buds to CK and/or GA. Changes in the sensitivity of the buds to CK (Turnbull and Hanke, 1985a) and GA (Suttle, 2004) have been also been described in potato. However, these studies demonstrated sensitivity changes after the establishment and subsequent release of endodormancy. In the current study, as described within Chapter 8, factors contributing to the sensitivity changes associated with the inception of endodormancy were explored. While other studies in *Zantedeschia* have reported an increase in endogenous ISCK levels coinciding with the release of endodormancy (D'Arth et al., 2007), given that ARCK were detected naturally in *Zantedeschia* (Chapter 9), quantification of endogenous ARCK during the inception and release of endodormancy in *Zantedeschia* may support the role of CK in the control of endodormancy.

Among several factors that could possibly contribute to changes in the sensitivity of the responding tissue (Firn, 1986), the buds in this case, differences in the uptake, availability and metabolism of [8-^{14}C] BAP were investigated as a first step towards understanding sensitivity changes of the buds to CK within the context of dormancy. With the advent of molecular/genetic approaches and the identification of receptors for CK (Inoue et al., 2001), other factors such as the receptivity of the hormone, affinity between the hormone and the receptor and the subsequent signal transduction cascade could be investigated in detail in the future.

10.2.3.1 Uptake, availability and metabolism of [8-^{14}C] BAP

An integral of changes in the uptake, availability and metabolism of [8-^{14}C] BAP (Chapter 8) contributed to the decline in the sensitivity of the buds to CK with the establishment of endodormancy, which manifested as a decline in branching observed in Chapter 7. The same factors therefore also contributed to the increase in branching observed following the application of BAP alone in phase 1 where the buds are predominantly controlled by apical dominance (Chapter 8). Perception of the signal is the fist step in signal transduction (Heyl and Schmulling, 2003), and the significant decline in the uptake of [8-^{14}C] BAP in phase 3 from both the leaves (Fig. 8.2A; B) following the application of unlabelled BAP (8.9 mM), constituted the first factor that contributed to sensitivity changes. In phase 1, a significant decline in the uptake of [8-^{14}C] BAP was observed even between the youngest leaf (leaf 1; Fig. 8.2A) and the penultimately youngest leaf (leaf 2; Fig. 8.2B). Therefore, it is not surprising that the uptake was lower in phase 3, during which time the natural senescence of leaves had commenced (Chapter 7).

The decline in the uptake of [8-^{14}C] BAP in phase 3, contributed to a concomitant decline in the availability of radioactivity to other plant parts (Fig. 8.3). Accordingly, a significant decline in the availability of radioactivity was observed in almost all subterranean plant parts (Fig. 8.3I-N) in phase 3, and especially the buds. Since the buds comprise the responding tissue, this difference in the availability of radioactivity to the buds affected by uptake and/or availability was considered to be amongst the defining factors that determines the branching response in phase 1 and the absence of the same in phase 3. Moreover, true to the observation that exogenously applied CK is directed to the buds during the early stages of growth (Abo-Hamed et al., 1984), the apical bud, other axillary buds and the upper region of the tuber where the buds reside, possessed the highest level of radioactivity following the application of BAP (8.9 mM) in phase 1 (refer Section 8.3.3.1).

As expected, the lower uptake of [8-^{14}C] BAP observed in both the leaves in phase 3 (Fig. 8.2A; B) corresponded to a high amount of unmetabolized [8-^{14}C] BAP remaining in the distal halves of the leaves where the treatments were applied (Fig. 8.6A3 (a); E3 (a)). In these parts in phase 1 (Fig. 8.6A1 (a)) *meta*-topolin (mT) was the major metabolite following the application of [8-^{14}C] BAP + BAP (8.9 mM). In the subterranean parts of the

plant, a significant change in the metabolic profile of [8-^{14}C] BAP was not observed within the three phases of growth. Adenosine monophosphate (AMP) and mT constituted the major metabolites (Fig. 8.6I (a)-N (a)). A qualitative change in the metabolic profiles of [^{3}H]-Z and [^{3}H]-iP was also not observed during different stages of dormancy in potato (Suttle, 2001). While a quantitative change in the metabolic profiles of [^{3}H]-Z and [^{3}H]-iP was also not observed in potato (Suttle, 2001), the amount of metabolic mT in the subterranean plant parts was different within the three phases of growth. In the upper region of the tuber, for example, the amount of mT was 6 times higher in phase 1 (Fig. 8.6 L1 (a)) than that in phase 3 (Fig. 8.6 L3 (a)). Given that mT is an active ARCK (Holub et al., 1998) and was found to stimulate branching more effectively than BAP (Kaminek et al., 1987), this difference in the amount of mT observed in the responding tissues between phases 1 and 3, was considered to be amongst the key factors that contributed to a decline in the sensitivity of the buds to CK with the establishment of endodormancy.

As observed after the application of [8-^{14}C] BAP + BAP (8.9 mM) (Fig. 8.2A; B), a significant decline in the uptake of [8-^{14}C] BAP in phase 3 from both the leaves was not observed following the sequential application of unlabelled GA_3 (1.4 mM) (Fig. 8.2A; B). Again, compared to the application of [8-^{14}C] BAP + BAP (8.9 mM) in phase 3, the sequential application of GA_3 caused a significant increase in the availability of radioactivity in the upper region of the tuber where the buds reside (Fig. 8.3L). In this part, the amount of metabolic mT was 4 fold higher in the upper region of the tuber (Fig. 8.6 L3 (b)) following the application of GA_3 in phase 3, compared to the application of BAP (Fig. 8.6K3 (a); L3 (a)). Combined together, these results strongly suggest the influence of CK and/or GA on the ontogenetic changes in the sensitivity of the buds with the establishment of endodormancy, which in turn was manifested as an increase in branching after the sequential application of BAP followed by GA_3 (BAP $\rightarrow$ GA_3) in phase 3 (Chapter 7). Further studies involving radiolabeled GA_3 may elucidate the influence of CK and/or GA on the sensitivity changes. Nevertheless, this result marks a new dimension in the cross-talk between CK and GA and hormonal cross-talk in general, and is definitely worth pursuing.

10.2.4 Topolins in *Zantedeschia*

An interesting result from the metabolic studies of [8-^{14}C] BAP conducted in *Zantedeschia* (Chapter 8) was the identification of mT as a metabolic product of BAP (Fig. 8.6; Table 8.2). However, this deduction was made based on the coincidence of one of the metabolic radioactive peaks of [8-^{14}C] BAP with the retention time of an authentic standard of mT (Table 8.1), and the identification of mT in the fraction corresponding to the authentic standard following the application of unlabeled BAP (8.9 mM), using LC-MS analyses (Table 8.2). Unequivocal evidence that mT was derived from BAP can be corroborated through the identification of the [8-^{14}C] group of BAP in metabolic mT. Given that initial results from metabolic studies in *Zantedeschia* have provided a strong lead in this direction, the current system can be exploited further to prove this metabolic pathway, probably using rigorous LC-MS analyses.

The identification of mT in the fraction corresponding to the authentic standard of mT following the application of unlabeled BAP (8.9 mM) using LC-MS analyses (Table 8.2), led to the assumption that mT could exist naturally in summer-flowering *Zantedeschia*. Subsequently, using enzyme linked immunosorbent assays (ELISA) (refer Section 9.2.4) and LC-MS analyses (refer Section 9.2.5) of fractions corresponding to 6 different ARCK in natural plants of *Zantedeschia* in phase 1 (refer Section 9.2.3), mT (Fig. 9.1c) and 6-benzylaminopurine riboside (BAR) (Fig. 9.1f) were identified (Table 9.1). This was in contrast to the identification of *ortho*-topolin riboside (oTR) naturally in fruits of winter-flowering *Zantedeschia aethiopica* (das Neves and Pais, 1980a). Given the differential biological activity of isomers of topolins in bioassays (Holub et al., 1998), spatial differences in the distribution of topolins is possible, where oT could be present in mature plants i.e., phase 3, in contrast to the presence of mT during early stages of the plant i.e., phase 1. Further studies should be aimed at constructing and comparing the natural profile of ARCK during the three phases of growth of *Zantedeschia*.

A comparison of the peak area corresponding to mT of the natural plant fraction, and that following the application of BAP highlighted the role of exogenous BAP in regulating mT content (Fig. 9.2). Whether BAP activated the endogenous mT biosynthetic pathway or mT was derived via metabolism of BAP still remains questionable. Quantification of natural

ARCK during the three phases of the growth cycle, and following the application of BAP has not been attempted before and is required in order to progress this line of research.

Collectively, unequivocal evidence that mT is derived via the metabolism of BAP has serious physiological and commercial implications. This would be a major step in elucidating the biosynthetic/metabolic pathway of ARCK, which in comparison to the ISCK group, is less understood (refer Section 2.3.1.2.1). Given that mT was found to be more effective in stimulating branching than BAP (Kaminek et al., 1987), and mT has already been recommended as an alternative to BAP as the CK source in micropropagation (refer Section 2.3.1.2.1), mT could possibly replace BAP in the stimulation of branching *in vivo* in the near future.

10.3 Hormonal control of flowering in Zantedeschia

Within the context of this thesis, two approaches were adopted to attain the commercial objective of increasing floral productivity in *Zantedeschia* (refer Section 1.6):

1. Mitigating the decline in flowering associated with long-term tuber storage and,
2. Increase the floral productivity via increasing the number of shoots/degree of branching.

Subsequently, the second approach was hypothesized to influence floral productivity via two mutually inclusive/exclusive mechanisms (refer Section 4.1):

i. An increase in branching would equate to an increase in the leaf area and LAD, which is directly related to the size/weight of the tuber (Funnell et al., 2002). Since the size/weight of the tuber is directly related to the floral productivity of *Zantedeschia* (Corr and Widmer, 1991; Naor et al., 2005a), an increase in tuber size/weight achieved by stimulation of branching would equate to an increase in floral productivity. Additionally, since the tubers of *Zantedeschia* are also commercial significant (refer Section 1.2), larger sized tubers would potentially fetch a higher price.
ii. Owing to its sympodial growth habit (refer Section 1.3), stimulation of flowering in a branched plant would trigger the flowering cascade, resulting in enhanced floral productivity.

10.3.1 Decline in flowering with long-term tuber storage

As hypothesized (refer Section 3.1), the decline in the total (Fig. 3.1) and primary (Fig. 3.2) flowering percentages between 4 and 6 month storage durations, corroborated a concomitant decline in the sensitivity of the buds to GA. Using the modeling approach proposed by Firn (1986) as a template (refer Section 2.2.1), this change in sensitivity between the 4 and 6 month storage durations was attributed to a decline in the affinity of GA_3 to the corresponding receptors (Fig. 3.1; 3.2). While a further decline in the flowering percentage was expected after 9 months of storage, in contrary to the hypothesis, an increase in the flowering percentage was observed between the 6 and 9 months storage durations (Fig. 3.1; 3.2).

At the outlook, this increase in the flowering percentage signaled a restoration in the sensitivity of the buds to GA after 9 months of storage. On closer observation however, a significant decline in the degree of branching was observed between the 4 (4.2 ± 0.2 shoots), 6 (4.8 ± 0.2 shoots) and 9 (3.5 ± 0.2 shoots) month storage durations (Table 3.1), and irrespective of the storage durations, on an average, approximately 2 flowers were produced per tuber (Table 3.2). Given that the dominant buds emerge and flower first (Funnell and Go, 1993), the population of predominantly dominant buds that emerged after 9 months of storage were naturally prone to flowering, which manifested as an increase in the flowering percentage (Fig. 3.1; 3.2).

Even with the commercial recommendation of GA application to increase flowering, only 60% of the emerging shoots are capable of flowering (Funnell and Go, 1993). Therefore, all shoots are not equally prone to flowering. Given that a variable population of buds exists in flowering-sized tubers of *Zantedeschia* (Funnell and Go, 1993), a prerequisite for further research is to classify the buds based on their flowering potential. Naor et al. (2005a) have suggested that the flowering potential of the buds declines with increasing distance from the apical bud. However, further studies should aim at a bud to bud comparison of the phenological events prior to flowering. This can be achieved by photographic tracking of the buds from the first growth cycle until flowering is achieved during the third growth cycle (Fig. 1.1).

10.3.2 Increasing floral productivity via increasing tuber size/weight

A direct relationship between tuber size/weight and floral productivity has been established (Corr and Widmer, 1991; Naor et al., 2005a). An increase in branching stimulated by the application of BAP in 1N NaOH during phase 1 of the growth cycle (Fig. 6.3), possibly by enhancing the leaf area and LAD, resulted in an increase in tuber size (Fig. 6.6a) and weight (Fig. 6.5) as proposed by Funnell et al. (2002). The positive correlation between the leaf area and LAD and its consequential effect on tuber size/weight was further vindicated by the application of a commercial formulation of BAP i.e., Cylex^{TM} (Valent BioSciences, USA). At higher concentrations, the application of Cylex^{TM} caused scorching of leaves not evident with the application of BAP in 1N NaOH (refer Section 6.3.1). Consequently, the weight of tubers from Cyclex treated plants at the end of the growth cycle was even lesser than that of the control (Fig. 6.5), which also reflected in the visual size of the tuber (Fig. 6.6b). This result, apart from corroborating the direct link between branching, leaf area and LAD, and tuber size/weight, also highlighted the importance of the formulation of BAP to be employed to stimulate branching in *Zantedeschia*. Though the effects of other CK formulations were not evaluated in this study, the formulation comprising BAP in 1N NaOH can be safely recommended for stimulating branching in *Zantedeschia*.

The tubers obtained following the application of BAP in 1N NaOH during the first growth cycle, when treated with the existing commercial recommendation of GA_3 alone (0.3 mM) in the second growth cycle, exhibited 30-60% higher floral productivity than the tubers treated with GA_3 alone (Fig. 6.7). On the contrary however, and further reiterating the direct relationship between tuber size/weight and floral productivity, the tubers obtained following the application of Cylex^{TM} at high concentrations in the first growth cycle (Fig. 6.5) resulted in lesser floral productivity compared to the existing commercial recommendation of GA_3 alone (0.3 mM) (Fig. 6.7). This strategy of increasing the floral productivity of *Zantedeschia* via increasing the tuber size/weight, which in turn was increased via stimulation of branching, has significant commercial implications for the cut flower industry. In view of the fact that the tubers of *Zantedeschia* are also commercially significant (refer Section 1.2), this result provides a double advantage to the growers.

10.3.3 Increasing floral productivity via shoot number

As hypothesized (refer Section 1.6), an increase in branching and subsequent stimulation of the sympodial flowering cascade means that the sequential order of application of BAP and GA_3 in group 1 i.e., (BAP → GA_3) (Fig. 7.2) which produced a higher degree of branching (Table 7.1) than the sequential order comprising GA_3 followed by BAP in group 2 (GA_3 → BAP) should have higher floral productivity. On the contrary however, the floral productivity was significantly lower in all the three phases of growth, compared to the order of application comprising GA_3 → BAP (Table 7.1). This order of application (GA_3 → BAP) consistently resulted in a significant increase in the percentage of flowering plants, compared to the sequential order of BAP → GA_3 (Table 7.2A). Also, within the series of experiments conducted within Chapter 3, the increased number of shoots after the 6 months storage duration (4.8 ± 0.2 shoots) did not result in a concomitant increase in floral productivity, compared to that after 9 months of storage (3.5 ± 0.2 shoots) (Table 3.2). In view of these results, the direct relationship between the number of shoots/degree of branching and floral productivity of *Zantedeschia* needs to be revisited. As observed earlier (refer Section 10.3.1), the factors that determine floral transition in the shoot need to be determined to fully understand this relationship.

Nevertheless, an increase in the floral productivity of *Zantedeschia* achieved via an increase in the tuber size (Chapter 6), and via the sequential application of GA_3 followed by BAP (GA_3 → BAP) (Chapter 7) fulfill the commercial objective of this thesis, and command further exploration, particularly with regard to the relationship between branching and flowering, hormonal control of branching and flowering in *Zantedeschia*, sensitivity changes of the buds to CK and/or GA with the establishment of endodormancy and identifying the role of topolins in the context of dormancy. The results thus obtained, can be incorporated into the bud outgrowth model involving strigolactones (Ferguson and Beveridge, 2009), and can contribute to the pool of evidence drawing common mechanisms between dormancy and flowering in plants (Horvath, 2009).

11. Appendices

Appendix 11.1. Composition of the bark-based growth medium (Daltons, New Zealand) used throughout this study for growing *Zantedeschia* cv. 'Best Gold'.

Ingredient	**Percentage composition (%)**
C.A.N - Fines A grade	50%
Fibre	30%
Pacific pumice (7 mm)	20%

Appendix 11.2. Regime and composition of the slow-release fertilizer mixed with the bark-based medium used throughout this study for growing *Zantedeschia* cv. 'Best Gold'.

Fertilizer regime per 100 litres of base media mix.

- Dolomite (Ravensdown fertilizers; New Zealand) 150g
- 8-9 month Osmocote (Scotts International B.V., Nijverheidsweg; The Netherlands) 200g
- 3-4 month Osmocote (Scotts International B.V., Nijverheidsweg; The Netherlands) 100g

Fertilizer ingredients

Name of fertilizer	Ingredients	Percentage of ingredients (%)
Dolomite	Magnesium	11.5
	Calcium	24.0
8-9 month Osmocote	Nitrogen	16
	Phosphorus	3.5
	Potassium	10.0
	Sulphur	2.4
	Magnesium	1.2
	Boron	0.02
	Copper	0.05
	Iron	0.4
	Manganese	0.06
	Molybdenum	0.02
	Zinc	0.015
3-4 month Osmocote	Nitrogen	15
	Phosphorus	4.8
	Potassium	10.8

Sulphur	3.0
Magnesium	1.2
Boron	0.02
Copper	0.05
Iron	0.4
Manganese	0.06
Molybdenum	0.02
Zinc	0.015

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Topolins: Current Research Status and Applications

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Abstract

Topolins are hydroxylated analogues of 6-benzylaminopurine (BAP). They belong to a group of cytokinins with an aromatic side chain, in contrast to the isoprenoid group which contain an aliphatic side chain. Though topolins have been identified in diverse plant species, knowledge on their biosynthesis, signal transduction and metabolism is limited. Recent research advances in the isoprenoid group can therefore provide a template to direct further research on topolins. In spite of their high biological activity, applications of topolins to date have focused primarily on their use as an alternative source of cytokinin in micropropagation. The review starts with a primer on the history, nomenclature and chemical structure of topolins. A list of topolins identified in different plant species is provided to substantiate their widespread occurrence. A brief description of recent findings on the biosynthesis and metabolism of the isoprenoid group is offered to compare and contrast existing information on topolins. Determination of the biological activity of the stereoisomers of topolins is described as influenced by their chemical structure and signal perception. An account of the existing and plausible applications of topolins in yield accumulation, alleviating dormancy and abiotic stress management is provided. Finally, the review expresses confidence that topolins will play a significant role in the emerging scenario that cytokinins could hold the key to the next green revolution.

1. Introduction

The last decade has been very exciting for plant hormonal research with significant advances in our understanding of the existing hormones (Santner and Estelle, 2009), discovery of new plant hormones (Gomez-Roldan et al., 2008), exploring the network of hormonal cross-talk (Ross et al., 2001; Rashotte et al., 2005; Weiss and Ori, 2007; Chandler, 2009), and establishing the role of this network in key developmental processes (Ferguson and Beveridge, 2009; Subbaraj et al., 2010). This progress has been largely facilitated by genetic (McCourt, 1999; Ashikari and Matsuoka, 2002; Gazzarrini and McCourt, 2003), analytical (Birkemeyer et al., 2003; Pan and Wang, 2009) and bioinformatics (Peng et al., 2008) approaches.

Cytokinins are hormones with a characteristic trait of promoting cell division (Miller et al., 1955). In plants, they regulate diverse developmental and physiological processes such as the regulation of root and shoot growth, branching, chloroplast development, leaf senescence, stress response and pathogen resistance (Heyl and Schmülling, 2003). Two major groups of cytokinins are known. The naturally occurring adenine derivatives and the synthetic phenylurea group comprising CPPU and thidiazuron. The naturally occurring cytokinins are further divided into two groups based on the chemical structure of the adenine N^6-side chain as the isoprenoid group (ISCKs) which carries an aliphatic side chain, and the aromatic group (ARCKs) which carries an aromatic side chain (Mok and Mok, 2003). The ISCK group is mainly represented by Z and iP type of cytokinins, whereas the ARCK group is mainly represented by BAP and its hydroxylated analogues called topolins (Strnad, 1997).

Fundamental knowledge on the chemistry, activity and function of cytokinins was derived from early research on the ISCK group (Staden and Davey, 1979; Letham and Palni, 1983). New wisdom on the molecular mechanisms of biosynthesis (Kakimoto, 2003), perception and signal transduction (Heyl and Schmülling, 2003), metabolism and transport (Kudo et al., 2010) of cytokinins has also been provided by the ISCK group. Research on the ARCK group, though not equivalent to the ISCK group, has progressed mainly due to the exclusive techniques developed for this group of cytokinins, which includes the development of specific antibodies (Strnad, 1996), improved extraction and purification methods (Hoyerová

Abbreviations	
ABA	Abscisic acid
AHK	Arabidopsis histidine kinase
AMP/ADP/ATP	Adenosine mono/di/triphosphate
ARCKs	Aromatic cytokinins
BA3G/BA7G/ BA9G	6-benzylaminopurine 3-glucoside/6-benzylaminopurine 7-glucoside/6-benzylaminopurine 9-glucoside
BAP/BAR/ BAR5'P	6-benzylaminopurine/ 6-benzylaminopurine riboside/ 6-benzylaminopurine riboside 5'-diphosphate
CBP	Cytokinin binding protein
CKX	Cytokinin oxidase/dehydrogenase
CPPU	N-phenyl-N'- [2-chloro-4-pyridyl] urea
CRE1	Cytokinin response
CYP375A	Cytochrome P450 monooxygenase
cZ	cis-Zeatin
DHZ	Dihydrozeatin
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
GA	Gibberellic acid
Gn1a	Grain number
HPLC	High performance liquid chromatography
iP/iPR/ iPRDP	Isopentenyl adenine/ Isopentenyl adenine riboside/ Isopentenyl ribose 5'-diphosphate
IPT	Isopentenyl transferase
ISCKs	Isoprenoid cytokinins
K/KR	Kinetin/kinetin riboside
LAD	Leaf area duration
LOG	Lonely guy
MemT/MemTR	Methoxy meta-topolin/ methoxy meta-topolin riboside
MeoT/MeoTR	Methoxy ortho-topolin/ methoxy ortho-topolin riboside
mRNA/tRNA	Messenger ribonucleic acid/transfer ribonucleic acid
MS	Mass spectrometry
mT/mTR/ mT9G/mTOG/ mTR5'P	Meta-topolin/meta-topolin riboside/ meta-topolin 9-glucoside/meta-topolin O-glucoside/ meta-topolin ribose 5'-diphosphate
oT/oTR/oT9G/ oTOG/oTR5'P	Ortho-topolin/ortho-topolin riboside/ ortho-topolin 9-glucoside/ortho-topolin O-glucoside/ortho-topolin ribose 5'-diphosphate
pT/pTR	Para-topolin/ para-topolin riboside
QTL	Quantitative trait loci
SAM	Shoot apical meristem
tZ/tZRDP	Trans-zeatin/ trans-zeatin ribose 5'-diphosphate
Z/ZR	Zeatin/ zeatin riboside

et al., 2006) and state-of-the-art HPLC and MS techniques (Tarkowski et al., 2009). These techniques have cumulatively enabled the identification of previously unidentified compounds of the ARCK group (Barciszewski et al., 2007; Huang et al., 2007).

Comparative studies on the signal perception (Spíchal et al., 2004), endogenous concentration (Baroja-Fernandez et al., 2002), metabolism (Alvarez et al., 2008) and bioactivity (Amoo et al., 2010) between ISCKs and ARCKs suggest that the structural differences between the two groups could have far reaching physiological implications than previously thought. Though progress has been made, clear gaps exist in our understanding of the role of topolins. The aim of this review therefore, is to provide an update on the current research status and applications of topolins with a perspective on potential areas of application.

2. History and Nomenclature

The isolation and identification of kinetin from herring sperm DNA (Miller et al., 1955), the first compound with cytokinin activity to be chemically identified (Skoog and Armstrong, 1970), sparked a race for the identification of compounds with similar activity in plants and the chemical synthesis of identical analogues (Skoog, 1994). While BAP was synthesized in the lab the same year as the discovery of kinetin (Skinner and Shive, 1955; Skoog, 1994), zeatin was identified as a natural cytokinin in corn (*Zea mays*) endosperm only eight years later (Letham, 1963).

For almost twenty years since its chemical synthesis, BAP and its analogues were considered to be synthetic, until a hydroxylated derivative of BAP was identified naturally in mature leaves of poplar (*Populus* × *robusta*) (Horgan et al., 1973). Subsequent identification of the same compound in fruits of *Zantedeschia aethiopica* (das Neves and Pais, 1980a) and its structural isomer in poplar (Strnad et al., 1997) with putative cytokinin-like activity in bioassays (Holub et al., 1998) provided substantial evidence for the prevalent occurrence of these type of compounds naturally. To distinguish these compounds from the existing ISCK group of cytokinins, the trivial name of 'populins' (from poplar) was adopted (Horgan et al., 1975). However, since populin was already in practice for another chemical salicin benzoate, the Czech name for poplar=topol, and therefore 'topolins' was adopted for the hydroxylated

analogues of BAP (Strnad, 1997).

Several systems of abbreviations have been proposed to unify the nomenclature of cytokinins and its myriad derivatives (Letham and Palni, 1983; Crouch et al., 1993; Kamínek et al., 2000). The convention used by Strnad (1997) shall be followed throughout this review, due to its simple representation of the positional isomers of topolins and their derivatives and due to the widespread usage of this system of abbreviations in topolin related literature. In this system, topolins are represented by the alphabet 'T'.

3. Chemical Structure

ARCKs (Figure 1a-d) possess an aromatic N^6-side chain, whereas the ISCKs (Figure 1e-h) possess an aliphatic side chain (Shaw, 1994). Topolins (Figure 1b-d) are differentiated from BAP (Figure 1a) by the presence of a hydroxyl group in the aromatic side-chain. Positional isomers of topolins are further differentiated by the presence of the hydroxyl group at the 2-(*ortho*), 3-(*meta*) or 4-(*para*) positions, and are thereby referred to as *ortho*-topolin (oT) (Figure 1b), *meta*-topolin (mT) (Figure 1c) and *para*-topolin (pT) (Figure 1d), respectively. In a few plants (Tarkowska et al., 2003), the presence of a methoxy group (OCH_3) at the *ortho*-(MeoT) and *meta*-(MemT) positions have also been reported.

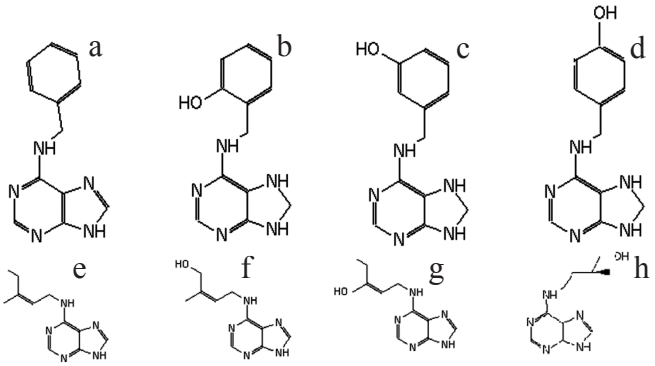


Figure 1: Chemical structures of key ARCKs and ISCKs represented by (a) BAP, (b) oT, (c) mT, (d) pT, (e) iP, (f) cZ, (g) tZ and (h) DHZ (From PMN: www.plantcyc.org)

Among the ISCKs, the zeatin group (Figure 1f-h) is distinguished from iP (Figure 1e) by the presence of a hydroxyl group in the side chain. The zeatin group is further categorized by the stereoisomeric position of the hydroxyl group as cZ (Figure 1f) where the hydroxyl group is present in the *cis* position, tZ (Figure 1g) where the hydroxyl group is present in the *trans* position, and DHZ (Figure 1h) where the hydroxyl group is saturated (Shaw, 1994).

4. Biosynthesis

At this point of time, knowledge on the biosynthesis of ARCKs is only speculative. In fact, credible knowledge on the biosynthetic pathway of ISCKs has been gathered only recently (Kakimoto, 2003). Two major modes of ISCK biosynthesis have been proposed: 1) via tRNA and 2) *de novo* synthesis of free cytokinins (Letham and Palni, 1983). In either mode, addition of the isopentenyl side-chain to an adenine base is considered to be the rate limiting step for cytokinin biosynthesis. The identification of the genes that encode the enzyme IPT, which catalyses this transfer in tRNA (Miyawaki et al., 2006; Sakamoto et al., 2006) and in the *de novo* pathway (Kakimoto, 2001; Sakamoto et al., 2006) has been crucial to the understanding of ISCK biosynthesis. tRNA-IPTs have been identified virtually in all organisms (Kamada-Nobusada and Sakakibara, 2009). However, the contribution of tRNA derived ISCKs to the total cytokinin pool is less than 40%, which suggests the significance of the *de novo* biosynthetic pathway (Kakimoto, 2003).

Detailed description of the *de novo* biosynthetic pathway of ISCKs has been dealt with elsewhere (Kakimoto, 2003; Kamada-Nobusada and Sakakibara, 2009). Briefly (Figure 2), considering ADP as a substrate for prenylation, IPT catalyses the transfer of the isopentenyl group from DMAPP to ADP

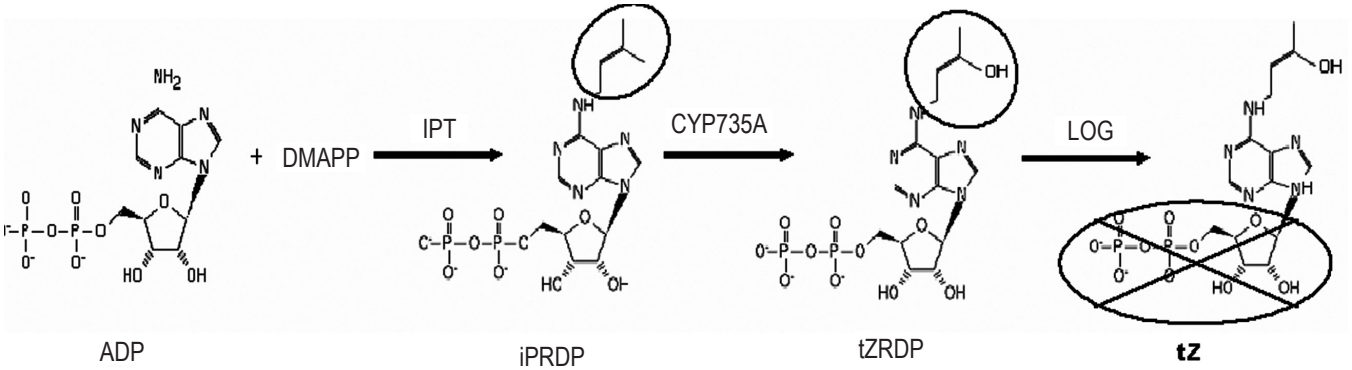


Figure 2: The proposed *de novo* biosynthetic pathway of ISCKs showing ADP as the preferred substrate for IPT to transfer the isopenetenly group from DMAPP resulting in the formation of iPRDP, followed by hydroxylation in the side chain catalysed by CYP735A resulting in tZRDP, and the release of the nucleotide group by the enzyme encoded by the LOG gene, finally resulting in the formation of tZ

resulting in the formation of iPRDP. The isopentenyl-nucleotide (iPRDP) then undergoes hydroxylation at the prenyl side-chain catalysed by CYP375A resulting in the formation of tZ-nucleotide (tZRDP). The *LOG* gene that encodes an enzyme with phosphoribohydrolase activity (Kurakawa et al., 2007) releases the ribose 5'-diphosphate moeity from tZ-nucleotide in the final step, resulting in the formation of tZ. ADP and ATP are the preferred substrates for prenylation in plants, whereas AMP is preferred by bacteria (Kakimoto, 2003).

Under the condition that no concrete evidence exists for the biosynthesis of ARCKs, it is not unreasonable to consider the *de novo* biosynthetic pathway of ISCKs as a template. With ADP/ATP as a substrate, a transferase enzyme may catalyse the transfer of a benzyl group to the N^6-position, resulting in BAR5'P. Subsequent hydroxylation of the benzyl side-chain, catalysed by CYP375A or its homolog may create the corresponding hydroxy benzyladenosine 5'-diphosphate, which ultimately results in the formation of topolins following the release of ribose 5'-diphosphate moeity by the enzyme encoded by the *LOG* genes or their homologues. Nevertheless, alternative biosynthetic pathways via tRNA or the metabolism of phenolics cannot be ruled out (Strnad, 1997).

5. Metabolism

Unlike the knowledge on biosynthesis, considerable information on the metabolism of ARCKs exists, largely derived from feeding experiments involving radiolabelled BAP (McCalla et al., 1962; Ramina 1979; Abo-Hamed et al., 1984; Zhang et al., 1987; Auer et al., 1992). However, substantial information on the metabolism of topolins per se is lacking, due to the limited studies that involve the tracking of radiolabelled topolins (Werbrouck et al., 1996). The metabolic events of cytokinins can be broadly classified into those involving 1) modifications of the adenine ring and 2) modifications of the side chain (Jameson, 1994).

5.1. Modifications of the adenine ring

5.1.1. Interconversion between the free base, nucleosides and nucleotides

The interconversion between the free base and their corresponding ribosides and ribotides/nucleotides (Figure 3) are features of adenine metabolism (Mok and Mok, 2003). Though the formation of the BAP ribotide (BAR5'P) is not a prerequisite for absorption in plants (Jameson, 1994), the biological activity of the free base is relatively higher than the corresponding ribosides and ribotides (Strnad, 1997). Given that the nucleotide acts as the substrate for cytokinin biosynthesis (Kakimoto, 2003), and the riboside and ribotide are capable of transport (Auer et al., 1992), this interconversion between the free base, nucleoside and nucleotides, could play a significant role in the

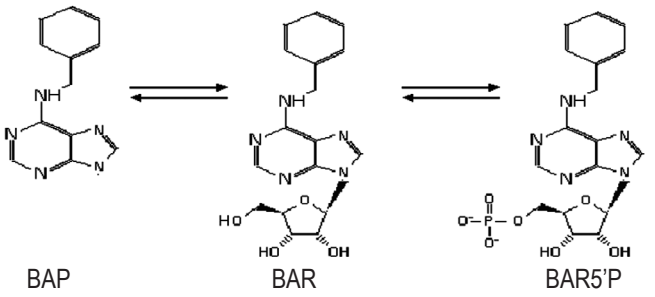


Figure 3: Forms of interconversion of cytokinins between the free base (BAP), the corresponding riboside (BAR) and ribotide (BAR5'P)

cytokinin turnover (Jameson, 1994).

5.1.2. Glucosylation

Glucosides are amongst the major metabolites of BAP (Werbrouck et al., 1995), and glucosylation can occur at the 3, 7 or 9 positions of the adenine moiety (Jameson, 1994) (Figure 4). Glucosides are considered to be storage forms of cytokinins (Strnad, 1997), and are therefore considered to be biologically inactive (Letham and Palni, 1983). The 9-glucoside is among the most prevalent metabolite of ARCKs identified in plants (Table 1).

5.1.3. N-alanyl conjugation

Conjugation of the amino acid alanine at the N^9-position of the adenine moiety has been detected as a metabolic product of both ISCKs (MacLeod et al., 1976) and ARCKs (Zhang et al., 1987). These metabolites not only possess enhanced stability, but are also capable of releasing free BAP, and are therefore considered biologically active (Jameson, 1994).

5.2. Modifications of the side chain

5.2.1. Hydroxylation

In the biosynthetic pathway of ISCKs (Figure 2), the addition and stereoisomeric position of the hydroxyl group determines the type of ISCK formed, which in turn determines its biological activity (Jameson, 1994). Likewise, it is logical to imagine a similar metabolic event for ARCKs, where hydroxylation at the *ortho*-, *meta*- or *para*- positions could determine the cor-

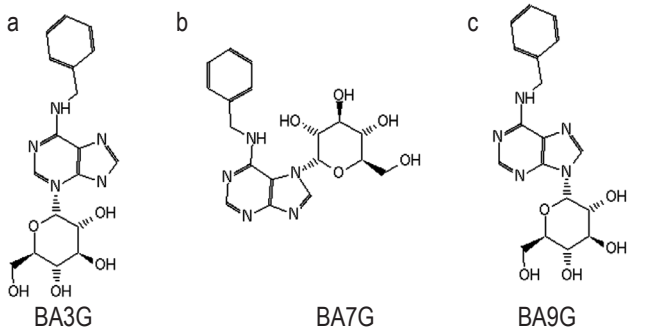


Figure 4: Derivatives of BAP showing glucosylation at the (a) N^3-, (b) N^7- and (c) N^9-positions of the adenine moiety

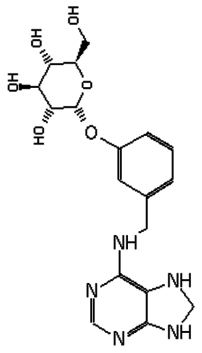
Table 1: Chronological order of the identification of ARCKs naturally in different plant species and plant parts			
Plant species	Plant part	ARCK identified	Reference
Poplar (*Populus* × *robusta* Schneid.)	Mature leaves	oTR	Horgan et al., 1973
Calla lily (*Zantedeschia* sp. K.*)*	Fruits	oTR, oT9G-2-methylthio analogue	Chaves das Neves and Pais, 1980a, b
Anise (*Pimpinella anisum* L.)	Cell culture	BAR	Ernst et al., 1983
Tomato (*Solanum lycopersicum* L.) plants infested with *Agrobacterium tumefaciens*	Crown galls of stems	BAP, BAR, BA9G	Nandi et al., 1989
Poplar	Young fully expanded leaves	oT, oTR, oT9G	Strnad et al., 1994
Oil palm (*Elaeis guineensis* Jacq.)	Shoots, inflorescence and embryos	BAP, BAR, BA9G, oT, oTR, oT9G, mT, mTR, mT9G	Jones et al., 1996
Poplar	Mature leaves	mT, mTR, mT9G	Strnad et al., 1997
Potato (*Solanum tuberosum* L.)	Leaf and stem	BAP, BAR, BA9G, oT, oTR, oT9G, mT, mTR, mT9G	Baroja-Fernandez et al., 2002
Chenopodium rubrum	Cell culture	oTOG, oTOG-2-methylthio analogue	Doležal et al., 2002
Arabidopsis (*Arabidopsis thaliana* L.) and poplar	Leaves	MeoT, MemT, MeoTR, MemTR	Tarkowská et al., 2003
Coconut (*Cocos nucifera* L.)	Immature inflorescence, SAM, spear leaf and embryo	BAP, BAR, BA9G, BAR5'P	Sáenz et al., 2003
Tagetes minuta	Dry achenes	BAP	Stirk et al., 2005
Gastrodia elata	Rhizomes	pTR	Huang et al., 2007
Beech (*Fagus sylvatica* L.)	Leaves and roots	BAP, BAR, BAR5'P, mT, mTR, mTR5'P, oT, oTR, oTR5'P	Winwood et al., 2007
Corn (*Zea mays* L.)	Xylem sap	BAP	Alvarez et al., 2008
Pea (*Pisum sativum* L.)	Roots	BAP, mT	Stirk et al., 2008

responding biological activity. However, in the feeding experiments conducted so far, the metabolism of radiolabelled BAP to any of the topolins has not been detected (Strnad, 1997). The detection of negligible amounts of free BAP in plants, suggests that the conversion of the BAP to its hydroxylated form could occur rapidly in plants (Strnad, 1997).

5.2.2. O-glucosylation

O-glucosides are abundant metabolites of *meta*-topolin and *ortho*-topolin capable of reversible sequestration (Strnad, 1997). In mT feeding experiments, mTOG (Figure 5) was a primary metabolite that degraded faster than BA9G, obtained with the application of BAP (Werbrouck et al., 1996). O-glucosides have been shown to hydrolyse during key developmental stages of plants, probably resulting in formation of the free bases (Jameson, 1994; Strnad, 1997).

Apart from the metabolites discussed above, other derivatives comprising modifications in the adenine and side chain moiety have been identified naturally in plants (Table 1). A 2-methylthio analogue of oT has been identified in fruits of *Zantedeschia aethiopica* (das Neves and Pais, 1980b) and methoxy derivatives of oT and mT have been identified in *Arabidopsis* and poplar (Tarkowská et al., 2003). Their significance in the metabolic events of topolins is yet to be determined. Considering the scope of this review, the main metabolic features of topolins have been briefly described here. A more elaborate description of the metabolic events of ARCKs has been described by Strnad (1997), and detailed analyses of the metabolism of ISCKs (Letham and Palni, 1983; Jameson, 1994; Mok and Mok, 2003) and the enzymes involved (Mok



mTOG

Figure 5: Derivative of mT showing O-glucosylation in the side chain moiety

and Martin, 1994) have been discussed elsewhere.

6. Natural Occurrence

Natural occurrence of topolins is not confined to plants. They have also been identified in lower order plants such as the moss *Physcomitrella patens* (Von Schwartzenberg et al., 2007) and sea-weed extracts (Stirk et al., 2003). Though feeding experiments using radiolabeled BAP (Strnad, 1997) and mT (Werbrouck et al., 1996) have provided several metabolites, wider knowledge on the derivatives of topolins has been obtained from the identification of topolins and/or their metabolites naturally in different plant species and plant parts (Table 1). The identification of topolins and its metabolites in a wide range of plant species (Table 1) suggests that the natural occurrence of ARCKs could be more widespread than previously imagined. Their identification in a range of plant parts also suggests that either these parts are capable of *de novo* synthesis and/or ARCKs or their precursors are capable of translocation.

Widespread occurrence of cytokinins was further corroborated by the expression of *Arabidopsis* IPT genes in several plant parts (Kudo et al., 2010). The conviction however, is that the roots are the primary site of biosynthesis (Staden and Davey, 1979; Letham, 1994). The spatial distribution of cytokinins may therefore occur due to a circulatory transport mechanism involving translocation via the xylem and phloem (Staden and Davey, 1979; Stirk and Van Staden, 2010). Accordingly, different isoforms of cytokinins are present in the xylem and phloem. Zeatin type ISCKs are predominantly found in the xylem, whereas the iP type is predominantly found in the phloem (Romanov, 2009). While the cross-talk between the different isoforms is ambiguous at this stage, the predominant expression of CYP375A in the roots (Takei et al., 2004), localization of *LOG* mRNA in the shoot meristem tips (Kurakawa et al., 2007), and the differential perception of the cytokinins by the receptors (Spíchal et al., 2004) suggest that elucidation of the different delivery mechanisms of cytokinins is still required to fully understand their role in plant development.

In general, the free bases, ribosides and ribotides are considered to be motile in comparison to the glucosides (Auer et al., 1992; Strnad, 1997). Due to the lack of sufficient feeding experiments and knowledge on the biosynthetic pathway of topolins, the mechanisms of transport remain unknown. However, radiolabelled tracking experiments have shown that BAP is capable of acropetal (Mozes and Altman, 1977) and basipetal (Black and Osborne, 1965) transport. This possibly explains the natural occurrence of a wide array of topolins in different plant species and different plant parts (Table 1).

7. Structure-Activity Relationship

A compound is suggested to possess cytokinin function largely based on the results of bioassays (Mok, 1994). Therefore, the identification of a cytokinin either naturally (Horgan et al., 1975; Strnad et al., 1994, 1997) or through chemical synthesis in the lab (Skoog et al., 1967; Doležal et al., 2007) is immediately followed by the description of its activity in bioassays. The most common bioassays used to determine cytokinin activity are the tobacco callus bioassay, wheat leaf senescence bioassay and the *Amaranthus* bioassay (Kaminek et al., 1987a; Holub et al., 1998; Doležal et al., 2007), though other bioassays such as the radish leaf expansion bioassay (Letham, 1971), soybean callus bioassay (Miller, 1961), and the release of lateral buds of peas from apical dominance (Kaminek et al., 1987a) have also been described. While the bioassays provide preliminary evidence of the biological activity of cytokinins, the sensitivity of bioassays could vary for different cytokinins (Holub et al., 1998; Sakakibara, 2006). Careful interpretation of the results from bioassays is therefore advised. The biological activity of cytokinins is determined by 1) chemical structure of the cytokinin comprising modifications in the side chain and the adenine ring, and 2) perception of the cytokinin signal by the receptors and subsequent initiation of the down stream reactions.

7.1. Chemical structure

7.1.1. Modifications of the side chain

Cytokinin activity is determined by the presence or absence of the hydroxyl groups and their stereoisomeric position in the side chain moiety (Sakakibara, 2006). Between iP and Z, Z with a hydroxyl group in the side chain was found to be at least 5 times more active than iP which lacks the hydroxyl group in a soybean callus bioassay (Manos and Goldthwaite, 1976). Within the Z group, the presence of the hydroxyl group in the *trans*-position in tZ showed higher biological activity in many bioassays than cZ, in which the hydroxyl group is present in the *cis*-position (Mok and Mok, 2003). Likewise in ARCKs, the presence of the hydroxyl group in topolins could render higher biological activity than BAP, and within the topolins, the stereoisomeric position of the hydroxyl group in the *ortho*/*meta*/*para* positions could determine the biological activity.

The high biological activity of hydroxylation at the *meta*-position of the aromatic side chain was revealed (Horgan et al., 1975) even before the identification of mT naturally (Strnad et al., 1997). In the tobacco callus and wheat leaf senescence bioassays, mT showed higher cytokinin activity than BAP and oT, and was on par with that of Z. However, in the more sensitive *Amaranthus* bioassay, the activity of mT was less than that of Z and BAP at their highest concentrations. Nevertheless, hydroxylation at the *para*- and *ortho*-positions consistently showed low biological activity in all the bioassays (Kaminek et al., 1987a; Holub et al., 1998).

The presence of the hydroxyl group in the side chain facili-

tates the formation of O-glucosides (Werbrouck et al., 1996), which are capable of rapid conversion to the free base when required (Strnad, 1997). oTOG, identified naturally in suspension cultures of *Chenopodium rubrum* (Doležal et al., 2002), showed only moderate biological activity in the *Amaranthus* bioassay, probably due to the conjugation in the *ortho*-position of the side chain. The methoxy derivatives of topolins (MeoT and MemT) identified naturally in *Arabidopsis* and poplar showed biological activity on par with BAP in the tobacco callus and *Amaranthus* bioassays. However, in the wheat leaf senescence bioassay, their activity was twice higher than that of BAP (Tarkowská et al., 2003).

7.1.2. Modifications of the adenine ring

An intact adenine ring is required for high cytokinin activity (Skoog et al., 1967), and therefore the free bases possess higher cytokinin activity than their corresponding metabolites (Sakakibara, 2006). Amongst the modifications of the adenine ring, ribosides and the 9-glucosides of topolins have been frequently identified naturally in different plant species (Table 1). Ribosylation of Z at the 9-position caused a 10-fold reduction in biological activity (Holub et al., 1998). A comparison of oTR, mTR and pTR with BAR, showed that oTR and pTR always exhibited low biological activity, whereas mTR showed high activity in all the bioassays tested (Kaminek et al., 1987a). Also, the free base mT showed higher biological activity than mTR in the *Amaranthus* bioassay, whereas their activity was the same in the wheat leaf senescence test (Holub et al., 1998). mTR and BAR showed higher biological activity than ZR in the tobacco callus bioassay (Holub et al., 1998), whereas MemTR showed higher activity than mTR and mT in the soybean callus bioassay (Amoo et al., 2010).

The general trend of the biological activity in the three bioassays was *meta*≥*ortho*≥*para* (Doležal et al., 2007). However, irrespective of the side chain, glucosylation at the 9-position of the adenine ring exhibited biological activity near zero in ARCKs (Holub et al., 1998) and poorer than the control in ISCKs (Letham and Palni, 1983).

7.2. Perception and signal transduction

The first step in the chain reaction that ultimately manifests as a biological response is the perception of the signal. The difference in the relative activities of different cytokinins in bioassays could therefore be due to the perception and downstream signal transduction processes. Three cytokinin receptors, CRE1/AHK4, AHK3 and AHK2, were identified in *Arabidopsis* (Inoue et al., 2001; Suzuki et al., 2001; Yamada et al., 2001) and the signal transduction of cytokinin was found to occur through a two-component signaling system involving a phosphorelay, prominent in bacteria and similar to the signal transduction of ethylene (Hwang and Sheen, 2001). A detailed discussion of cytokinin signaling can be found elsewhere (Heyl and Schmülling, 2003; Werner and Schmülling, 2009; Keiber and Schaller, 2010). Most bioassays measure cytokinin activity over a long duration, while cytokinins are capable of rapid degradation. A bacterial system to determine cytokinin activity rapidly was therefore developed, which involves expression of the cytokinin receptors in *E. coli* (Suzuki et al., 2001; Yamada et al., 2001).

In this system, ISCKs had higher activity than ARCKs in both CRE1/AHK4 and AHK3 receptors expressed in *E. coli*. Among the ARCKs, mT showed highest activity in both the receptors, with about 30% activity in CRE1/AHK4 and 80% activity in AHK3 (tZ showed 100% activity in both receptors), followed by BAP which showed 7.7% activity and 23.6% activity in CRE1/AHK4 and AHK3 respectively, and oT which showed no activity whatsoever. The corresponding ribosides showed no activity in CRE1/AHK4, whereas in AHK3 the activity was in the same order as the free bases. The O- and N-glucosides of ISCKs tested in this system also showed no activity with both the receptors (Spíchal et al., 2004).

Testing these compounds *in planta* using a reporter gene-based bioassay in *Arabidopsis* (D'Agostino et al., 2000) however corroborated the results obtained from bioassays. mT and BAP showed high activity on par with tZ, with oT and the corresponding ribosides expressing relatively less activity. O-glucosides showed high activity in this assay, probably because of breakdown to the free base *in planta*. This assay however integrates responses from several cytokinin pathways, and therefore cannot provide conclusive evidence on cytokinin activity (Spíchal et al., 2004). Based on the variety of results obtained with ARCKs in different bioassays, between the two receptors tested in the bacterial system, and the reporter gene-based bioassay, it seems that ARCK activity involves unidentified mechanisms. This could possibly involve exclusive sensing mechanisms for ARCKs (Doležal et al., 2007) which could belong to a group of unidentified CBPs (Keim and Fox, 1980; Strnad, 1997), or the involvement of the receptor AHK2 which was not tested in the bacterial system (Spíchal et al., 2004).

8. Applications of Topolins

8.1. Cytokinin source in micropropagation

The global market potential for tissue culture plants is expected to be worth 15 b US$ annum^{-1} (Govil and Gupta, 1997), and the major share of this volume is contributed by plants for cut flowers and pot plants (Prakash, 2007). Since its chemical synthesis, BAP has been the most preferred cytokinin component of the tissue culture industry to stimulate shooting in vitro, owing to its high activity and affordable price (Werbrouck et al., 1995). However, BAP has several inherent drawbacks which includes inhibition of root formation (Werbrouck et al.,

1995), high abnormality index (Amoo et al., 2010) and non-maintenance of histogenic stability (Bogaert et al., 2004). This leads to acclimatization problems and poor regeneration ex vitro, resulting in high production costs (Prakash, 2007). These problems of the tissue culture industry can partly be resolved by using alternative sources of cytokinins. Topolins were therefore tested as viable alternatives for BAP in the micropropagation of *Spathiphyllum* sp. (Werbrouck et al., 1996). Since then they have been used successfully for the micropropagation of several plant species such as turmeric (Salvi et al., 2002), potato (Baroja-Fernandez et al., 2002), plantain (Roels et al., 2005), *Aloe* (Bairu et al., 2007), banana (Bairu et al., 2008) and sea oats (Valero-Aracama et al., 2010).

The inhibition of root formation by BAP during the micropropagation of *Spathiphyllum* sp. was attributed to the formation of BA9G in the basal part of the callus, which remained unmetabolized for several weeks (Werbrouck et al., 1995). On the contrary, when mT was used, root formation was not inhibited and the O-glucoside of mTR was the major metabolic product (Werbrouck et al., 1996). Localized accumulation of mTR is avoided by rapid translocation to other plant parts, and the presence of the riboside group could prevent glucosylation at the 9-position (Kaminek et al., 1987a). The O-glucoside is also capable of sequestration to its free base when required in contrast to the 9-glucoside which is a storage form (Strnad, 1997). During the micropropagation of *Barleria greenii* (Amoo et al., 2010), BAP reported the highest abnormality index measured as a ratio of abnormal adventitious shoots to normal ones, whereas all the topolins tested (mT, mTR and MemTR) had an abnormality index lower than that of the control. BAP has been reported to cause phytotoxicity in plants (Bogaert et al., 2004), also probably due to the sessile nature of BA9G (Amoo et al., 2010). Maintenance of the histogenic composition of leaves is essential during the micropropagation of chimeras. In a *Petunia* leaf-variegated chimera (Bogaert et al., 2004), MemTR was superior to BAP in the maintenance of histogenic composition, and produced only a small number of albinos and green shoots than BAP. Other benefits of topolins in micropropagation include their anti-senescence activity in *Rosa* shoots in vitro (Bogaert et al., 2004) and alleviation of hyperhydricity of *Aloe* shoots in vitro (Bairu et al., 2007).

Topolins have overcome some of the serious drawbacks inherent to BAP, the traditional cytokinin source in micropropagation for more than fifty years. This advantage of topolins combined with increased shoot production in vitro (Amoo et al., 2010) provides an effective alternative source of cytokinins for the micropropagation industry. However, the beneficial effects of topolins are not universal. In *Vaccinium* sp. (Meiners et al., 2007) and azaleas (Mertens et al., 1996), tZ was superior to mT, and in wild service tree (Malá et al., 2009), BAP was better than mT and MemTR in promoting adventitious shoot production. Also, mTR was shown to inhibit rooting in some plant species (Werbrouck, 2008). Further research on the metabolic properties of topolins and extensive evaluation in other plant species is suggested. Albeit, the choice of cytokinins could determine the success or failure of the micropropagation of a plant species (Werbrouck, 2008), and topolins are very promising in this area of application.

8.2. Increase in agricultural yield/biomass

Manipulation of the genes that regulate GA biosynthesis (Salamini, 2003) resulted in the development and widespread adoption of short and sturdy dwarf varieties of wheat and rice, characterized by resistance to lodging by wind and rain and effective in utilization of fertilizers to produce high yield, which led to the green revolution (Borlaug, 1983). The global population has multiplied several folds since then, and is expected to reach 8.9 b by 2050. To feed this ever increasing population, the productivity of food crops has to increase by 50% (Sakamoto, 2006).

Cytokinins influence several yield related components such as the delay of senescence, resistance of plants to various forms of stress, respiration, source-sink activity and alleviation of apical dominance (Kamínek, 1992). Exogenous application of cytokinin therefore increased the yields of corn, rice, pepper, cucumber and cantaloupe (Mayeux et al., 1983). In cereals, exogenously applied topolins increased the grain weight plant^{-1}, elongation of the ears, number of grains head^{-1}, weight of grain head^{-1}, leaf area, leaf area duration and accumulation of nutrients in the grains, thereby increasing the harvest index of the plant (Hradecká and Petr, 1992; Trčková et al., 1992).

The net photosynthesis of a plant bears a direct relationship to the biomass produced, which in turn alters the source-sink allocation within the plant, ultimately influencing the harvest index of the plant (Sakamoto, 2006). Cytokinins are well known to regulate photosynthetic processes of the plant, either directly by increasing chlorophyll synthesis (Kuraishi et al., 1992) and/or indirectly by mediating light responses (Argueso et al., 2009). Winter rye plants treated with mTR had increased leaf area and LAD (Hradecká and Petr, 1992). In geophytes such as sugar beet, foliar application of mTR delayed the senescence of leaves by increasing the chlorophyll content and the net photosynthetic rate of the leaves. This, possibly by altering the source-sink distribution, resulted in increased root biomass and hence total biomass of the plant (Čatský et al., 1996).

The formation of O-glucoside in preference to N-glucosides (Werbrouck et al., 1996), combined with rapid translocation (Kaminek et al., 1987a) and degradation of the corresponding metabolites (Bairu et al., 2008) could provide topolins the advantage of being used to increase agricultural yield in prefer-

ence to BAP. Recently, a QTL that increases grain number, *Gn1* was identified in rice (Ashikari et al., 2005). It was found to encode a gene for CKX, which irreversibly degrades cytokinins (Mok and Mok, 2003). Transgenic rice plants with antisense CKX genes had low levels of CKX, resulting in an increase in grain number (Ashikari et al., 2005). CKX is selective in identifying substrates for degradation. ISCKs with a double bond in the side chain are preferred substrates for catalytic cleavage of the side chain by CKX, compared to ARCKs and O-glucosides (Strnad, 1997). This immunity of ARCKs to CKX degradation could have a major impact on the cytokinin pool of the plant, and ARCKs could be directly involved in increasing the grain number of transgenic *Gn1* plants.

Cereals and geophytes constitute the major volume of food crops. Given that the exogenous application of topolins influences the yield parameters of cereals and geophytes, the results described above are indicative of the potential application of topolins. However, very few studies have reported the influence of topolins on yield related parameters. Further research in this area is recommended.

8.3. Alleviating dormancy

Dormancy is the temporary suspension of visible outgrowth of any structure containing a meristem and can be caused by factors outside the bud (para-dormancy/apical dominance), intrinsic factors within the bud (endo-dormancy) or environmental factors (eco-dormancy) (Lang, 1987). The inception and release of dormancy, which comprises overlapping phases of para- and/or endo- and/or eco-dormancy, involves decision making in the SAM orchestrated by hormonal cross-talk (Subbaraj et al., 2010). Cytokinins are historically known to stimulate branching by alleviating apical dominance (Sachs and Thimann, 1967). This process is primarily controlled by an antagonistic relationship with auxin, mainly synthesized in the apical bud, capable of regulating the local synthesis of cytokinins (Tanaka et al., 2006) and/or cytokinin export from the roots (Bangerth, 1994). Cross-talk with other hormones such as GA (Weiss and Ori, 2007) and strigolactones (Ferguson and Beveridge, 2009) also plays a vital role in the control of branching.

This ability of cytokinins to stimulate branching is of great use to increase the production of propagules or cuttings for many plant species in commercial nurseries. Exogenous application of mTR was more effective than BAP in increasing the number of cuttings in *Poinsettia* and *Gerbera*. The detrimental effects of BAP such as the inhibition of root formation and phytotoxicity were not observed following the application of mTR (Kaminek et al., 1987b). Floral productivity of calla lilies is directly related to the number of branches plant^{-1}. Therefore, the stimulation of branching by the exogenous application of BAP could have commercial implications (Subbaraj et al., 2010).

The role of cytokinins in the control of endo-dormancy is still poorly understood (Horvath, 2009). After the onset of endo-dormancy, BAP failed to stimulate branching in calla lilies (Subbaraj et al., 2010), whereas in potato, exogenous application of cytokinin released buds from endo-dormancy (Turnbull and Hanke, 1985). The establishment of endo-dormancy could coincide with a decline in the sensitivity of the buds to cytokinins (Subbaraj et al., 2010) and/or a change in the endogenous cytokinin concentration (Turnbull and Hanke, 1985). The endogenous cytokinin pool of potato cv. 'Kennebec', a cultivar which expressed high viability in vitro largely comprised ARCKs (92%), in contrast to cv. 'Jaerla', a cultivar with low viability, which comprised up to 57% of ISCKs (Baroja-Fernandez et al., 2002). Alleviating bud dormancy of food crops, by stimulating branching/tillering and extending the period of active growth, can have an indirect influence on the yield/biomass, and topolins have a definite role in the control of dormancy.

8.4. Management of abiotic stress response

Cytokinins are mainly synthesized in the roots (Letham, 1994) and are involved in signaling water status and available nutrient information or the lack of it to the shoot (Argueso et al., 2009). Therefore, under conditions of abiotic stress, for example drought, cytokinin signaling aids in retaining the photosynthetic capacity of the shoot (Werner and Schmülling, 2009). One of the initial reactions on the exposure of plants to drought conditions is enhanced CKX activity (Havlova et al., 2008) causing a reduction in ISCK content in the xylem sap (Schachtman and Goodger, 2008). ABA, which controls stomatal conductance of leaves (Davies et al., 2005) is also synthesized in the roots, and was found to increase CKX gene expression (Brugiere et al., 2003). Concomitant with the decline in endogenous ISCK level, a surge in endogenous BAP content was noticed in drought induced maize plants (Alvarez et al., 2008). As mentioned earlier, the CKX activity by reducing the concentration of ISCKs, could allow the ARCKs to be directly involved in retaining the photosynthetic activity of drought induced plants.

The cytokinin signaling pathway is also affected by cold and salt stress conditions. Cold stress rapidly down regulated the expression of the three cytokinin receptors. While *AHK2* and *AHK4* were down-regulated in *Arabidopsis* plants exposed to osmotic or salt stress, *AHK3* was up-regulated (Argueso et al., 2009). Such changes in cytokinin receptor activity have also been described in other plants. Therefore, apart from the regulation of the endogenous cytokinin pool, receptor activity could also be regulated in response to abiotic stress. How topolins manage abiotic stress control under the emerging scenario of climate change and efficient water use, is a topic worth further

investigation.

8.5. Other applications

Floral transition of *Sinapis alba* was marked by a transient increase in the influx of endogenous cytokinins to the shoot, which may probably be used for the subsequent formation and differentiation of floral organs (Lejeune et al., 1994). However in *Chenopodium rubrum*, floral differentiation was inhibited by the application of BAP (Blažková et al., 2001). Visible manifestation of flowering could involve factors beyond the control of cell division. While BAP did not stimulate the differentiation of floral primordia in calla lilies, it facilitated the visible manifestation of floral initiation stimulated by GA (Subbaraj et al., 2010). By promoting cell division and the ensuing DNA synthesis, the application of topolins directed the initiation of germination in seeds of *Tagetes minuta* (Stirk et al., 2005), and also enhanced the subsequent cotyledon growth (Palavan-Ünsal et al., 2002). Physiological and morphological changes in response to biotic stress are mediated by cytokinins, which involves changes in the cell division pattern caused by the pathogen/symbiont. Several microbes such as *Erwinia herbicola*, *Pseudomonas syringae*, *Rhodococcus fascians*, *Plasmodiophora brassicae* use this mechanism to infect host plants (Werner and Schmülling, 2009). In the classic plant-pathogen interaction involving *Agrobacterium tumefaciens*, BAP and its derivatives were identified in the crown galls of infested tomato plants (Nandi et al., 1989). The role of cytokinins in the nodulation process of nitrogen-fixing bacteria is also being investigated (Argueso et al., 2009).

That topolins have functional roles beyond cell division control was hypothesized even at the time of its discovery, when Horgan et al. (1975) detected oTR in mature, fully expanded leaves of poplar that had ceased cell division. The transient increase in topolin levels on exposure to light was suggestive of a role in leaf expansion (Hewett and Wareing, 1973).

In plant tissue culture, cytokinins have the ability to induce callus growth, a group of undifferentiated cells that proliferate incessantly in a disorganized manner. Since this mechanism is similar to that of cancer in animal cells, a role for cytokinin in the control of cancer was suggested (Doležal et al., 2007). This was followed by reports that demonstrated the ability of cytokinin bases to induce cell differentiation in human cancer cells (Ishii et al., 2003). Consequently, numerous derivatives of both ISCKs and ARCKs were chemically synthesized to test their ability as anti-cancer drugs (Doležal et al., 2006, 2007). Ribosides were more effective in regulating cancer formation at lower concentrations than the free bases (Voller et al., 2010). While the anti-cancer activity of iPR, KR and BAR was confirmed, oTR which showed minimal biological activity in plant bioassays (Kaminek et al., 1987a; Holub et al., 1998), had the highest anti-cancer activity in a panel of cancer lines tested (Voller et al., 2010).

9. Conclusion

The early identification of the ISCK group of cytokinins naturally (Letham, 1963), prior to the ARCK group (Horgan et al., 1973), has been advantageous for the progress of research in the ISCK group. Nevertheless, recent advances in the elucidation of the biosynthesis (Kakimoto, 2003), metabolism and transport (Kudo et al., 2010), and signal transduction (Heyl and Schmülling, 2003) of the ISCK group has provided an inventory of information to compare and contrast the existing information on ARCKs. Therefore, an account of the current research status of the ARCK group was assumed to be timely and beneficial to identify further areas of research. In spite of its high biological activity (Holub et al., 1998), the application of topolins in plant science has primarily focused on its use as an alternative cytokinin source in micropropagation, with a few reports on enhancing yield parameters (Hradecká and Petr, 1992; Trčková et al., 1992), and stimulation of branching (Kaminek et al., 1987b). This review was therefore aimed to provide a compilation of existing information on topolins with simultaneous reference to the recent developments in ISCK research, and to propose potential areas of application.

The identification of topolins in a broad range of plant species suggests that the natural occurrence of this type of cytokinins is more widespread than initially thought. Though the biosynthetic pathway of ISCKs seems to be an ideal template for ARCK biosynthesis, where the key enzymes in ISCK synthesis could play similar roles in ARCK synthesis, the lack of identification of the basic components such as the substrate and side chain donor, suggest an exclusive biosynthetic pathway for ARCKs. The relative susceptibility of ISCKs to CKX degradation (Strnad, 1997) and the metabolism of topolins to O-glucosides in preference to the N-glucosides of BAP (Werbrouck et al., 1995, 1996) highlight the significance of structural differences between the ISCK and ARCK groups and within the ARCK group, respectively. The differential perception of the ISCK and ARCK groups by the cytokinin receptors also corroborates an exclusive sensing and signal transduction strategy for ARCKs (Spíchal et al., 2004). Overall, key aspects of the biosynthesis, metabolism and signal transduction of ARCKs still remain ambiguous, and require further investigation.

As mentioned earlier, studies on the applications of topolins are limited, and have been confined to its recommendation as an alternative source of cytokinin to BAP in micropropagation (Amoo et al., 2010). In other plant developmental processes, topolins are either reported to play a direct (Kaminek et al., 1987b; Trčková et al., 1992) or indirect role (Ashikari et al., 2005; Alvarez et al., 2008) by affecting the endogenous cytokinin pool. Overall, this review expresses confidence that topolins

have wider physiological roles than currently reported.

10. Perspective

A better understanding of the vital aspects of ISCK biosynthesis and signal transduction has led to the development of genetically engineered transgenic plants involving key biosynthetic/metabolic genes. A list of the transgenic plants developed and the cytokinin genes involved have been listed by Ma (2008). Mostly, this involves enhanced cytokinin production via overexpression of IPT genes (Kakimoto, 2003) or silencing the CKX gene (Ashikari et al., 2005). The resulting transgenic plants have been assessed for their effects on cytokinin mediated developmental processes such as seed production, leaf and flower anti-senescence, stress adaptation and fruit development (Ma, 2008). While the use of such transgenic plants has several benefits, over production of cytokinins could have detrimental effects on plants such as phytotoxicity. Either spatial and/or temporal expression of the respective genes or application of cytokinins as a chemical spray at the desired concentration and stage of growth of the plant would enable controlled cytokinin action.

The diminishing land area available for agriculture combined with the burgeoning global population has underlined the need to improve the productivity of food crops. Cytokinins are known to affect several yield related parameters of plants. While regulation of GA synthesis was employed for the first green revolution, scope for the next green revolution relies largely on the use of cytokinins (Sakakibara, 2006). In that emerging scenario, the salient features of topolins such as their high biological activity, easy chemical synthesis at affordable prices and minimal deleterious effects on plants, would establish them as the ideal source of cytokinins.

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Dormancy and Flowering Are Regulated by the Reciprocal Interaction Between Cytokinin and Gibberellin in *Zantedeschia*

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Abstract Floral productivity of *Zantedeschia* is dependent on the conversion rate of buds to shoots, which is controlled by varying intensities of para- (apical dominance), endo- (dormancy), and ecodormancy. We present evidence of cross-talk between cytokinin and gibberellin in their complementary roles to alleviate bud dormancy and enhance flowering in a perennial geophyte. We assessed the impact of cytokinin and gibberellin, applied alone and in sequential combinations, on bud fate during three phases along the ontogeny of growth, which coincide with the progressive transition of buds from apical dominance to dormancy. Given that cytokinin can stimulate branching and gibberellin can induce flowering in *Zantedeschia*, we measured these phenotypic responses as parameters of bud commitment. The efficacy of cytokinin alone to stimulate branching declined with the transition to dormancy (phase $1 = 3.8 \pm 0.2$ shoots; phase $3 = 1.0 \pm 0.3$ shoots). To sustain branching during this transition, a sequential application of gibberellin was necessary. Gibberellin alone failed to stimulate branching. The efficacy of gibberellin alone to stimulate flowering diminished with the transition to dormancy. Any flowering during this transition occurred only after the sequential application of cytokinin. Cytokinin alone failed to stimulate flowering. Alleviating bud dormancy and enhancing flowering in *Zantedeschia*, achieved by the reciprocal cross-talk between cytokinin and gibberellin, contributes to the pool of evidence drawing common mechanisms between dormancy and flowering and may have commercial implications.

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Introduction

Calla lilies (*Zantedeschia* sp. Family: Araceae) are gaining commercial importance worldwide as a cut flower and potted flowering plant (Kuehny 2000) and are New Zealand's second most exported cut-flower crop (Funnell and others 2002). A herbaceous perennial geophyte, the growth habit of *Zantedeschia* is sympodial (Funnell 1993), where floral transformation (spathe and spadix) of the apical bud triggers a flowering cascade in one or two axillary buds below it (Halligan and others 2004). This is, however, preceded by the development of buds to shoots, so that the floral productivity of *Zantedeschia* is a direct consequence of the conversion rate of buds to shoots.

The conversion rate of buds to shoots is controlled by an inherent developmental program (Naor and others 2005a), which in turn is executed via different degrees of para- (apical dominance) and endodormancy (dormancy) (Lang 1987; Faust and others 1997). Physical removal of the apical bud (Thimann and Skoog 1933) promotes axillary bud outgrowth (branching) in *Zantedeschia* (Clark and others 1987), thereby demonstrating the existence of apical dominance. Though the progressive establishment of bud dormancy is acknowledged (Corr and Widmer 1988; Naor and others 2005a), the transition of buds from apical dominance to dormancy is not visually represented. To predict the onset and duration of dormancy, a heat-unit accumulation model was developed (Carrillo Cornejo and others 2003).

The growth cycle of *Zantedeschia* is marked by a unique set of morphological attributes. There is a period of active

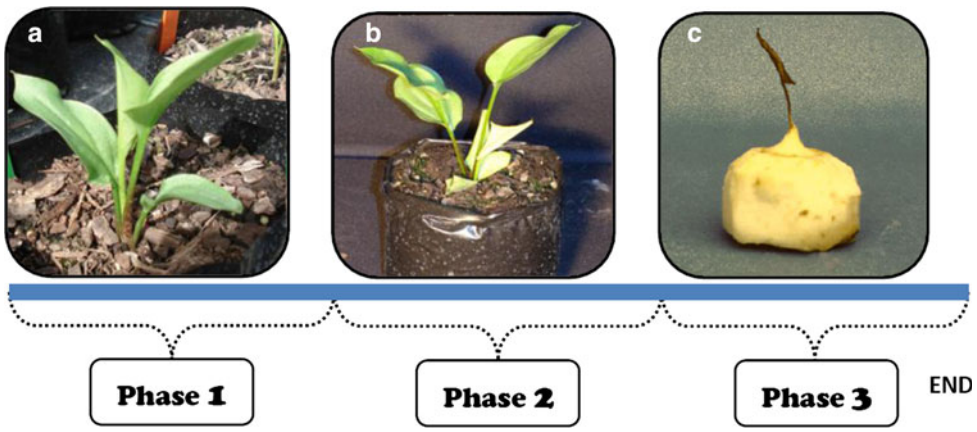

Fig. 1 Single-shoot system of *Zantedeschia* cv. 'Best Gold' showing the three developmental phases of the growth cycle, demarcated based on visual clues. Plant in (**a**) phase 1, with four leaves, during its active growth period, (**b**) phase 2, with seven leaves, after the cessation of new leaf production, and (**c**) phase 3, after the formation of an atrophied leaf, as revealed by removing the last emerged leaf

growth in which there is continuous production of new leaves, followed by the cessation of new leaf production, then the formation of an atrophied leaf (senescence before development) in the axil of the last emerged leaf (Carrillo Cornejo and others 2003; Halligan and others 2004), and subsequently the end of the growth cycle. Using these visual signposts, we divided the growth cycle of *Zantedeschia* into three phases: phase 1 comprises the active growth period prior to the cessation of new leaf production (Fig. 1a); phase 2, from the cessation of new leaf production until the formation of an atrophied leaf (Fig. 1b); and phase 3, from the formation of an atrophied leaf through to the end of the growth cycle (Fig. 1c). These phases hypothetically track the transition of buds from apical dominance to dormancy.

Cytokinins are thought to act antagonistically to terminally derived auxin (Sachs and Thimann 1967; Bangerth and others 2000) and promote branching in many plant species (Miller 1961), including monocots (Hussey 1976). In earlier studies involving *Zantedeschia* tubers (Naor and others 2005a) and plantlets in vitro (Ngamau 2001; Naor and others 2005a), the application of the cytokinin 6-benzyl aminopurine (BAP) was reported to stimulate branching. Although the role of cytokinin in apical dominance and shoot branching is widely acknowledged, the role of cytokinin in dormancy is still poorly understood (Horvath 2009). Gibberellins influence several plant processes (Stowe and Yamaki 1959; Hedden and Kamiya 1997), including the transition to flowering (Boss and others 2004). In *Zantedeschia*, irrespective of meristem size and age, the application of gibberellin dictates floral differentiation (Corr and Widmer 1987; Naor and others 2005b).

Cross-talk between cytokinin and gibberellin has been implicated in diverse plant processes, including cell differentiation and meristem fate (Horvath and others 2003; Weiss and Ori 2007). At different developmental stages meristem fate is regulated by reciprocal interactions between both of them, where cytokinin inhibits gibberellin biosynthesis (Jasinski and others 2005) and gibberellin inhibits cytokinin synthesis (Greenboim-Wainberg and others 2005). A significant consequence of regulating the meristem fate by means of this cross-talk involves alleviating bud dormancy in a range of woody plant species and geophytes (Wareing and Saunders 1971; Suttle 2004).

Regulating the meristem fate depends on the mode of manipulating this hormonal cross-talk. Simultaneous application of cytokinin and gibberellin completely arrested axillary bud outgrowth in cuttings of *Solanum andigena*, whereas their individual application promoted the development of buds either as orthotropic shoots or diageotropic stolons, respectively (Woolley and Wareing 1972). In *Zantedeschia*, although the simultaneous application of cytokinin and gibberellin as Promalin (Valent BioSciences Corp., Walnut Creek, CA, USA) enhanced the total number of flowers per plant, the proportion of flowering buds was not significantly enhanced compared to the application of gibberellin alone (Funnell and others 1991), and branching was not significantly enhanced compared to the application of cytokinin alone (Funnell and MacKay 1988). A sequential application of cytokinin followed by gibberellin on suppressed buds of soy bean was found to be more successful in promoting branching compared to the simultaneous application of cytokinin and gibberellin or the application of cytokinin alone (Ali and Fletcher 1971).

Considering the existing evidence on the simultaneous application of cytokinin and gibberellin in *Zantedeschia* and evidence from other plant species that a greater response was obtained with sequential application, the sequential application of these two hormones to *Zantedeschia* was examined in the current experiment. Studies on the complementary roles of cytokinin and gibberellin and the effects of sequential applications of cytokinin

followed by gibberellin and vice versa on the meristem fate are limited. Although the reciprocal interaction between cytokinin and gibberellin on meristem fate is evident at the biosynthesis and signal transduction levels, phenotypic expression of this cross-talk has been scarcely reported. Given that in *Zantedeschia* these effects are phenotypically manifested as branching and flowering responses, the objective of the present study was to examine the roles of cytokinin and gibberellin, applied alone and in sequential combinations, in regulating bud dormancy and flowering in *Zantedeschia* as affected by (1) the three developmental phases of the growth cycle, (2) the sequential order of application, and (3) the concentration of each hormone.

Materials and Methods

Plant Material and Culture

Zantedeschia cv. 'Best Gold' is less prone to branching (D'Arth and others 2007), and the seedlings produce only one primary shoot (single-shoot system) during their first vegetative growth cycle (Funnell and Go 1993) (Fig. 1). Therefore, any axillary bud outgrowth can be easily associated with the apical bud. This advantage of the single-shoot system was employed in the current study.

Seeds of *Zantedeschia* cv. 'Best Gold' were germinated at 20 ± 2°C until plumule emergence. Seedlings were then transplanted at a uniform depth in 2.8 l polythene planter bags with a commercial growing medium (Daltons, New Zealand), containing 4.2 g of dolomite (Ravensdown Fertilisers, New Zealand), 0.9 N–0.2P–0.6 K g of 8–9-month Osmocote and 0.4 N–0.01P–0.3 K g of 3–4-month Osmocote (Scotts International B.V., Nijverheidsweg, The Netherlands) per bag. The plants were grown in a glasshouse at the Plant Growth Unit, Massey University, Palmerston North, New Zealand (40°20′S). The glasshouse was maintained at a minimum temperature of 15°C and vented at 19°C. The planter bags were placed on drained benches with capillary matting, and an irrigation frequency was maintained through drippers which supplied 50–60 ml of water per plant per day. Temperature within the glasshouse was monitored using multiple sensors and recorded every 10 min using a Squirrel 1200 data meter/logger (Grant Instruments Ltd., Cambridgeshire, UK). Accumulation of degree-days was calculated as described by Carrillo Cornejo and others (2003).

Three Developmental Phases and Treatment Application

Pilot studies were conducted to demarcate the three developmental phases based on an integral of the number of leaves present on the primary shoot, the presence of an atrophied leaf, calendar days, and degree-days. Seedlings normally produced 7 ± 2 leaves before the cessation of new leaf production. Therefore, in the current study, within each of the three phases (Fig. 1), seedlings with 4 ± 1 leaves (~85 days after sowing, ~1520 degree-days) were selected for phase 1; seedlings with 7 ± 1 leaves (~155 days after sowing, ~2700 degree-days) after the cessation of new leaf production and prior to the formation of an atrophied leaf, were selected for phase 2; and seedlings with 2 ± 1 leaves remaining (~210 days after sowing, ~3700 degree-days) after the formation of an atrophied leaf were selected for phase 3.

The effective range of concentrations of cytokinin and gibberellin to be applied was also determined from pilot studies. In the current study, cytokinin as BAP at 0, 2.2, 4.4, or 8.9 mM (0, 500, 1000, or 2000 mg l^{-1}, respectively) and gibberellin as GA_3 at 0, 0.3, 0.9, or 1.4 mM (0, 100, 300, or 500 mg l^{-1}, respectively) were applied as foliar sprays until runoff, at one of the four concentrations of each hormone. All concentrations of BAP and GA_3 (OlChemIm Ltd., Czech Republic) were dissolved in 15 ml of 1 N NaOH and 10 ml of 95% ethanol, respectively, and were then made up to the required volume with distilled water. Tween 20 (Sigma CAS No. 9005-64-5) was added as a surfactant. The control treatments comprised all components minus BAP and GA_3, respectively.

BAP and GA_3 were applied in two sequential orders which makes for two groups: group 1 (BAP → GA_3), where BAP was applied first (period 1) followed by the application of GA_3 (period 2) (Fig. 2a), and group 2 (GA_3 → BAP), where GA_3 was applied first (period 1) followed by the application of BAP (period 2) (Fig. 2b). Within any period, two sprays of each hormone were applied, with the intratime interval between two consecutive sprays of the same hormone and the intertime interval between the application of each hormone being 10 days (Fig. 2).

Experimental Design and Statistical Analysis

For each of the three developmental phases, the 16 combinations of the concentrations of BAP and GA_3 within each group were arranged in a 4 × 4 crossover design (Fletcher and John 1985). Eight individual plant replicates were used for each of the 16 combinations within a group (8 replicates × 16 combinations × 2 groups = 256 plants per phase).

The plants were maintained in three rows of benches along the length of the glasshouse and were apportioned equally to each phase, across the three rows, as they progressed along the ontogeny of growth from phase 1 to phase 3. For each phase, all 32 combinations of the concentrations of BAP and GA_3 (16 combinations × 2 groups)

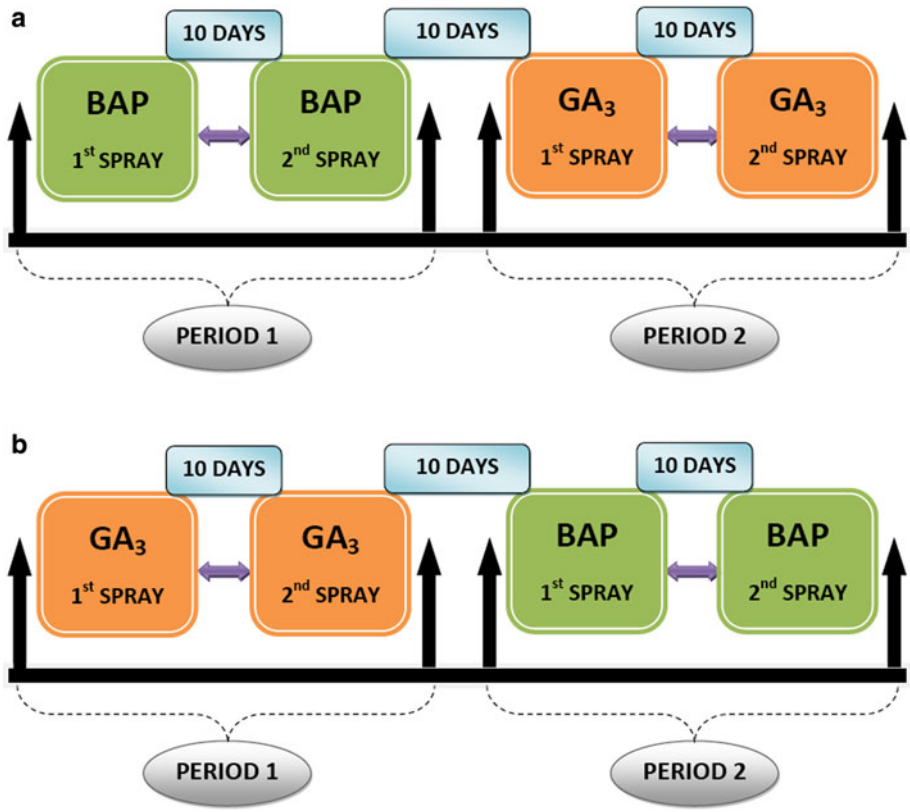

Fig. 2 Pictorial representation of the sequential order and timing of the application of each combination of BAP and GA_3 concentration used within each developmental phase of the study. **a** Group 1 (BAP → GA_3): two foliar sprays of BAP in period 1 were followed by two sprays of GA_3 in period 2. **b** Group 2 (GA_3 → BAP): two foliar sprays of GA_3 in period 1 were followed by two sprays of BAP in period 2. Two sprays of each hormone were applied within any period, with the intratime interval between two consecutive sprays being 10 days and the intertime interval between each hormonal application being 10 days

were assigned to the 256 plants in a completely randomized manner. Pilot studies and temperature sensors at multiple locations did not reveal any positional or temperature gradients across the glasshouse.

Analyses of variance for the branching and flowering data, tests of proportions for the percentage of flowering plants and primary and axillary flowering data, means ± standard errors, mean separation tests and contrasts between the two groups, and the three phases and four concentrations of each hormone were carried out using PROC GLM of SAS version 9.2 (SAS Institute, Cary, NC, USA) and MS Excel (Microsoft Corp., Redmond, WA, USA).

Observations

For each phase, the number of visible axillary shoots per plant (branching) at a minimum height of 1 cm above the growing medium were counted at the start of the experiment (time-zero), after the application of either BAP alone in group 1 or GA_3 alone in group 2 (10 days after period 1), and after the sequential application of GA_3 or BAP, respectively (10 days after period 2) (Fig. 2). The total number of visible flowers per plant (flowering) partitioned as those emerging from primary and axillary shoots was counted as and when they appeared. A floral stem was characterized by a peduncle bearing a spadix. A final count of the flowers, if present, was recorded 75 days after the last foliar spray.

Histological Examination

Floral differentiation at the apical and three axillary buds along the longest axis of the tuber, representing incremental phyllotactic distance and age, was determined using a stereomicroscope. For each phase, untreated plants (control), plants treated with the highest concentrations of BAP (8.9 mM) followed by GA_3 (1.4 mM) in group 1, and those treated vice versa for group 2 were destructively harvested for bud dissections at time-zero, 10 days after period 1, and 10 days after period 2. For each time of dissection, three individual plant replicates were used for control and treatments applied in groups 1 and 2.

Results

Overview

The plastochron of untreated (control) plants of *Zantedeschia* cv. 'Best Gold' grown under the conditions described above was 10 days. Continuous leaf production was observed until the formation of 7–8 leaves. Subsequently,

new leaf production ceased and withering of existing leaves commenced, starting with the older leaves. With two leaves remaining in the primary shoot, an atrophied leaf was noticed in the axil of the last emerged leaf, which was followed by the withering of all leaves and thus the end of the growth cycle.

Visible axillary bud outgrowth, marked by the initial emergence of one to two cataphylls, was normally observed 10 days after two consecutive applications of BAP at a 10-day interval. One to three ovate leaves unfurled subsequently. In group 2 ($GA_3 \rightarrow$ BAP), however, axillary shoots with longer petioles bearing lanceolate leaves were observed. During the flowering growth cycle, visible flowering is normally observed within 60 days after GA_3 application. Because the current study was undertaken during the vegetative growth cycle, flowering time was sporadic and large variability in the flowering data was observed (Fig. 5d–f). Flowers produced in this study were mostly malformed, denoted by the presence of a short peduncle and deformed spathes.

Branching

Time-Zero

At the beginning of each developmental phase, all plants comprised only the primary shoot, that is, axillary shoots were absent.

10 Days After Period 1

In phase 1 (Fig. 3a), increased branching occurred with increasing concentrations of BAP ($P < 0.0001$). This ability to stimulate branching declined gradually ($P < 0.0001$) for all concentrations of BAP in phases 2 and 3 (Fig. 3a). For example, branching stimulated by the highest concentration of BAP (8.9 mM), declined from 3.8 ± 0.2 shoots ($n = 32$) in phase 1 to 1.0 ± 0.3 shoots ($n = 32$) in phase 3.

In phase 1, though not concentration dependent (Fig. 3b), the application of GA_3 alone was sufficient to stimulate branching, at least by an additional shoot ($P < 0.05$). However, in phases 2 and 3, GA_3 alone had no effect on branching. At 10 days after period 1, axillary shoots were not observed in the control plants.

10 Days After Period 2

At this time (Fig. 4a–c), the stimulating effect of BAP alone on branching was more prolific than that noted at 10 days after period 1 (Fig. 3a) in all three phases and across all concentrations of BAP. For example, 6.6 ± 0.5 shoots per plant ($n = 8$) were stimulated by 8.9 mM in phase 1 at 10 days after period 2 (Fig. 4a) compared with

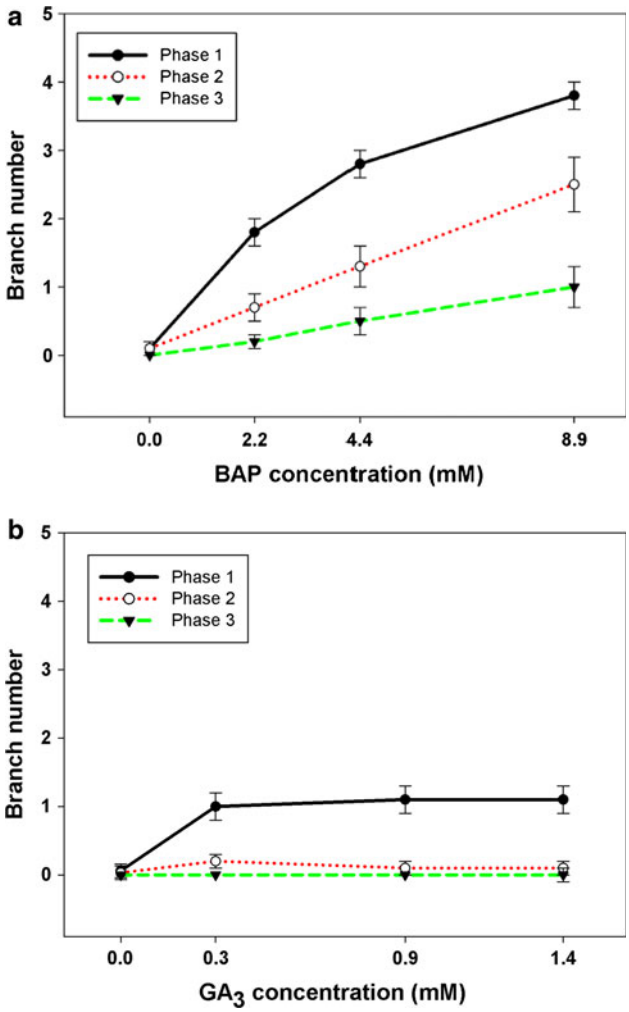

Fig. 3 Number of axillary shoots per plant ± standard error observed during the three developmental phases of the growth cycle of *Zantedeschia* cv. 'Best Gold' at 10 days after period 1 after the application of **a** BAP alone in group 1 and **b** GA_3 alone in group 2 ($n = 32$)

3.8 ± 0.2 shoots ($n = 32$) observed at 10 days after period 1 (Fig. 3a). At 10 days after period 2, 1 ± 0.2 axillary shoots were observed in the control plants. In phase 1 (Fig. 4a), at 2.2 mM, a sequential application of GA_3 (BAP $\rightarrow$ GA_3) was effective in stimulating branching significantly compared to the application of BAP alone ($P < 0.05$). However, for the other concentrations of BAP (4.4 and 8.9 mM), a sequential application of GA_3 (BAP $\rightarrow$ GA_3) failed to enhance branching significantly.

GA_3 alone did not stimulate branching significantly in phase 1 (Fig. 4d). Any increase in branching ($P < 0.0001$) observed in group 2 occurred only after the sequential application of BAP ($GA_3 \rightarrow$ BAP). Compared to the branching stimulated by all concentrations of GA_3 alone (1.8 ± 0.17; $n = 32$), significant ($P = 0.05$) incremental branching was observed with increasing concentrations of a sequential application of BAP ($GA_3 \rightarrow$ BAP), with

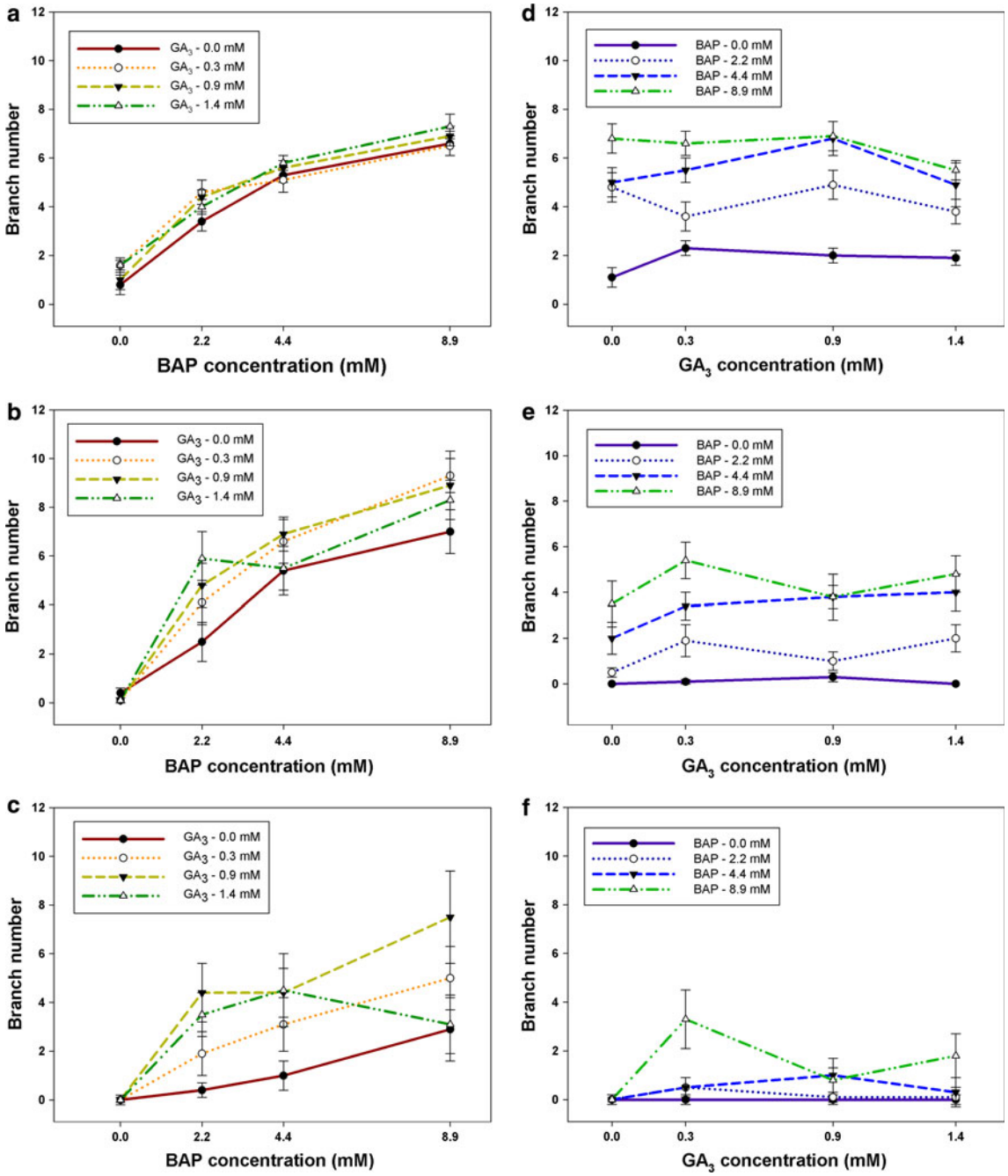

Fig. 4 Number of axillary shoots per plant ± standard error observed during the three developmental phases of the growth cycle of *Zantedeschia* cv. 'Best Gold' at 10 days after period 2 after the sequential application of BAP followed by GA_3 in group 1 (BAP → GA_3) in **a** phase 1, **b** phase 2, **c** phase 3, and after the sequential application of GA_3 followed by BAP in group 2 (GA_3 → BAP) in **d** phase 1, **e** phase 2, and **f** phase 3 ($n = 8$)

4.25 ± 0.28, 5.5 ± 0.35, and 6.4 ± 0.29 shoots ($n = 32$ each) stimulated by 2.2, 4.4, and 8.9 mM concentrations of BAP, respectively. A comparison of branching between the two sequential orders of application (group 1 versus group 2), showed that the order of application was insignificant in eliciting a branching response in phase 1 (Table 1A).

In phase 2, with the progressive decline in the branching effect of BAP alone (Fig. 3a), a sequential application of GA_3 (BAP → GA_3), compared to the application of BAP alone, significantly ($P < 0.02$) and marginally ($P < 0.1$) enhanced branching following the application of BAP at 2.2 and 8.9 mM concentrations, respectively (Fig. 4b), but it failed to enhance branching significantly ($P > 0.1$) following the application of BAP at 4.4 mM. However, a comparison between branching stimulated by each concentration of BAP, combining all concentrations of GA_3

Table 1 Effects of the sequential orders of application of BAP and GA_3 on (A) the number of axillary shoots (branching) and (B) flowers (flowering) in *Zantedeschia* cv. 'Best Gold' during the three developmental phases

	Phase 1	Phase 2	Phase 3
(A) Branching (number of axillary shoots per plant)			
Group 1 (BAP → GA_3)	4.40 a	4.73 a	2.54 a
Group 2 (GA_3 → BAP)	4.51 a	2.27 b	0.41 b
(B) Flowering (total number of flowers per plant)			
Group 1 (BAP → GA_3)	0.63 a	0.05 a	0.00 a
Group 2 (GA_3 → BAP)	1.41 b	0.30 b	0.15 b

Different *small letters* indicate significant differences ($\alpha = 0.05$) between the two sequential orders of application. Mean separation by DMRT ($n = 128$)

($n = 32$), revealed significant ($P = 0.05$) incremental branching with increasing concentrations of BAP, where 4.3 ± 0.48, 6.1 ± 0.44, and 8.3 ± 0.50 shoots were stimulated by 2.2, 4.4, and 8.9 mM concentrations of BAP, respectively (Fig. 4b).

In phase 3, the stimulating effect of BAP alone declined further (Fig. 3a). Compared to BAP applied alone, the sequential application of GA_3 in group 1 (Fig. 4c) significantly increased branching for 2.2 mM ($P < 0.01$) and 4.4 mM ($P < 0.03$) concentrations of BAP, and marginally increased ($P < 0.1$) branching for BAP at 8.9 mM concentration. However, a significant ($P = 0.05$) increase in branching was caused by the sequential application of 8.9 mM BAP followed by 0.9 mM GA_3 (Fig. 4c).

As in phase 1 (Fig. 4d), GA_3 alone failed to provoke a significant branching response in phases 2 and 3 (Fig. 4e, f), and branching in group 2 occurred only after the sequential application of BAP. This ability of BAP to stimulate branching when applied sequentially after GA_3 (GA_3 → BAP) also diminished gradually ($P < 0.05$) from phase 1 to phase 3. BAP at 8.9 mM, which stimulated 6.5 ± 0.3 shoots in phase 1 across all concentrations of GA_3 (Fig. 4d), stimulated only 1.5 ± 0.7 shoots in phase 3 (Fig. 4f). Overall, a comparison of branching between the two sequential orders of application (group 1 versus group 2) in phases 2 and 3 (Table 1A) showed that group 1 (BAP → GA_3) stimulated more branching than group 2 (GA_3 → BAP).

Flowering

Visible Flowering

In phase 1, the application of GA_3 alone was sufficient to induce a linear flowering response ($P < 0.05$) (Fig. 5d). However, compared to the application of GA_3 alone, the sequential application of BAP in group 2 (GA_3 → BAP) (Fig. 5d) enhanced the flowering response ($P < 0.05$). For example, the application of GA_3 at 1.4 mM followed by BAP at 8.9 mM in phase 1 (Fig. 5d) produced 3.5 ± 0.8 flowers ($n = 8$) per plant compared to 1.4 ± 0.4 flowers ($n = 8$) obtained with the application of GA_3 alone at 1.4 mM. In phases 2 and 3 (Fig. 5e, f), the efficacy of GA_3 alone to provoke flowering was depleted. Any flowering observed in these phases was only after the sequential application of BAP in group 2. For example, no flowering occurred in phases 2 (Fig. 5e) and 3 (Fig. 5f), even with the highest concentration of GA_3 (1.4 mM) applied alone, whereas 1.9 ± 0.9 and 0.9 ± 0.4 flowers per plant were induced, respectively ($n = 8$), after the sequential application of BAP at 8.9 mM.

BAP alone, regardless of the concentration, had no effect on flowering during all three phases of growth (Fig. 5a-c). Flowering in group 1 (BAP → GA_3), therefore, occurred only after the sequential application of GA_3. In phase 1, increased flowering occurred with increasing concentrations of GA_3 ($P < 0.001$) (Fig. 5a), and the ability of GA_3 to provoke flowering in group 1 diminished as the buds progressed toward phase 2 (Fig. 5b) and was completely eliminated in phase 3 (Fig. 5c). Overall, a comparison of the total number of flowers per plant produced by the two sequential orders of application (group 1 versus group 2) in the three phases of growth (Table 1B) showed that group 2 (GA_3 → BAP) always produced significantly ($P = 0.05$) higher numbers of flowers than group 1 (BAP → GA_3).

The percentage of plants induced to flowering (minimum of 1 flower), though progressively diminishing from phase 1 to phase 3 (Table 2A), was also significantly ($P = 0.01$) higher in group 2 than in group 1 ($n = 128$). In group 2, the flowers emerging from the primary shoot contributed significantly more ($P = 0.01$) to the total flower count than in group 1 for phases 1 and 3 (Table 2B). Consequently, the percentage of flowers emerging from axillary shoots in phase 1 (72.8%) was significantly ($P = 0.01$) higher than that from the primary shoot (27.2%) in group 1 and that from axillary shoots in group 2 (52.2%). However, in phase 2, though axillary flowering was the main contributor for the total flower count, the differences between axillary and primary flowering within each group and axillary flowering between the two groups were not statistically significant ($P > 0.1$). Among the flowering plants, the total number of flowers (primary and axillary) per plant (Table 2C) was also higher in group 2 along the three phases, but was significantly ($P = 0.05$) higher than that in group 1 only in phases 1 and 3.

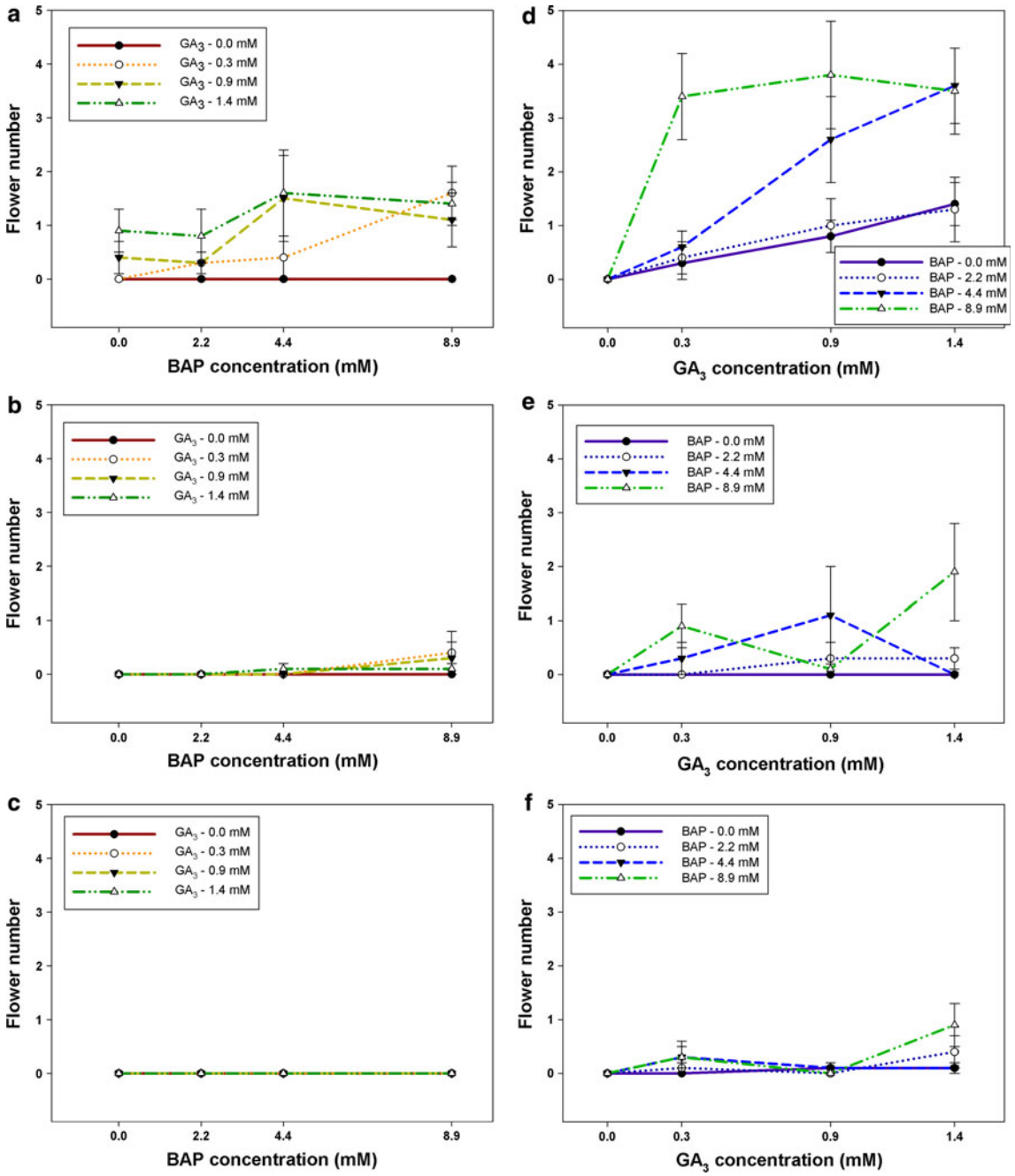

Fig. 5 Number of flowers per plant ± standard error observed during the three developmental phases of the growth cycle of *Zantedeschia* cv. 'Best Gold' after the sequential application of BAP followed by GA_3 in group 1 (BAP → GA_3), in **a** phase 1, **b** phase 2, **c** phase 3, and after the sequential application of GA_3 followed by BAP in group 2 (GA_3 → BAP) in **d** phase 1, **e** phase 2, and **f** phase 3 ($n = 8$)

Histological Examination

Floral differentiation was identified by the formation of a smooth elongated apex and sometimes by the presence of a primordial spadix. Floral differentiation at the apical and three axillary buds, which represents increasing phyllotactic distance and age, was not noticed in untreated (control) plants ($n = 3$) at any of the three observation times (time-zero, 10 days after period 1, and 10 days after period 2), during all three phases of growth (Table 3). At 10 days after period 1, a floral primordium was noticed in the apex of the apical bud during the three phases only in plants treated with the highest concentration of GA_3 (1.4 mM) ($n = 3$) in group 2. At 10 days after period 2,

Table 2 Effects of the sequential orders of application of BAP and GA_3 on (A) the percentage of flowering plants, (B) percentage of flowers emerging from the primary and axillary shoots, and (C) the total number of flowers per flowering plant in *Zantedeschia* cv. 'Best Gold' during the three developmental phases

	Phase 1	Phase 2	Phase 3
(A) Percentage of flowering plants ($\alpha = 0.01$)			
Group 1 (BAP → GA_3)	29.7 a	3.1 a	0.0 a
Group 2 (GA_3 → BAP)	47.7 b	13.3 b	10.2 b
(B) Percentage of primary (and axillary) flowers ($\alpha = 0.01$)			
Group 1 (BAP → GA_3)	27.2 a (72.8 a)	42.9 a (57.1 a)	0.0 a (0.0 a)
Group 2 (GA_3 → BAP)	47.8 b (52.2 b)	44.7 a (55.3 a)	68.4 b (31.6 b)
(C) Number of flowers per flowering plant ($\alpha = 0.05$)			
Group 1 (BAP → GA_3)	2.1 a	1.8 a	0.0 a
Group 2 (GA_3 → BAP)	3.0 b	2.2 a	1.5 b

Different *small letters* indicate significant differences ($\alpha = 0.01$ or 0.05) between the two sequential orders of application. Mean separation by DMRT and test of proportions by two-tailed *t* test

Table 3 Effect of the sequential orders of application of BAP and GA_3 on floral differentiation at the apical and three axillary buds representing incremental age and phyllotactic distance observed upon histological examination at time-zero, 10 days after period 1, and 10 days after period 2 in *Zantedeschia* cv. 'Best Gold' during the three developmental phases

	Phase 1				Phase 2				Phase 3			
	Axillary buds			Apical bud	Axillary buds			Apical bud	Axillary buds			Apical bud
	1	2	3		1	2	3		1	2	3	
Time-zero												
Control												
10 days after period 1												
Control												
Group 1 (BAP)												
Group 2 (GA_3)				+				+				+
10 days after period 2												
Control												
Group 1 (BAP → GA_3)	+	+	+	+	+	+	+	+	+	+	+	+
Group 2 (GA_3 → BAP)	+	+	+	+	+	+	+	+	+	+	+	+

+ denotes the presence of a floral primordium at the apex of the bud observed on histological examination ($n = 3$)

floral differentiation was noticed in the apical and all three axillary buds of the three phases with the application of BAP at 8.9 mM and GA_3 at 1.4 mM concentrations ($n = 3$), irrespective of the sequential order of application (groups 1 and 2) (Table 3).

Discussion

The Three Developmental Phases and the Progressive Establishment of Dormancy

The developmental program in autonomous geophytes such as *Zantedeschia* is controlled primarily by innate mechanisms (Naor and Kigel 2002). Physiological and/or morphological representation of these underlying mechanisms is lacking and, therefore, a chronological analysis of the developmental changes would provide a better understanding of these processes (Naor and others 2008). These developmental changes are different episodes in the life of the shoot apical meristem (SAM), which passes through distinct phases during its post-embryonic growth (Poethig 1990). Though the cessation of new leaf production and the formation of an atrophied leaf cannot be regarded as definitive indicators of the onset of dormancy (Halligan and others 2004), to our understanding, the progression from an active growth period characterized by continuous production of new leaves to the cessation of new leaf production and the formation of an atrophied leaf reflects a series of developmental changes in the SAM. These steps also act as useful landmarks in the ontogeny of *Zantedeschia*. These changes in the SAM, manifested as visual

clues, formed the basis of our partition of the growth cycle of *Zantedeschia* into three developmental phases.

A simple diagnostic tool to test the onset of dormancy is decapitation, which would fail to trigger branching during bud dormancy. Likewise, the establishment of bud dormancy can also be diagnosed by the deteriorating sensitivity of the buds to cytokinin, indicated by the branching response. A change in sensitivity is regarded as a change in the magnitude of an induced response (branching in this study) by an organ (bud) to a known amount of exogenous hormone (BAP) (Firn 1986). We showed that in phase 1 (Fig. 3a), where buds are predominantly controlled by apical dominance, a linear branching response can be stimulated with increasing concentrations of BAP. However, as the buds progressed toward phases 2 and 3 (Fig. 3a), there was a concomitant decline in the magnitude of the branching response with the same concentrations of BAP. Therefore, the transition of buds from apical dominance to dormancy could in part result in the loss in sensitivity of the buds to cytokinin (effect), or a loss in the sensitivity of the buds to cytokinin could signal the establishment of dormancy (cause). This study, therefore, highlights the significance of the changes in sensitivity of the buds to cytokinin as a key determinant in the progressive establishment of dormancy. This study also corroborates the parallel inception of dormancy during the three developmental phases of *Zantedeschia*, which vindicates our classification of the growth cycle based on the visual landmarks (Fig. 1).

Alleviating Bud Dormancy in *Zantedeschia*

Hormonal signals are vital for altering the developmental pathway of the SAM and, therefore, maintaining the flexibility of the bud meristems (Traas and Vernoux 2002). The differential response of buds to exogenous cytokinin at different developmental stages was noted in soy bean when Ali and Fletcher (1970) showed that the application of cytokinin alone was sufficient to stimulate branching in 8-day-old seedlings. However, in 16-day-old seedlings, cytokinin alone was not sufficient to stimulate branching after mitosis had ceased. A sequential application of gibberellin was required for sustained axillary bud outgrowth. A role for cytokinin in promoting mitosis and a role for gibberellin in enhancing shoot elongation was emphasized.

In the current study, as noted in soy bean, BAP alone was sufficient to alleviate bud dormancy, predominantly governed by apical dominance in phase 1 (Fig. 3a). In phase 2, after the cessation of new leaf production, presumably mitosis had ceased and, therefore, the developmental pathway of the SAM was altered. The efficacy of BAP alone to alleviate bud dormancy had diminished, and a sequential application of GA_3 in group 1 was required to sustain branching (Fig. 4b). In phase 3, after the formation of an atrophied leaf, which marked another cornerstone in the transition toward stronger bud dormancy, the efficacy of BAP alone to stimulate branching declined further, and the dependence on a sequential application of GA_3 to alleviate bud dormancy intensified further (Fig. 4c). Therefore, along the three developmental phases, with continuing modifications in the developmental pathway of the buds, the progressive establishment of dormancy synchronized with the gradual decline in the efficacy of BAP to alleviate bud dormancy and a concomitant increase in the need for a sequential application of GA_3.

In two different studies in *Zantedeschia*, endogenous cytokinin (D'Arth and others 2007) and gibberellin (Naor and others 2008) levels were found to be higher in sprouting buds than in dormant buds, which in the present study correspond to buds in phases 1 and 3, respectively. In phase 1, the application of BAP alone, interacting with endogenous gibberellins, may have been sufficient to elicit a branching response, which deemed the order of application of BAP immaterial in phase 1 (Table 1A). This also explains why the sequential application of GA_3 in group 1 failed to enhance the branching response significantly in phase 1 (Fig. 4a). This theory would also help to explain the increased branching, by at least one additional shoot, measured with the application of GA_3 alone in phase 1 (Fig. 3b). The application of GA_3, interacting with endogenous cytokinin, may have been sufficient to provoke a mild branching response in phase 1, which eventually declined with the establishment of dormancy in phases 2 and 3 (Fig. 3b). In dormant buds (phase 3), after the decrease in endogenous cytokinin and gibberellin levels (D'Arth and others 2007; Naor and others 2008), the application of BAP, by promoting mitosis, may have catalyzed the transformation of the dormant bud to its responsive state, and the sequential application of GA_3, by promoting shoot elongation, may have catalyzed the subsequent transition to a growing shoot (Ferguson and Beveridge 2009). The key to alleviating bud dormancy in *Zantedeschia*, therefore, involves cross-talk between BAP and GA_3, where GA_3 enhanced branching only when applied sequentially after BAP (BAP $\rightarrow$ GA_3) and had no effect on branching when applied alone (Table 4).

Enhancing Flowering in *Zantedeschia*

Gibberellin evokes different flowering responses in different plant species (Levy and Dean 1998) and collaborates with other signals such as photoperiod, vernalization, and autonomous control to culminate in floral initiation (Boss and others 2004). Gibberellin mediates the transition of activated buds, regardless of size and age, from a vegetative to a floral state in *Zantedeschia* (Naor and others

Table 4 Summary of results highlighting the cross-talk between BAP and GA_3 in their complementary roles on branching and flowering of *Zantedeschia* cv. 'Best Gold'

Hormone application	Branching	Flowering
BAP	Stimulated alone; efficacy declined with onset of dormancy	No effect
GA_3	No effect	Stimulated alone; efficacy declined with onset of dormancy
Group 1 (BAP → GA_3)	GA_3 complementary to BAP when applied sequentially	Attributed solely to GA_3 component; efficacy declined with onset of dormancy
Group 2 (GA_3 → BAP)	Attributed solely to BAP component; efficacy declined with onset of dormancy	BAP complementary to GA_3 when applied sequentially

2005b). In this study we have shown that in phase 1, where the buds are considered to be in an active state, the application of GA_3 alone induced a linear flowering response in group 2 (Fig. 5d). However, with the establishment of dormancy in phases 2 and 3, GA_3 alone failed to evoke any visible flowering response (Fig. 5e, f). Histological examination after the application of the highest concentration of GA_3 (1.4 mM) alone at 10 days after period 1, however, revealed a floral primordium in the apex of the apical bud in phases 2 and 3 (Table 3). In *Zantedeschia* plantlets grown in vitro and induced to floral transition with the application of gibberellin, inflorescence development ceased at later stages of growth, resulting in premature abortion (Naor and others 2004). Therefore, the floral differentiation in *Zantedeschia*, dictated by the application of gibberellin, does not guarantee visible manifestation of flowering. Apart from the size and age of the bud, flowering competence may involve a series of events exclusive of gibberellins (Mutasa-Gottgens and Hedden 2009). In *Zantedeschia*, day-neutral to flowering (Funnell 1993), this could involve a change in sensitivity of the buds to gibberellins implemented by the same innate mechanisms that govern the developmental program.

Though cytokinins are not frequently mentioned in flowering discussions, evidence supporting the definitive role of cytokinins in promoting mitosis and subsequent differentiation of floral organs is available for *Sinapis alba* (Lejeune and others 1994) and *Chenopodium* sp. (Machácková and others 1993). In *Zantedeschia* plantlets in vitro, cytokinin alone failed to induce inflorescence development, but it interacted with gibberellin, resulting in floral development (Naor and others 2004). In this study, the application of BAP alone, irrespective of concentration, had no effect on visible flowering (Fig. 5a–c) or floral differentiation in the apex (Table 3) during all three developmental phases.

However, when BAP was applied after GA_3 in group 2, the total number of flowers per plant (Table 1B) and the percentage of flowering plants (Table 2A) were significantly enhanced in all three phases, even in phases 2 and 3 where the efficacy of GA_3 alone to initiate flowering was entirely depleted (Fig. 5e, f). The percentage of flowering plants (Table 2A) and the number of flowers per plant (Table 1B) produced in group 1 (BAP → GA_3), can be attributed solely to the gibberellin factor, which progressively diminished with the establishment of dormancy in phases 2 and 3 (Fig. 5b, c), as observed in group 2 (Fig. 5e, f). Histological examination of apical and axillary buds at 10 days after period 2 (Table 3) revealed that floral differentiation had occurred in all the buds, irrespective of the order of application of BAP and GA_3 (group 1 or 2) and the phyllotactic distance and the age of the axillary buds. Visible manifestation of the differentiated primordia was, however, more predominant in group 2 (Fig. 5e, f).

The primary shoot possessed the largest number of flowering buds (Naor and others 2005b). In the current study, the percentage of the flowers emerging from the primary shoot was significantly higher in group 2 than in group 1 (Table 2B). Histological examination at 10 days after period 1 (Table 3) also revealed floral differentiation only in the apical bud of plants treated with GA_3 (group 2). Presumably, after the floral "switch" was turned on by the initial application of GA_3, the sequential application of BAP may have rekindled mitosis at the SAM, resulting in differentiation of floral organs, which triggered an increase in percentage of flowers emerging from the primary shoot and, therefore, the total flower number (Tables 1B, 2C) and percentage of flowering plants in group 2. The key to enhanced flowering in *Zantedeschia*, therefore, involves cross-talk between BAP and GA_3, where BAP enhanced floral productivity only when applied sequentially after GA_3 (GA_3 → BAP), and had no effect on flowering when applied alone (Table 4).

Hormonal Cross-talk Regulates Dormancy and Flowering in *Zantedeschia*

Cross-talk in plant physiology refers to signal integration from multiple hormone inputs, which could be either direct, indirect, or coregulatory, resulting in a common biological output (Chandler 2009). In this study we have shown that the application of BAP followed by GA_3 (BAP → GA_3) was the key to alleviating bud dormancy

(branching), and the application of GA_3 followed by BAP ($GA_3 \rightarrow$ BAP) was the key to enhanced flowering in *Zantedeschia*. The application of GA_3 alone had no effect on branching and the application of BAP alone had no effect on flowering. We assume that GA_3 complements the branching process initiated by BAP through its well-known role in shoot elongation (Hedden and Kamiya 1997), and BAP complements the flowering process initiated by GA_3 through its well-known role in mitosis (Campbell and others 1996). The respective biological outputs, branching and flowering, are more likely to be the result of coregulation between the independent pathways of cytokinin and gibberellin. Cross-talk between cytokinin and gibberellin and their reciprocal interaction in dormancy release or enhanced flowering have not been previously reported in a single plant species. The current findings, where alleviating bud dormancy (branching) and enhanced flowering were depicted as two facets of regulating meristem fate in day-neutral *Zantedeschia*, would contribute to the model, drawing common mechanisms between dormancy and flowering in plants (Horvath 2009).

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